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Mobilization of Opioid-containing Polymorphonuclear Cells by Hematopoietic Growth Factors and Influence on Inflammatory Pain

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Background: Leukocytes can control inflammatory pain by secretion of opioid peptides, stimulated by cold-water swimming or local injection of corticotropin-releasing factor, and subsequent activation of opioid receptors on peripheral sensory neurons. This study investigated whether mobilization of polymorphonuclear cells (PMN) by granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) enhances immigration of opioid-containing PMN and peripheral opioid analgesia in rats with Freund complete adjuvant–induced hind paw inflammation.

Methods: In circulating PMN of rats treated with G-CSF+SCF and sham-treated rats, opioid peptide content was measured by radioimmunoassay. Expression of adhesion molecules (CD62L, CD49d, CD18), *in vitro* migration in the Boyden chamber, and infiltrating leukocytes were analyzed by flow cytometry. Chemokine messenger RNA transcription was quantified by LightCycler polymerase chain reaction. Paw pressure threshold was measured at baseline, after cold-water swimming, and after injection of corticotropin-releasing factor.

Results: G-CSF+SCF treatment increased circulating PMN (11-fold, $P < 0.05$). Mobilized PMN had decreased content of β-endorphin but not of Met-enkephalin per cell, down-regulation of CD62L, up-regulation of CD49d (but no change in CD18), and reduced migration toward higher chemokine concentrations (all $P < 0.05$). In the paw, one of four chemokine messenger RNAs was significantly expressed during the first 2 h of inflammation ($P < 0.05$), immigration of PMN and opioid-containing cells was slightly increased (1.5-fold, $P < 0.05$), and baseline paw pressure threshold, as well as paw pressure threshold increases induced by corticotropin-releasing factor and cold-water swimming, were unchanged ($P > 0.05$).

Conclusions: G-CSF+SCF mobilized circulating opioid-containing PMN but had a minor influence on cell migration and peripheral analgesia, probably because of the low expression of chemokines in the inflamed paw and one of the decreased β-endorphin content in PMN.

OPIOIDS are the most powerful drugs for the control of severe postoperative and chronic pain in humans.[1] In addition to systemic therapy, analgesia can be produced by local injection of morphine in patients undergoing knee surgery or with chronic arthritis.[2] In the synovium, both opioid receptors on sensory neurons and opioid-containing leukocytes are present,[3] and these opioid peptides contribute to postoperative analgesia.[4] Local opioid-mediated analgesia is devoid of the central side effects of systemic opioid administration (*e.g.*, respiratory depression and nausea).

Studies in models of inflammation have elucidated the underlying mechanisms of peripheral opioid analgesia: polymorphonuclear cells (PMN), monocytes, and lymphocytes are present at the site of an inflammation and contain opioid peptides, such as Met-enkephalin and β-endorphin.[5] Opioid-mediated analgesia can be induced in rats by stress (*e.g.*, cold-water swimming [CWS]) or by local injection of corticotropin-releasing factor (CRF),[6,7] and it can be antagonized by naloxone or by anti-opioid peptide antibodies.[8] Conversely, inhibition of migration of opioid-containing leukocytes significantly impairs peripheral endogenous analgesia.[9,10] During the early phase of inflammation, only relatively few opioid-containing leukocytes (mostly PMN) are present. With the duration of inflammation, the number of opioid-containing leukocytes as well as CWS–induced analgesia increases progressively, whereas hyperalgesia at baseline, *i.e.*, pain sensitivity before liberation of opioid peptides by CWS or CRF, is unchanged.[11] Based on these findings, we hypothesized that increased migration of opioid-containing PMN into inflamed tissue could increase analgesia, particularly at early stages.

Hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF), mobilize PMN and their precursors from the bone marrow.[12] For example, treatment with G-CSF has been shown to enhance recruitment of PMN to the inflamed lung after intratracheal challenge with bacteria or lipopolysaccharide.[13,14] Although G-CSF treatment alone is effective, the mobilization of circulating PMN is maximized by using a combined treatment of G-CSF and stem cell factor (SCF).[15,16]

Recruitment of PMN to inflamed sites is a multistep process involving chemotaxis as well as cell adhesion (fig. 1): chemokines are produced at the site of inflammation and are subsequently presented on the luminal surface of the endothelium.[17] Circulating PMN recognize most of these chemokines through a single receptor (CXCR2),[18] and receptor binding triggers enhanced expression of adhesion molecules.[17,19] These molecules reg-
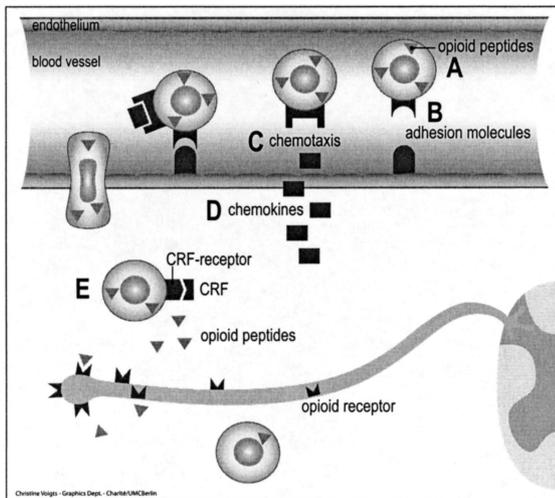
Fig. 1. Opioid-containing leukocytes: recruitment into the inflamed paw and peripheral opioid-mediated analgesia. Several steps influence the coordinated migration of opioid-containing leukocytes to a site of inflammation: (*A*) the absolute number of opioid-containing leukocytes in the circulation and the content of opioid peptides per cell (*i.e.*, β-endorphin and Met-enkephalin; fig. 2), (*B*) the expression of adhesion molecules on circulating leukocytes (*i.e.*, selectins [CD62L, L-selectin], integrins [CD18, β_2 integrin], and immunoglobulin superfamily [CD49d, VLA-4]; these molecules interact with adhesion molecules on the inflamed endothelium, leading to leukocyte rolling, sticking, and transmigration; fig. 3B), (*C*) the ability of circulating opioid-containing leukocytes to migrate in direction to a stimulus (*i.e.*, chemotaxis by chemokines, *e.g.*, macrophage inflammatory protein-2; fig. 3C), and (*D*) the expression of these chemokines in the inflamed paw (table 2). (*E*) These factors determine the absolute number of opioid-containing leukocytes in the inflamed paw (fig. 5A). Intraplantarly injected corticotropin-releasing factor (CRF) induces the release of opioid peptides from leukocytes in the inflamed paw, leading to peripheral opioid-mediated analgesia.

ulate subsequent rolling, sticking, and endothelial transmigration of PMN.[20] Consistently, blockage of adhesion molecules inhibits migration of opioid-containing leukocytes to the site of inflammation and significantly decreases CWS- and CRF-induced peripheral analgesia.[9,10]

The current study was designed to elucidate mechanisms to enhance the recruitment of opioid-containing PMN to sites of painful inflammation. These PMN have previously been shown to be of major importance in peripheral opioid analgesia.[11] To understand the pathways governing the migration of these PMN to inflamed tissue, we analyzed the expression of adhesion molecules, the migratory capacity of PMN, and the production of chemokines. More specifically, we examined whether PMN mobilization by G-CSF and SCF treatment (1) increases the number of circulating PMN and their opioid peptide content, (2) influences the expression of adhesion molecules (L-selectin, integrins CD18 and CD49d) and the chemotaxis of circulating PMN, and (3) enhances immigration of opioid-containing leukocytes and peripheral opioid analgesia in rats with unilateral hind paw inflammation.

