Ccl2 and Ccl3 Mediate Neutrophil Recruitment via Induction of Protein Synthesis and Generation of Lipid Mediators

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Objective—Although the chemokines monocyte chemoattractant protein-1 (Ccl2/JE/MCP-1) and macrophage inflammatory protein-1α (Ccl3/MIP-1α) have recently been implicated in neutrophil migration, the underlying mechanisms remain largely unclear.

Methods and Results—Stimulation of the mouse cremaster muscle with Ccl2/JE/MCP-1 or Ccl3/MIP-1α induced a significant increase in numbers of firmly adherent and transmigrated leukocytes (>70% neutrophils) as observed by in vivo microscopy. This increase was significantly attenuated in mice receiving an inhibitor of RNA transcription (actinomycin D) or antagonists of platelet activating factor (PAF; BN 52021) and leukotrienes (MK-886; AA-861). In contrast, leukocyte responses elicited by PAF and leukotriene-B4 (LTB4) themselves were not affected by actinomycin D, BN 52021, MK-886, or AA-861. Conversely, PAF and LTB4, but not Ccl2/JE/MCP-1 and Ccl3/MIP-1α, directly activated neutrophils as indicated by shedding of CD62L and marked upregulation of CD11b. Moreover, Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited leakage of fluorescein isothiocyanate dextran as well as collagen IV remodeling within the venular basement membrane were completely absent in neutrophil-depleted mice.

Conclusions—Ccl2/JE/MCP-1 and Ccl3/MIP-1α mediate firm adherence and (subsequent) transmigration of neutrophils via protein synthesis and secondary generation of leukotrienes and PAF, which in turn directly activate neutrophils. Thereby, neutrophils facilitate basement membrane remodeling and promote microvascular leakage. (Arterioscler Thromb Vasc Biol. 2009;29:1787-1793.)

Key Words: leukocyte | migration | chemokines | permeability | basement membrane

L eukocyte recruitment from the microvasculature to sites of inflammation is a key event in both innate and adaptive immunity. In this process, a diversity of adhesion molecules, proteases, and chemokines are involved regulating the sequential steps of leukocyte rolling, firm adherence, and transmigration.1,2

Chemokines are small molecules (8 to 14 kDa) which can be classified into C, CC, CXC, and CX3C chemokines according to the arrangement of their N-terminal cysteine residues. Increased levels of chemokines and their respective receptors have been found in numerous pathological conditions. According to the current paradigm, chemokine receptors on circulating leukocytes are supposed to interact with chemokines presented on the venular endothelium. These interactions immediately activate leukocyte integrins which, in turn, facilitate firm adherence and transmigration of leukocytes.3–5

In the past years, particularly CC chemokines have been extensively studied in various inflammatory pathologies. Concluding from these studies, CC chemokines such as monocyte chemoattractant protein-1 (Ccl2/JE/MCP-1) and macrophage inflammatory protein-1α (Ccl3/MIP-1α) have been suggested to exclusively mediate the migration of monocytes and lymphocytes.3–5 However, there is a growing body of evidence that Ccl2/JE/MCP-1 and Ccl3/MIP-1α are also critically involved in the recruitment of neutrophils.6,7 The underlying mechanisms, however, remain largely unclear.

Recently, it has been reported that both Ccl2/JE/MCP-1 and Ccl3/MIP-1α are able to induce the release of lipid mediators such as leukotriene-B4 (LTB4).8–10 The functional relevance of endogenously generated lipid mediators including prostaglandins, leukotrienes, and PAF for each single step of the recruitment process of neutrophils elicited by Ccl2/JE/
MCP-1 and Ccl3/MIP-1α is not clear. Furthermore, the role of de novo protein synthesis in these responses has not yet been investigated.