Materials and Methods

Animals and Induction of Inflammation

Animal protocols were approved by the animal care committee of the State of Berlin (Landesamt für Arbeitsschutz, Gesundheitsschutz und Technische Sicherheit, Berlin, Germany) and were in accordance with the guidelines of the International Association for the Study of Pain.[21] Male Wistar rats weighing 140–200 g were injected with 150 μl Freund complete adjuvant (FCA; Calbiochem, La Jolla, CA) into the right hind paw (intraplantar) and developed an inflammation confined to the inoculated paw. Experiments were conducted at 2 h after injection of FCA unless stated otherwise. For all injections, rats were briefly anesthetized with halothane using an open circuit without nitrous oxide.

PMN Mobilization by Hematopoietic Growth Factors

Rats were randomly distributed into cages by the animal caretakers, who did not participate in the experiments and were not informed about the study protocol. Before the distribution of rats, cages were marked with letters (A or B) for either the control or the treatment group. Over 5 consecutive days before FCA inoculation, rats were injected subcutaneously once daily with a combination of 50 μg/kg recombinant rat SCF (Amgen Inc., Thousand Oaks, CA) and 100 μg/kg recombinant human G-CSF (filgrastim; Amgen) in 100 μl glucose 5% (Braun-Melsungen, Melsungen, Germany), as described previously.[15] Rats in the control group were injected subcutaneously with solvent (100 μl glucose 5%).

Fluorescence-activated Cell Staining

Blood Cells. Heparinized blood (20 units/ml, Braun-Melsungen) was obtained by cardiac puncture from deeply anesthetized rats, fixed and lysed as described by the manufacturer (FACS lysing solution®; BD Biosciences, Heidelberg, Germany). PMN and mononuclear cells (including monocytes and lymphocytes) were identified by forward–sideward scatter characteristics. For adhesion molecule staining, blood was incubated with fluorescein isothiocyanate–conjugated monoclonal antibodies (mAbs; all at 20 μg/ml and all from BD Biosciences) for 15 min at room temperature in the dark and further processed as described at the beginning of this paragraph. The following antibodies were used: hamster anti-rat CD62L (L-selectin) antibody (isotype: immunoglobulin [Ig] G, clone HRL-1), mouse anti-rat CD49d (VLA-4) mAb (isotype: IgG_{2a}, clone MRα4-1), and mouse anti-rat CD18 mAb (isotype: IgG_1, clone WT.3). Controls were incubated with the appropriate isotype-matched antibodies: hamster IgG antibody (clone B81-3), mouse IgG_{2a}, and mouse IgG_1. The intensity of staining was measured by geometric mean fluorescence intensity (MFI). To correct for background nonspecific staining,

the MFI of the isotype-matched control mAb was subtracted from the MFI of the adhesion molecule mAb.

Paw Tissue. Paw tissue was dissected from the inflamed paw, leaving the deep flexor tendon *in situ*. The overlying skin was removed and the tissue was cut into small fragments, enzymatically digested for 60 min, and pressed through a nylon filter to obtain single-cell suspensions as previously described.[11,22] All samples were stained with Cy-Chrome (BD Biosciences Pharmingen, San Diego, CA) -conjugated mouse anti-rat CD45 mAb (against all hematopoietic cells; 4 μg/ml). For intracellular staining, cells were fixed with 1% paraformaldehyde, permeabilized with saponin buffer, and stained with the following mAbs: mouse anti-rat ED1 fluorescein isothiocyanate (monocytes/macrophages, 2 μg/ml; Serotec, Oxford, England), mouse anti-rat RP-1 phycoerythrin (PMN, 12 μg/ml; BD Biosciences), and mouse anti-rat CD3 phycoerythrin (lymphocytes, T cells, 4 μg/ml; BD Biosciences). Intracellular opioid peptides were stained using the pan-opioid mouse 3E7 (subtype IgG$_{2a}$; Gramsch Laboratories, Schwabhausen, Germany), an isotype-matched murine mAb as a control, and secondary antibodies.[11,22]

Quantification of Cells and Analysis of Data. Fluorescence-activated cell staining events from fluorescent TruCOUNT® beads (BD Biosciences) and from stained cells were collected simultaneously as previously described (70,000 events/paw and 20,000 events in the cell migration assay).[11,22] The number of cells per tube was calculated in relation to the known number of fluorescent TruCOUNT® beads. Data were analyzed using CellQuest software (BD Biosciences).

Perfusion, Cell Separation, and Radioimmunoassay

After sternotomy, rats were perfused through the left ventricle with 0.9% NaCl containing 2.7 mM EDTA at room temperature (Sigma Chemicals, Taufkirchen, Germany) using an automatic roller pump (Reglo Analog; Ismatec, Glattbrugg, Switzerland). A total of 120 ml perfusate was collected from the right ventricle by passive drainage. After dextran sedimentation (Amersham Biosciences, Uppsala, Sweden) to remove erythrocytes, the cell suspension was layered on a two-phase Percoll (Amersham Biosciences) gradient (58.6% Percoll with 0.15 M NaCl and 78% with 0.18 M NaCl) (Amersham Biosciences, Freiburg, Germany) and centrifuged at 700g for 40 min. PMN were further purified by negative selection with mouse anti-rat CD3 fluorescein isothiocyanate and anti-rat CD4 fluorescein isothiocyanate mAbs using magnetic cell separation (MACS; Miltenyi, Bergisch-Gladbach, Germany) as described previously,[11] and cell pellets were frozen until further usage. Cell purity was determined by staining with phycoerythrin-conjugated anti-RP-1 (PMN marker) mAb and analyzed by flow cytometry. Cell pellets were thawed, reconstituted with radioimmunoassay buffer, and lysed by repetitive freeze–thaw–sonication cycles. Met-enkephalin and β-endorphin content was measured using commercially available kits (Bachem, Weil am Rhein, Germany) as previously described.[11] According to the manufacturer, the cross-reactivity of the anti-Met-enkephalin antibody is 0.1% to human β-endorphin and that of the anti-rat β-endorphin antibody is 0% to Met-enkephalin. The sequence of Met-enkephalin is identical in rats and humans.

Chemotaxis Assay

To remove erythrocytes, peripheral blood leukocytes were purified by dextran sedimentation and subsequent hypotonic lysis. The chemokine macrophage inflammatory protein-2 (MIP-2; PeproTech, London, England) was dissolved in complete medium (Roswell Park Memorial Institute 1640 medium; Life Technologies, Paisley, Scotland) containing 100 U/ml penicillin, 10 mg/ml streptomycin, and 10% fetal calf serum (all from Invitrogen, Groningen, The Netherlands) at a dose range of 10^{-6} to 10^{-10} M and was placed into the lower well of a Boyden chamber (Costar, Bodenheim, Germany). After trypan blue staining and counting, 4×10^5 cells in 100 μl complete medium were added to the upper well, separated from the lower well by a cell permeable nylon membrane. After incubation for 60 min at 37°C and 5% CO$_2$, the migrated cell suspension from the lower well was stained with mouse anti-rat CD45 Cy-Chrome (BD Biosciences Pharmingen) Ab (4 μg/ml) and was quantified by TruCOUNT® beads. A migration index was calculated by dividing the number of migrating cells toward MIP-2 by the number of migrating cells under control conditions (medium alone).

Polymerase Chain Reaction

mRNA Isolation and cDNA Synthesis. Subcutaneous plantar paw tissue was dissected, and a piece of 25–35 mg from the central inflamed part of the paw was homogenized in Trizol (Invitrogen) as previously described.[23] To synthesize complementary DNA (cDNA), RNA was annealed to 0.125 μM oligodesoxythymidine for 3 min at 65°C and transcribed in the presence of reverse-transcriptase buffer, 0.01 M dithiothreitol, 1 mM deoxynucleoside triphosphate, 10 U avian myeloblastosis virus reverse transcriptase, and 4 U ribonuclease inhibitor (all from Roche Diagnostics, Mannheim, Germany, except dithiothreitol, Sigma Chemicals) at 42°C for 2 h.

Chemokine mRNA Semiquantification by Light-Cycler PCR. Polymerase chain reaction (PCR) was performed with DNA Sybr green following the manufacturer's instructions (Roche Diagnostics) using 10 μM of both sense and antisense primers (listed in the next paragraph). cDNA was amplified in a four-step-per-cycle PCR consisting of 95°C for 30 s, primer-specific annealing, elongation, and a fluorescence detection (temperature step [Tm]) step (table 1). In pilot experiments, the temperature was determined just below the product-specific melting temperature, and this Tm was incorpo-

Table 1. Instrument Settings for LightCycler Polymerase Chain Reaction

Parameters	Chemokines				
	RPL-19	KC	MIP-2	LIX	CINC-2
NIH accession number	X82202	U85628	X65647	U90448	D87927
No. of cycles	29	35	33	40	35
Annealing temperature, °C	69	69	69	62	61
Annealing time, s	3	3	3	4	3
Elongation temperature, °C	72	72	72	72	72
Elongation time, s	14	8	5	7	9
Fluorescence detection (Tm), °C	89	87	85	79	87

The parameters of the polymerase chain reaction for each chemokine and the housekeeping gene are summarized.

CINC-2 = cytokine-induced chemokine; KC = keratinocyte-derived chemokine; LIX = lipopolysaccharide-induced chemokine; MIP-2 = macrophage inflammatory protein 2; NIH = National Institutes of Health; RPL-19 = ribosomal protein L-19 (housekeeping gene as reference gene); Tm = temperature step.

rated into the PCR for detection of fluorescence. Chemokine messenger RNA (mRNA) was quantified using duplicate samples and a standard dilution with a known number of copies for a specific PCR product as described previously.[23] Ribosomal protein L19 (RPL-19) was used as a reference gene for quantification.

PCR Primers. Primers were intron spanning, and genomic amplification was excluded by agarose visualization. The following primers were used: RPL-19 (sense [SE] 5' AAT CGC CAA TGC CAA CTC TCG and antisense [as] 5' TGC TCC ATG AGA ATC CGC TTG); keratinocyte-derived chemokine (SE 5' AAT GAG CTG CGC TGT CAG TGC and as 5' TTG GGG ACA CCC TTT AGC ATC); MIP-2 (SE 5' CAA CCA TCA GGG TAC AGG GGT and as 5' GTC CTG GAG GGG TCA CCG T); lipopolysaccharide-induced chemokine (SE 5' CCA AGG TGG AAG TCA TAG C and as 5' GCT TTG TTT TCT TAT TTT CAC TGC); cytokine-induced chemokine 2 (SE 5' CAG CCT TCA GGG ACT GTT G and as 5' AGC TGG ACT TGT CAC TCT TC).

Measurement of Analgesia

Nociceptive thresholds were assessed using the paw pressure algesiometer as previously described (modified Randall-Selitto test; Ugo Basile, Comerio, Italy).[8,11,22] Briefly, pressure was applied to the dorsal surface of the hind paw. The average pressure required to illicit paw withdrawal, the paw pressure threshold (PPT), was determined by three consecutive trials both on the inflamed and on the contralateral noninflamed hind paw. PPT determination was performed before treatment (baseline) and 1 min after CWS[8,11] or 5 min after intraplantar injection of CRF.[10] Previous dose–response analysis of CRF injection revealed a peak effect at 8 ng that was fully antagonized by intraplantar but not subcutaneous naloxone, demonstrating peripheral opioid-mediated antinociception (data not shown; subcutaneous injections were performed at a remote location on the back to exclude any systemic effect). Data are presented as percent of maximum possible effect (calculated as $[PPT_{treated} - PPT_{pretreated}]/[250 - PPT_{pretreated};$ a cutoff value of 250 g was used in all experiments to exclude tissue damage).[22] All experiments were per-

formed by an examiner blinded to the treatment protocol.

Experimental Protocols

In each experiment, rats were randomly assigned to one of two groups that were injected with G-CSF+SCF (treatment) in 5% glucose or with the solvent only (5% glucose as a control) as described above (Material and Methods, PMN Mobilization by Hematopoietic Growth Factors). On the fifth day and 3 h after the last injection, an examiner blinded to the group assignment injected all rats with FCA intraplantarly and performed the subsequent experiments (removal of paw tissue or behavioral experiments; number of animals per group are given in the figure legends): (1) determination of circulating PMN by flow cytometry and of opioid peptide content by radioimmunoassay; (2) expression of adhesion molecules on circulating PMN by flow cytometry; (3) chemotaxis of circulating PMN in the Boyden chamber; (4) quantification of chemokine mRNA in the inflamed paw by LightCycler (Synthegen, Houston, TX) PCR; (5) quantification of leukocyte subpopulations and of opioid-containing leukocytes in the inflamed paw by flow cytometry; and (6) measurement of analgesia in behavioral experiments before and after CWS or injection of CRF.

Statistics

Normally distributed data were analyzed by *t* test. If the data lacked equal variance or normality, the Mann–Whitney rank sum test was used. Comparisons of multiple time points were analyzed by analysis of variance (ANOVA) or ANOVA on ranks with a *post hoc* comparison by the Dunnett and Dunn method, respectively. Migration indexes were compared by two-way ANOVA. A *P* value less than 0.05 was considered significant. Data are expressed as mean ± SEM.