In addition to leukocyte migration, Ccl2/JE/MCP-1 and Ccl3/MIP-1α have been implicated in the control of microvascular permeability. Moreover, Ccl3/MIP-1α has recently been demonstrated to induce remodeling of the perivascular basement membrane, a process which might promote microvascular leakage during inflammatory conditions. The contribution of neutrophils to these events, however, has not yet been studied.

Therefore, the objective of the present study was to analyze (1) the role of de novo protein synthesis and secondary generation of lipid mediators such as prostaglandins, leukotrienes, and PAF for each single step of the leukocyte recruitment process, and (2) the functional relevance of neutrophils for basement membrane remodeling as well as for the regulation of microvascular permeability in Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited inflammation.

Methods

To elucidate the mechanisms underlying neutrophil responses as well as changes in microvascular permeability elicited by the CC chemokines Ccl2/JE/MCP-1 and Ccl2/MIP-1α as well as the lipid mediators PAF and LTB4, in vivo microscopy on the cremaster muscle of anesthetized male BALB/c mice was performed 3 hours after intrascrotal application of the respective inflammatory stimuli. At the end of the in vivo test period, tissues were collected, fixed, and immunostained for collagen IV (confocal microscopy), CD45, Gr-1, or F4/80 (light microscopy). In addition, differential leukocyte counts as well as leakage of Evans blue were analyzed in the cremaster muscle treated with vehicle or actinomycin D (A; scale bar 20 μm). Leukocyte responses in mice treated with actinomycin D (B and D), BN 52021, MK-886, AA-861, indomethacin (C and E), or vehicle undergoing stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α (mean±SEM; n=6 per group; P<0.05 vs untreated; *P<0.05 vs vehicle; §P<0.05 vs indomethacin).

Figure 1. Ccl2/JE/MCP-1–stimulated postcapillary venules in the cremaster muscle treated with vehicle or actinomycin D (A; scale bar 20 μm). Leukocyte responses in mice treated with actinomycin D (B and D), BN 52021, MK-886, AA-861, indomethacin (C and E), or vehicle undergoing stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α (mean±SEM; n=6 per group; P<0.05 vs untreated; *P<0.05 vs vehicle; §P<0.05 vs indomethacin).

step of the leukocyte recruitment process, in vivo microscopy in the mouse cremaster muscle was used (Figure 1A). As is well known, the surgical preparation of the cremaster muscle induced leukocyte rolling in postcapillary venules. No significant differences were observed in numbers of rolling leukocytes among all experimental groups (data not shown). In contrast, after 3 hours of stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α, leukocyte firm adherence was significantly elevated as compared to unstimulated controls. This increase was almost completely abrogated in mice treated with actinomycin D as well as significantly decreased in mice receiving BN 52021, MK-886, or AA-861, respectively. Interestingly, Ccl2/JE/MCP-1– or Ccl3/MIP-1α–elicited firm adherence of leukocytes was not significantly altered in indomethacin-treated mice (Figure 1B and 1C).

In control animals, only few transmigrated leukocytes were found within the interstitial tissue. In contrast, stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α induced a significant elevation in numbers of transmigrated leukocytes as compared to unstimulated controls. This increase was almost completely abolished in animals receiving actinomycin D and significantly diminished in animals treated with BN 52021, MK-886, or AA-861, but was not altered in indomethacin-treated animals (supplemental Figure 1).

To further characterize the effect of actinomycin D, BN 52021, MK-886, AA-861, and indomethacin on each single
Phenotyping Transmigrated Leukocytes

To identify the phenotype of transmigrated leukocytes in the cremaster muscle, immunostaining for CD45 (common leukocyte antigen), Gr-1 (neutrophils/monocytes), and F4/80 (monocytes/macrophages) of cremasteric tissue samples was performed. In response to Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, or LTB₄, more than 80% of transmigrated leukocytes were positive for Gr-1 and ≈ 20% of transmigrated leukocytes were positive for F4/80, respectively (data not shown).