Results

PMN Mobilization and Opioid Peptide Content

Treatment with G-CSF+SCF significantly increased the number of circulating PMN (fig. 2A; PMN: 11-fold in-
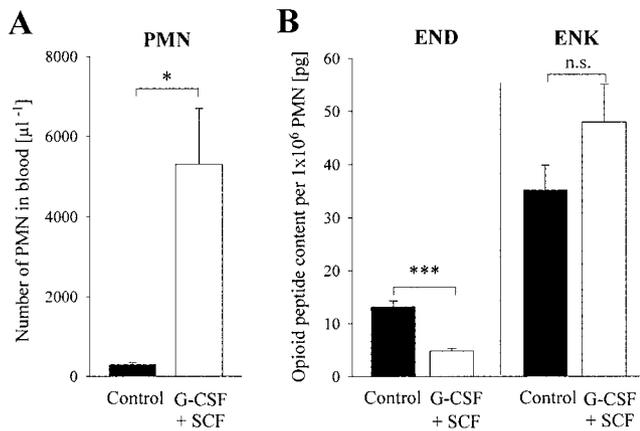
Fig. 2. Number of circulating polymorphonuclear cells (PMN) and opioid peptide content after granulocyte colony-stimulating factor (G-CSF) stem cell factor (SCF) treatment. (*A*) Rats (n = 6) were subcutaneously injected with solvent (control; *black bar*) or with G-CSF+SCF (*white bar*) for 5 days. Immune cell subpopulations of circulating blood were quantified by flow cytometry. Circulating PMN were significantly increased (* $P <$ 0.05, Mann–Whitney test). (*B*) In isolated PMN of rats treated with G-CSF+SCF, β-endorphin (END) content measured by radioimmunoassay was significantly decreased (*** $P <$ 0.001), whereas Met-enkephalin (ENK) content was unchanged ($P >$ 0.05 [not significant (n.s.)], n = 9 or 10, both *t* test).

crease; $P <$ 0.05, Mann–Whitney rank sum test) but not of mononuclear cells (all leukocytes except PMN, *i.e.*, mostly lymphocytes; control: 1,487 ± 182 *vs.* 2,548 ± 510 cells/μl blood; $P >$ 0.05, *t* test). For measurement of opioid peptide content, PMN were isolated from circulating blood with a purity of 90 ± 1% (control) and 95 ± 1% (G-CSF+SCF) as determined by flow cytometric staining with the mAb RP-1. Cells were more than 95% viable by trypan blue exclusion. In the PMN fraction, β-endorphin content per cell was significantly decreased and Met-enkephalin content was unchanged in rats treated with G-CSF+SCF (fig. 2B; $P <$ 0.001, $P =$ 0.148, respectively, *t* test).

Adhesion Molecule Expression and Migratory Capacity of Mobilized PMN

In rats treated with G-CSF+SCF, the MFI of CD62L staining was significantly lower, whereas the MFI of CD49d was significantly higher on circulating PMN (figs. 3A and B; $P <$ 0.05, $P <$ 0.001, *t* test). CD18 expression was unchanged ($P >$ 0.05, *t* test). The *in vitro* cell migration was not influenced by G-CSF+SCF treatment at low MIP-2 concentrations but was significantly lower in rats treated with G-CSF+SCF than in controls at concentrations of 10^{-6} to 10^{-8} M (fig. 3C; $P <$ 0.05, two-way ANOVA).

Chemokine mRNA Expression in the Inflamed Paw

mRNA expression of all four chemokines significantly increased with the duration of inflammation (table 2; all $P <$ 0.05, ANOVA). With the exception of keratinocyte-
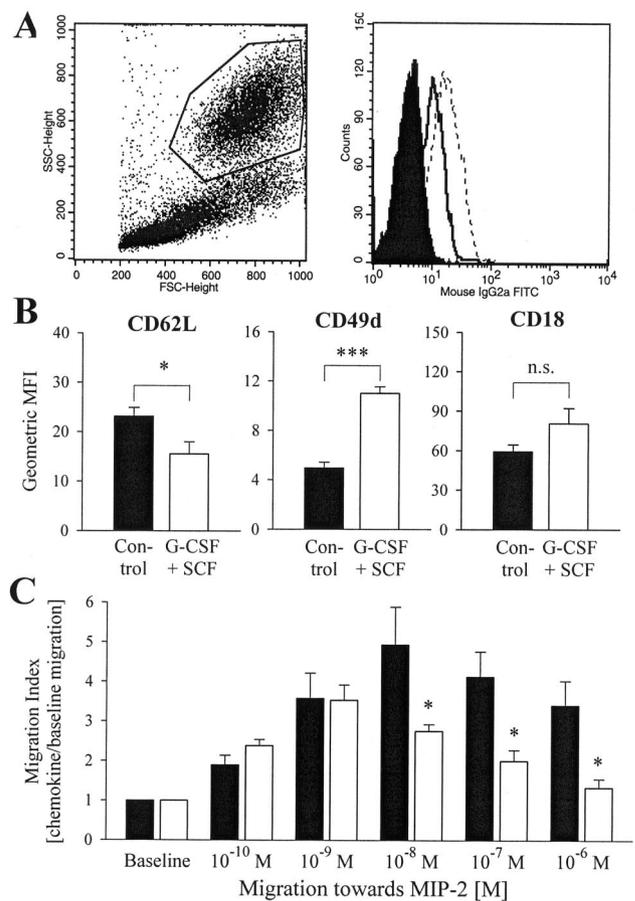

Fig. 3. Effects of granulocyte colony-stimulating factor (G-CSF) stem cell factor (SCF) treatment on adhesion molecule expression and on chemotaxis. (*A*) Using flow cytometry, circulating polymorphonuclear cells (PMN) were identified by size and granularity (sideward scatter [SSC] *vs.* forward scatter [FSC]; *left, black lined gate*) and were analyzed for adhesion molecule expression (*right*, isotype-matched control antibody [mouse immunoglobulin (Ig) G2a, *black area*], adhesion molecule antibody in control rats [*thick black line*] and in rats treated with G-CSF+SCF [*dashed line*], and fluorescein isothiocyanate (FITC)–conjugated antibodies were used). (*B*) PMN of control animals (solvent; *black bar*) expressed significantly more CD62L and less CD49d than PMN of animals treated with G-CSF+SCF (*white bar*; determined as geometric mean fluorescence intensity [MFI]; * $P <$ 0.05 and *** $P <$ 0.001, respectively, n = 5). CD18 expression was unaffected by this treatment ($P >$ 0.05 [not significant], all *t* test). (*C*) *In vitro* migration of PMN toward the chemokine macrophage inflammatory protein-2 (MIP-2) was measured in a Boyden chamber in control rats and rats treated with G-CSF+SCF (*black* and *white bars*, respectively, n = 5). A migration index relative to baseline migration was calculated. Dose-dependent migration was observed in both sets of animals at low MIP-2 concentrations, but animals treated with G-CSF–SCF had significantly less PMN migration at high MIP-2 concentrations (* $P <$ 0.05, two-way analysis of variance).

derived chemokine, this increase did not attain significance during the first 2 h after FCA inoculation.

PMN Migration into the Inflamed Paw and Peripheral Analgesia

Flow cytometric analysis of immune cell subpopulations in the inflamed paw showed that total hematopoi-

Table 2. Chemokine mRNA Expression in the Inflamed Paw

Chemokines	Duration of Inflammation, Hours after FCA Injection			
	0	1	2	12
KC	< 10	780 ± 170*	3,460 ± 880*	7,170 ± 1,190*
CINC-2	< 10	100 ± 60	60 ± 20	4,450 ± 1,220*
LIX	< 10	< 10	20 ± 10	2,120 ± 360*
MIP-2	< 10	10 ± 4	30 ± 10	3,400 ± 630*

Chemokine and reference gene messenger RNA (mRNA) (RPL-19) were quantified by LightCycler polymerase chain reaction from paw tissue at various time points after injection of Freund's complete adjuvant (FCA) (n = 5 rats per time point). Chemokine mRNA copy numbers were calculated per 10,000 RPL-19 mRNA copies and expressed as mean ± SEM.

* $P < 0.05$, analysis of variance on ranks, each chemokine compared to control (0 h FCA).

CINC-2 = cytokine-induced chemokine 2; KC = keratinocyte-derived chemokine; LIX = lipopolysaccharide-induced chemokine; MIP-2 = macrophage inflammatory protein-2; RPL-19 = ribosomal protein L-19 (housekeeping gene as reference gene).

etic (CD45$^+$) cells and PMN were significantly increased in rats treated with G-CSF+SCF (figs. 4A and B; both $P <$ 0.05, t test; few CD45$^+$ cells were detected in the noninflamed contralateral paw, n = 3). G-CSF+SCF treatment was significantly less effective to mobilize PMN to the inflamed paw than to expand PMN in blood (1.4-fold *vs.* 11-fold; figs. 2A and 4A; $P < 0.001$, Mann–Whitney
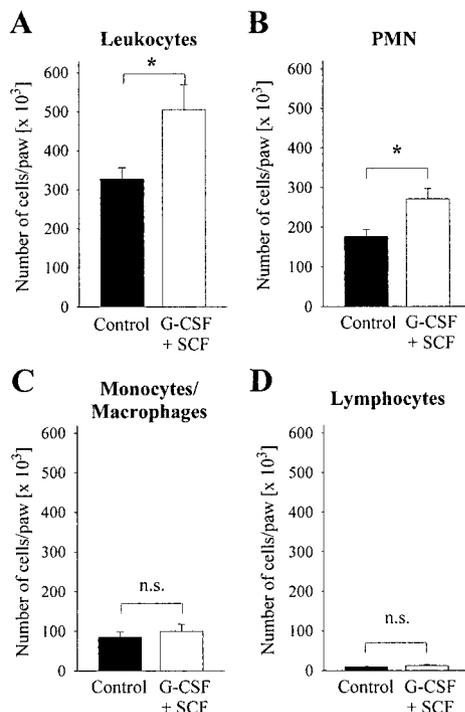

Fig. 4. Recruitment of polymorphonuclear cells (PMN) to a local site of inflammation. At 2 h after Freund complete adjuvant inoculation, significantly more leukocytes (*A*, CD45$^+$ cells) and PMN (*B*, RP-1$^+$ cells, both * $P < 0.05$) were present in the inflamed paw of rats treated with granulocyte colony-stimulating factor (G-CSF) stem cell factor (SCF) (*black bar* = solvent; *white bar* = G-CSF+SCF; n = 6 or 7) compared to controls. Monocytes/macrophages (*C*, ED1$^+$ cells) and lymphocytes (*D*, CD3$^+$ cells) were unchanged (both $P > 0.05$ [not significant], all t test).
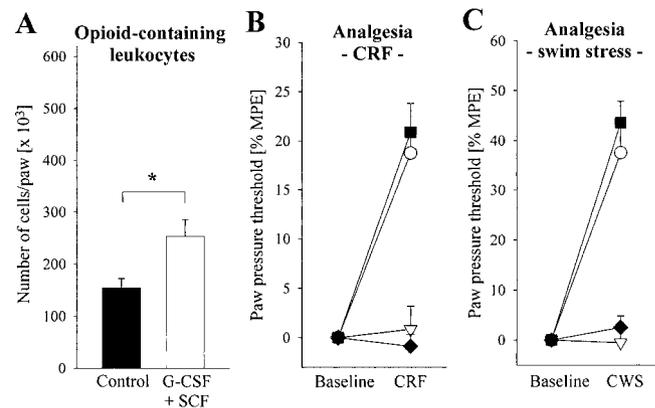

Fig. 5. Opioid-containing immune cells and peripheral opioid analgesia in the inflamed paw after mobilization of polymorphonuclear cells (PMN). (*A*) Flow cytometric analysis showed that opioid-containing leukocytes (3E7$^+$ cells) in the paw were significantly increased by granulocyte colony-stimulating factor (G-CSF) stem cell factor (SCF) treatment (*black bar* = solvent; *white bar* = G-CSF+SCF; * $P < 0.05$, t test; n = 6 or 7). (*B*) Injection of corticotropin-releasing factor (CRF) elicited similar elevations of paw pressure threshold in control animals (solvent; *open symbols*) and in animals treated with G-CSF+SCF (*filled symbols*) in the inflamed paw (*square* and *circle*; $P >$ 0.05 [not significant], t test; n = 7). Cold-water swimming stress (CWS; *C*) produced similar paw pressure threshold increases in treated and untreated animals ($P > 0.05$ [not significant], t test; n = 7). Neither CRF nor CWS resulted in increases in the paw pressure threshold in the noninflamed, contralateral paws (*B* and *C*; *inverted triangle* = controls; *diamond* = animals treated with G-CSF+SCF; all $P > 0.05$ [not significant], t test).

test). No changes occurred in the number of monocytes/macrophages or lymphocytes (figs. 4C and D; both $P >$ 0.05, t test). Opioid-containing cells were significantly increased by 1.5-fold in the inflamed paw of rats treated with G-CSF+SCF (fig. 5A; $P < 0.05$, t test); the percentage of opioid-containing leukocytes did not differ among groups (control 46.8 ± 2.4%, G-CSF+SCF 50.2 ± 2.2% of CD45$^+$ cells, $P > 0.05$, t test). Baseline PPTs in the inflamed or noninflamed paw were not significantly changed by hematopoietic growth factor treatment (inflamed: control 38.6 ± 3.2 g *vs.* G-CSF+SCF 37.9 ± 3.6 g; noninflamed paw: control 64.5 ± 1.6 g *vs.* G-CSF+SCF 67.9 ± 1.7 g; both $P > 0.05$, t test). Both CRF injection and CWS lead to a significant PPT increase in the inflamed paw of both control rats and rats treated with G-CSF+SCF (figs. 5B and C; $P < 0.05$, t test), which was not significantly different between rats treated with G-CSF–SCF and control rats ($P > 0.05$, t test).

Discussion

In this study, we examined whether endogenous peripheral opioid analgesia in early FCA inflammation can be augmented by systemic immunomodulation. We demonstrated that mobilization of PMN by the hematopoietic growth factors G-CSF and SCF induced a major increase in PMN in the circulation. Mobilized PMN had decreased

β-endorphin but unchanged Met-enkephalin contents and minor changes in the expression pattern of the adhesion molecules CD62L (L-selectin), CD18 (β₂ integrin), and CD49d (VLA-4). The *in vitro* migration ability of mobilized PMN was intact in response to low doses but impaired in response to high doses of the chemokine MIP-2. Chemokine mRNA transcription in the paw was hardly detectable in early inflammation. The *in vivo* migration of opioid-containing leukocytes to the inflamed paw was slightly increased, and endogenous peripheral analgesia was unaltered.