Phenotyping of leukocytes extravasated to the peritoneal cavity was performed by May-Grunwald-Giemsa staining of cell preparations from the peritoneal lavage. Three hours after stimulation with Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, or LTB₄ (73.8±6.2%; 83.3±3.2%; 82.8±3.9%; 87.6±3.7%, respectively), the majority of extravasated leukocytes were neutrophils. Only a small proportion of extravasated leukocytes was found to be monocytes/macrophages (supplemental Figures I and II).

Effect of Actinomycin D, BN 52021, MK-886, and AA-861 on Leukocyte Recruitment Elicited by PAF or LTB₄

Using in vivo microscopy in the mouse cremaster muscle, we further investigated the effect of actinomycin D, BN 52021, MK-886 (B and D), or vehicle undergoing stimulation with PAF and LTB₄ (mean±SEM; n=6 per group; #P<0.05 vs untreated).

**Figure 2.** Leukocyte responses in the cremaster muscle of mice treated with actinomycin D (A and C), BN 52021, MK-886 (B and D), or vehicle undergoing stimulation with PAF and LTB₄ (mean±SEM; n=6 per group; #P<0.05 vs untreated).

Effect of Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, and LTB₄ on Cell Surface Expression of CD11b/Mac-1 and CD62L/L-Selectin on Murine Neutrophils

The effect of Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, and LTB₄ (1 and 100 ng ml⁻¹, respectively) on the expression of the adhesion molecules CD11b/Mac-1 and CD62L/L-selectin on murine neutrophils was analyzed by flow cytometry (Figure 3; supplemental Table II). Incubation with Ccl2/JE/MCP-1 did not significantly alter the expression of CD11b/Mac-1 and CD62L/L-selectin on the surface of neutrophils. Stimulation with Ccl3/MIP-1α only weakly increased expression of CD11b/Mac-1 (significant only at 100 ng) while shedding of CD62L/L-selectin was not altered. In contrast, incubation with PAF (significant only at 100 ng) or with LTB₄ dose-dependently enhanced cell surface expression of CD11b/Mac-1 and shedding of CD62L/L-selectin (significant at 100 ng). The potent neutrophil stimulant PMA (10 ng ml⁻¹) elicited a similar profile of responses to that detected with PAF and LTB₄.

Effect of Cromolyn on Leukocyte Recruitment as Well as on Expression of E-Selectin Elicited by Ccl2/JE/MCP-1 and Ccl3/MIP-1α

Activated tissue mast cells in the mouse cremaster muscle are predominantly located in close proximity to postcapillary venules (Figure 4A). Therefore, the effect of an inhibitor of mast cell degranulation, cromolyn, on Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited leukocyte responses was evaluated in the cremaster muscle. Although no significant differences were detected in numbers of rolling leukocytes among experimental groups (data not shown), the Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited elevation in firm adherence and (subsequent) transmigration of neutrophils was almost completely abolished in cromolyn-treated animals (Figure 4B). Because the cremaster muscle is suggested to contain a low total number of mast cells as compared to other tissues, effects of cromolyn on Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited
neutrophil responses were also analyzed in the peritonitis assay. Similarly to our previous results, Ccl2/JE/MCP-1– and Ccl3/MIP-1α–elicited extravasation of leukocytes to the peritoneal cavity was significantly diminished in cromolyn-treated animals (supplemental Figure I).

Role of Neutrophils for Ccl2/JE/MCP-1– and Ccl3/MIP-1α–Elicited Microvascular Permeability

As a measure of microvascular permeability, leakage of FITC dextran to cremasteric tissue was determined. Stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α induced a significant elevation in the leakage of FITC dextran as compared to unstimulated controls, respectively (Figure 5A through 5C). This elevation of FITC dextran leakage was significantly diminished in animals treated with BN 52021 and almost completely abolished after treatment with MK-886 (Figure 5B) or actinomycin D (Figure 5A).