Endogenous opioid-mediated peripheral analgesia has been demonstrated in early stages of an experimental inflammation[9,11] as well as immediately after surgery in humans.[4] In circulating blood of Wistar rats, lymphocytes constitute the predominant population (60–70% of all leukocytes, 20–30% PMN, 5–10% monocytes[24]). In contrast, PMN are the major leukocyte and opioid-containing population in the inflamed paw during early inflammation (2 and 6 h after intraplantar FCA; of all leukocytes: 60–70% PMN, 20–25% monocytes/macrophages, < 5% lymphocytes[11]). Importantly, because changes in opioid peptide plasma concentrations do not correlate with analgesia,[25,26] an expansion of opioid-containing leukocytes in the blood will not predict the degree of pain control. In contrast, opioid-containing leukocytes in the inflamed paw induce peripheral opioid analgesia correlating with the number of opioid-containing cells.[8,11] During early inflammation (2 h after intraplantar FCA), opioid-containing leukocytes are present in lower absolute numbers, and peripheral analgesia is significantly weaker than in late inflammation (96 h after intraplantar FCA),[11] suggesting that analgesia could potentially be augmented by increasing migration of opioid-containing leukocytes to the inflamed paw. Previous studies have shown that recruitment of leukocytes by intraplantar injection of a single stimulatory agent (*e.g.*, leukotriene B4, formyl-methionyl-leucyl-phenylalanine, or nerve growth factor) induced pain that could be abolished by PMN depletion.[27–29] On the contrary, in a model of peritonitis (*i.e.*, acetic acid–induced writhing), pain was enhanced in mice depleted of PMN but not of monocytes/macrophages, indicating that PMN conferred analgesia, whereas activation of resident cells (rather than PMN) enhanced inflammatory pain.[29,30] Furthermore, PMN have been shown to contain opioid peptides such as β-endorphin and Met-enkephalin[5,31] and to express CRF receptors.[32,33] Activation of CRF receptors mediates opioid peptide release from leukocytes *in vitro*[34,35] and peripheral opioid analgesia *in vivo*.[6,7]

In concurrence with previous studies,[15,16] we have now demonstrated— using flow cytometric quantification—that combined G-CSF+SCF treatment was highly effective in increasing the number of circulating PMN (fig. 2A). Because the 3E7 mAb used for flow cytometry does not permit distinction between β-endorphin and Met-enkephalin, we measured opioid peptide content by radioimmunoassay in purified circulating PMN: cellular β-endorphin content was significantly decreased and Met-enkephalin content was unchanged in mobilized PMN (fig. 2B), which may be explained by observations that G-CSF+SCF treatment induces large numbers of immature PMN[15] and has differential effects on granular protein content.[36] To examine the potential of these cells to migrate to inflamed sites, we first characterized their expression of adhesion molecules. Migration of PMN requires rolling (mediated by selectins) and sticking (mediated by integrins).[20] Blockage of CD62 (selectins) by fucoidin has been shown to reduce migration of opioid-containing leukocytes to the inflamed paw and to essentially abolish analgesia.[9] Although previous studies have demonstrated that mobilization of circulating PMN by G-CSF is highly effective, whether these cells change the expression of adhesion molecules remains controversial.[37] In addition, expression of adhesion molecules has not been studied in combined G-CSF+SCF treatment. This strategy mobilizes an even higher degree of immature PMN than G-CSF treatment alone,[15] which might have a more pronounced effect on the adhesion molecule expression pattern. Similar to studies using G-SCF treatment in humans,[37] we demonstrate here that CD62L expression was only reduced to a minor extent (by 25%; fig. 3B) in rats treated with G-CSF+SCF. This is unlikely to contribute to a reduced capacity to migrate because even in the complete absence of CD62L in genetically deficient mice, PMN migration was reduced by only 50%.[38] The second stage of adhesion involves PMN sticking mediated by CD18 and CD49d expression. In the current study, mobilized PMN had normal CD18 and increased CD49d expression (fig. 3B), as has been shown in human PMN after G-CSF treatment.[39] Taken together, adhesion molecule expression is slightly altered in PMN mobilized by G-CSF+SCF but seems adequate for migration of opioid-containing leukocytes to an inflammatory site. Therefore, enhancement of adhesion molecule expression would not be likely to further augment analgesia.

In addition to adhesion, PMN migration requires intact chemotaxis. A decreased chemotactic capacity of G-CSF–mobilized PMN has been demonstrated in the majority of previous studies.[36,37,40] In rodents, recruitment is largely mediated by the chemokine receptor CXCR2 on PMN.[18,41,42] Because the four known ligands of the CXCR2 receptor (MIP-2, keratinocyte-derived chemokine, lipopolysaccharide-induced chemokine, and cytokine-induced chemokine 2)[43,44] all induce PMN recruitment at similar molar concentrations (10^{-8} M),[45–47] we examined *in vitro* chemotaxis toward one representative CXCR2 ligand (MIP-2) in the current study. We observed a chemotactic defect of mobilized PMN at higher chemokine concentrations ($\geq 10^{-8}$ M; fig. 3C).

The question arises whether these concentrations are physiologically relevant *in vivo*. Chemokines are produced within inflamed tissue, but they exert their chemotactic effects while being bound to glycosaminoglycans on the endothelium in the circulation.[17] This concentration cannot be determined *in vivo*. In an inflammatory model in mice, chemokine concentrations were determined in an air pouch 2 h after injection of carrageenan, and a concentration of 10^{-8} to 10^{-9} M was measured.[48] In accordance with this and other studies,[48,49] we detected only very low levels of chemokine transcription at early stages of inflammation (1 and 2 h *vs.* 12 h after FCA; table 2) by PCR. Taken together, our results suggest that mobilized PMN show a moderately reduced capacity to migrate toward the chemokine MIP-2 at concentrations potentially relevant *in vivo*, and only one of four chemokines known to recruit PMN is significantly expressed in early inflammation. These data indicate that augmentation of chemokine expression in the inflamed paw may be a promising strategy to enhance recruitment of opioid-containing PMN and analgesia.

In contrast to our findings in peripheral blood (11-fold increase), PMN mobilization by G-CSF+SCF resulted only in a modest increase (1.4-fold increase) in the number of PMN in the inflamed paw, without significant changes in the number of monocytes/macrophages or of lymphocytes (fig. 4). Previous studies have shown that G-CSF treatment increased the number of circulating PMN (by 3- to 10-fold),[50-52] but the effect on cell migration to inflamed tissue was variable: G-CSF treatment effectively increased PMN recruitment to the inflamed lung,[13,14,52] but PMN recruitment was decreased to human skin or to the cerebrospinal fluid in experimental meningitis.[40,53] In line with the modest increase of PMN in the inflamed paw by G-CSF+SCF treatment, the number of opioid-containing leukocytes increased to a minor extent (fig. 5A). Consistently, CWS- and CRF-induced analgesia were unchanged by PMN mobilization (figs. 5B and C). Because baseline hyperalgesia was unaltered, the slightly increased number of PMN at the site of inflammation in rats treated with G-CSF+SCF did not intensify pain.

Taken together, the lack of change in analgesia could be attributed to two factors: First, chemokine transcription during the early stage of inflammation is low (table 2), and mobilized PMN have an impaired ability to migrate (fig. 3C). This would explain the rather modest effect of PMN mobilization by G-CSF+SCF treatment in the paw compared to the circulation (figs. 2A and 4B). Second, although the number of opioid-containing ($3E7^+$) cells modestly increased in the paw examined by flow cytometry (fig. 5A), this does not reflect opioid peptide content per cell. In circulating PMN, β-endorphin content per cell was significantly decreased. Because our previous studies in later stages of inflammation

indicate that β-endorphin is the major peptide responsible for peripheral opioid analgesia,[8] this may contribute to the net lack of effect on analgesia observed here. In addition, opioid-mediated analgesia in early inflammation might be limited by an insufficient permeability of the perineural barrier to opioid peptides.[54] Finally, low levels of opioid receptor expression or G-protein coupling on the peripheral sensory nerve may account for the unchanged analgesic efficacy.[55,56]

In summary, our study examined an approach to augment peripheral endogenous analgesia. Treatment with hematopoietic growth factors was effective in expanding the number of circulating PMN, but these cells had a significantly decreased content of β-endorphin. In contrast to circulating blood, G-CSF+SCF increased the number of opioid-containing leukocytes at the site of an inflammation only to a minor extent, and it did not alter peripheral opioid-mediated analgesia. Future strategies to augment the recruitment of opioid-containing PMN and to enhance endogenous analgesia should be directed at increasing chemokine production but not at enhancing the expression of adhesion molecules.

References

1. Aubrun F, Langeron O, Quesnel C, Coriat P, Riou B: Relationships between measurement of pain using visual analog score and morphine requirements during postoperative intravenous morphine titration. ANESTHESIOLOGY 2003; 98: 1415-21

2. Kalso E, Smith L, McQuay HJ, Andrew Moore R: No pain, no gain: Clinical excellence and scientific rigour—lessons learned from IA morphine. Pain 2002; 98:269-75

3. Stein C, Pflüger M, Yassouridis A, Hoelzl J, Lehrberger K, Welte C, Hassan AH: No tolerance to peripheral morphine analgesia in presence of opioid expression in inflamed synovia. J Clin Invest 1996; 98:793-9

4. Stein C, Hassan AH, Lehrberger K, Giefing J, Yassouridis A: Local analgesic effect of endogenous opioid peptides. Lancet 1993; 342:321-4

5. Przewlocki R, Hassan AH, Lason W, Epplen C, Herz A, Stein C: Gene expression and localization of opioid peptides in immune cells of inflamed tissue: Functional role in antinociception. Neuroscience 1992; 48:491-500

6. Schäfer M, Carter L, Stein C: Interleukin 1 beta and corticotropin-releasing factor inhibit pain by releasing opioids from immune cells in inflamed tissue. Proc Natl Acad Sci U S A 1994; 91:4219-23

7. Schäfer M, Mousa SA, Zhang Q, Carter L, Stein C: Expression of corticotropin-releasing factor in inflamed tissue is required for intrinsic peripheral opioid analgesia. Proc Natl Acad Sci U S A 1996; 93:6096-100

8. Stein C, Hassan AH, Przewlocki R, Gramsch C, Peter K, Herz A: Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. Proc Natl Acad Sci U S A 1990; 87:5935-9

9. Machelska H, Cabot PJ, Mousa SA, Zhang Q, Stein C: Pain control in inflammation governed by selectins. Nat Med 1998; 4:1425-8

10. Machelska H, Mousa SA, Brack A, Schopohl JK, Rittner HL, Schäfer M, Stein C: Opioid control of inflammatory pain regulated by intercellular adhesion molecule-1. J Neurosci 2002; 22:5588-96

11. Rittner HL, Brack A, Machelska H, Mousa SA, Bauer M, Schäfer M, Stein C: Opioid peptide expressing leukocytes: Identification, recruitment, and simultaneously increasing inhibition of inflammatory pain. ANESTHESIOLOGY 2001; 95: 500-8

12. Semerad CL, Poursine-Laurent J, Liu F, Link DC: A role for G-CSF receptor signaling in the regulation of hematopoietic cell function but not lineage commitment or differentiation. Immunity 1999; 11:153-61

13. Nelson S, Summer W, Bagby G, Nakamura C, Stewart L, Lipscomb G, Andresen J: Granulocyte colony-stimulating factor enhances pulmonary host defenses in normal and ethanol-treated rats. J Infect Dis 1991; 164:901-6

14. Zhang P, Bagby GJ, Kolls JK, Welsh DA, Summer WR, Andresen J, Nelson S: The effects of granulocyte colony-stimulating factor and neutrophil recruitment on the pulmonary chemokine response to intratracheal endotoxin. J Immunol 2001; 166:458-65

15. Ulich TR, del Castillo J, McNiece IK, Yi ES, Alzona CP, Yin SM, Zsebo KM: Stem cell factor in combination with granulocyte colony-stimulating factor (CSF)

or granulocyte-macrophage CSF synergistically increases granulopoiesis in vivo. Blood 1991; 78:1954–62

16. Roeder I, de Haan G, Engel C, Nijhof W, Dontje B, Loeffler M: Interactions of erythropoietin, granulocyte colony-stimulating factor, stem cell factor, and interleukin-11 on murine hematopoiesis during simultaneous administration. Blood 1998; 91:3222–9

17. Middleton J, Patterson AM, Gardner L, Schmutz C, Ashton BA: Leukocyte extravasation: Chemokine transport and presentation by the endothelium. Blood 2002; 100:3853–60

18. Shibata F, Konishi K, Nakagawa H: Identification of a common receptor for three types of rat cytokine-induced neutrophil chemoattractants (CINCs). Cytokine 2001; 12:1368–73

19. Lum AF, Green CE, Lee GR, Staunton DE, Simon SI: Dynamic regulation of LFA-1 activation and neutrophil arrest on intercellular adhesion molecule 1 (ICAM-1) in shear flow. J Biol Chem 2002; 277:20660–70

20. Wagner JG, Roth RA: Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol Rev 2000; 52:349–74

21. Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983; 16:109–10

22. Schmitt TK, Mousa SA, Brack A, Schmidt DK, Rittner HL, Welte M, Schäfer M, Stein C: Modulation of peripheral endogenous opioid analgesia by central afferent blockade. ANESTHESIOLOGY 2003; 98:195–202

23. Brack A, Rittner HL, Younge BR, Kaltschmidt C, Weyand CM, Goronzy JJ: Glucocorticoid-mediated repression of cytokine gene transcription in human arteritis-SCID chimeras. J Clin Invest 1997; 99:2842–50

24. Foster PA, Wicks S, Foster M, Brain SD: Cellular pathology changes in rat skin following intradermal injection of nerve growth factor: Neutrophil-dependent and -independent events. J Pathol 2002; 197:245–55

25. Leonard TM, Klem SA, Asher MA, Rapoff MA, Leff RD: Relationship between pain severity and serum beta-endorphin levels in postoperative patients. Pharmacotherapy 1993; 13:378–81

26. Bragdon EE, Light KC, Costello NL, Sigurdsson A, Bunting S, Bhalang K, Maixner W: Group differences in pain modulation: Pain-free women compared to pain-free men and to women with TMD. Pain 2002; 96:227–37

27. Levine JD, Lau W, Kwiat G, Goetzl EJ: Leukotriene B4 produces hyperalgesia that is dependent on polymorphonuclear leukocytes. Science 1984; 225:743–5

28. Levine JD, Gooding J, Donatoni P, Borden L, Goetzl EJ: The role of the polymorphonuclear leukocyte in hyperalgesia. J Neurosci 1985; 5:3025–9

29. Bennett G, al-Rashed S, Hoult JR, Brain SD: Nerve growth factor induced hyperalgesia in the rat hind paw is dependent on circulating neutrophils. Pain 1998; 77:315–22

30. Ribeiro RA, Vale ML, Thomazzi SM, Paschoalato AB, Poole S, Ferreira SH, Cunha FQ: Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. Eur J Pharmacol 2000; 387:111–8

31. Vindrola O, Padros MR, Sterin-Prync A, Ase A, Finkielman S, Nahmod V: Proenkephalin system in human polymorphonuclear cells: Production and release of a novel 1.0-kD peptide derived from synenkephalin. J Clin Invest 1990; 86:531–7

32. Iavicoli S, Lopez-Perez E, Buehring GC, Thomas HA, Wei ET, Kishimoto T: Bipolar-shape response of human neutrophils to corticotropin-releasing factor. Eur J Pharmacol 1998; 349:301–6

33. Radulovic M, Dautzenberg FM, Sydow S, Radulovic J, Spiess J: Corticotropin-releasing factor receptor 1 in mouse spleen: Expression after immune stimulation and identification of receptor-bearing cells. J Immunol 1999; 162:3013–21

34. Cabot PJ, Carter L, Gaiddon C, Zhang Q, Schäfer M, Loeffler JP, Stein C: Immune cell-derived beta-endorphin: Production, release, and control of inflammatory pain in rats. J Clin Invest 1997; 100:142–8

35. Cabot PJ, Carter L, Schäfer M, Stein C: Methionine-enkephalin-and dynorphin A-release from immune cells and control of inflammatory pain. Pain 2001; 93:207–12

36. Leavey PJ, Sellins KS, Thurman G, Elzi D, Hiester A, Silliman CC, Zerbe G, Cohen JJ, Ambruso DR: In vivo treatment with granulocyte colony-stimulating factor results in divergent effects on neutrophil functions measured in vitro. Blood 1998; 92:4366–74

37. Spiekermann K, Roesler J, Emmendoerffer A, Elsner J, Welte K: Functional features of neutrophils induced by G-CSF and GM-CSF treatment: Differential effects and clinical implications. Leukemia 1997; 11:466–78

38. Steeber DA, Campbell MA, Basit A, Ley K, Tedder TF: Optimal selectin-mediated rolling of leukocytes during inflammation in vivo requires intercellular adhesion molecule-1 expression. Proc Natl Acad Sci U S A 1998; 95:7562–7

39. Hakansson L, Hoglund M, Jonsson UB, Torsteinsdottir I, Xu X, Venge P: Effects of in vivo administration of G-CSF on neutrophil and eosinophil adhesion. Br J Haematol 1997; 98:603–11

40. Ostergaard C, Benfield T, Gesser B, Kharazmi A, Frimodt-Moller N, Espersen F, Lundgren JD: Pretreatment with granulocyte colony-stimulating factor attenuates the inflammatory response but not the bacterial load in cerebrospinal fluid during experimental pneumococcal meningitis in rabbits. Infect Immun 1999; 67:3430–6

41. Dunstan CAN, Salafranca MN, Adhikari S, Xia Y, Feng L, Harrison JK: Identification of two rat genes orthologous to the human interleukin-8 receptors. J Biol Chem 1996; 271:32770–6

42. Frendeus B, Godaly G, Hang L, Karpman D, Lundstedt AC, Svanborg C: Interleukin 8 receptor deficiency confers susceptibility to acute experimental pyelonephritis and may have a human counterpart. J Exp Med 2000; 192:881–90

43. Rovai LE, Herschman HR, Smith JB: The murine neutrophil-chemoattractant chemokines LIX, KC, and MIP-2 have distinct induction kinetics, tissue distributions, and tissue-specific sensitivities to glucocorticoid regulation in endotoxemia. J Leukoc Biol 1998; 64:494–502

44. Amano H, Oishi K, Sonoda F, Senba M, Wada A, Nakagawa H, Nagatake T: Role of cytokine-induced neutrophil chemoattractant-2 (CINC-2) alpha in a rat model of chronic bronchopulmonary infections with Pseudomonas aeruginosa. Cytokine 2000; 12:1662–8

45. Shibata F, Konishi K, Kato H, Komorita N, al-Mokdad M, Fujioka M, Nakagawa H: Recombinant production and biological properties of rat cytokine-induced neutrophil chemoattractants, GRO/CINC-2 alpha, CINC-2 beta and CINC-3. Eur J Biochem 1995; 231:306–11

46. Frevert CW, Farone A, Danaee H, Paulauskis JD, Kobzik L: Functional characterization of rat chemokine macrophage inflammatory protein-2. Inflammation 1995; 19:133–42

47. Frevert CW, Huang S, Danaee H, Paulauskis JD, Kobzik L: Functional characterization of the rat chemokine KC and its importance in neutrophil recruitment in a rat model of pulmonary inflammation. J Immunol 1995; 154:335–44

48. Garcia-Ramallo E, Marques T, Prats N, Beleta J, Kunkel SL, Godessart N: Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. J Immunol 2002; 169:6467–73

49. Endlich B, Armstrong D, Brodsky J, Novotny M, Hamilton TA: Distinct temporal patterns of macrophage-inflammatory protein-2 and KC chemokine gene expression in surgical injury. J Immunol 2002; 168:3586–94

50. Ulich TR, del Castillo J, Yi ES, Yin S, McNiece I, Yung YP, Zsebo KM: Hematologic effects of stem cell factor in vivo and in vitro in rodents. Blood 1991; 78:645–50

51. Briddell RA, Hartley CA, Smith KA, McNiece IK: Recombinant rat stem cell factor synergizes with recombinant human granulocyte colony-stimulating factor in vivo in mice to mobilize peripheral blood progenitor cells that have enhanced repopulating potential. Blood 1993; 82:1720–3

52. Ogino M, Majima M, Kawamura M, Hatanaka K, Saito M, Harada Y, Katori M: Increased migration of neutrophils to granulocyte-colony stimulating factor in rat carrageenin-induced pleurisy: Roles of complement, bradykinin, and inducible cyclooxygenase-2. Inflamm Res 1996; 45:335–46

53. Price TH, Chatta GS, Dale DC: Effect of recombinant granulocyte colony-stimulating factor on neutrophil kinetics in normal young and elderly humans. Blood 1996; 88:335–40

54. Antonijevic I, Mousa SA, Schäfer M, Stein C: Perineurial defect and peripheral opioid analgesia in inflammation. J Neurosci 1995; 15:165–72

55. Hassan AH, Ableitner A, Stein C, Herz A: Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. Neuroscience 1993; 55:185–95

56. Schäfer M, Imai Y, Uhl GR, Stein C: Inflammation enhances peripheral mu-opioid receptor-mediated analgesia, but not mu-opioid receptor transcription in dorsal root ganglia. Eur J Pharmacol 1995; 279:165–9