In addition, leakage of FITC dextran to the cremaster muscle was analyzed in neutrophil-depleted animals. After 3 hours of stimulation with Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, or LTB4, there was a significant increase in the leakage of FITC dextran to the perivascular tissue in animals treated with an isotype control antibody as compared to unstimulated controls (Figure 5C and 5D). This increase was completely abolished in animals treated with an anti–Gr-1 antibody.

In separate experiments, leakage of Evans blue into the peritoneal cavity was analyzed. In response to Ccl2/JE/MCP-1– or Ccl3/MIP-1α–elicited Evans blue leakage was significantly reduced in mice rendered neutropenic by using either the anti–Gr-1-antibody RB6–8C5 or the anti–Ly-6G antibody 1A8 (supplemental Figure III).

Role of Neutrophils for Ccl2/JE/MCP-1– and Ccl3/MIP-1α–Elicited Collagen IV Remodeling

To investigate the expression profile of collagen IV within the perivascular basement membrane, immunofluorescence...
Neutrophil Recruitment by CC Chemokines

Reichel et al

Discussion

In the past years, the role of CC chemokines has been extensively studied in numerous pathological conditions. As a result of these studies, CC chemokines such as Ccl2/JE/MCP-1 and Ccl3/MIP-1α have been widely implicated in the recruitment of monocytes and lymphocytes, but not of neutrophils.3–5 Interestingly, however, there is a growing body of in vivo evidence that Ccl2/JE/MCP-1 and Ccl3/MIP-1α are also critically involved in the recruitment of neutrophils.6–7 The underlying mechanisms remained largely unclear.

In the present study, the role of de novo synthesis of proteins for rolling, firm adherence, and transmigration of neutrophils in response to Ccl2/JE/MCP-1- and Ccl3/MIP-1α was analyzed by using the RNA transcription inhibitor actinomycin D. Our in vivo data demonstrate that coadministration of actinomycin D with either Ccl2/JE/MCP-1 or Ccl3/MIP-1α almost completely abolished firm adherence and (subsequent) transmigration of neutrophils, indicating that Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited neutrophil recruitment is strictly dependent on de novo generation of proteins.

Proteins controlling neutrophil recruitment in response to Ccl2/JE/MCP-1 and Ccl3/MIP-1α might include adhesion molecules, proteases, and cytokines. Ccl2/JE/MCP-1 and Ccl3/MIP-1α are suggested to induce the expression of phospholipase A₂, cyclooxygenase, 5-lipooxygenase, or lysopAF acetyl transferase, enzymes facilitating the synthesis of lipid mediators including prostaglandins, leukotrienes, and PAF.13–16 Therefore, the involvement of endogenously generated prostaglandins, leukotrienes, and PAF in Ccl2/JE/MCP-1- and Ccl3/MIP-1α-dependent neutrophil responses was analyzed by using pharmacological antagonists of these lipid mediators, respectively. Here, we show that sequentially generated leukotrienes and PAF play crucial roles for Ccl2/JE/MCP-1- and Ccl3/MIP-1α-induced firm adherence and (subsequent) transmigration of neutrophils. Interestingly, blockade of prostaglandin synthesis had no effect on Ccl2/JE/MCP-1- and Ccl3/MIP-1α-elicited neutrophil responses. Previously, it has been reported that generation of LTBA₂ requires the presence of Ccl2/JE/MCP-1 and Ccl3/MIP-1α in the inflamed peritoneal and pleural cavity.9,10 Moreover, the number of neutrophils in the peritoneal exudate of Ccl3/MIP-1α-stimulated mice was found significantly reduced on blockade of leukotrienes.8 Hence, the above in vivo results strongly suggest that Ccl2/JE/MCP-1 and Ccl3/MIP-1α initiate an inflammatory cascade mediating intravascular adherence and (subsequent) transmigration of neutrophils through induction of de novo protein synthesis and an endogenous generation of lipid mediators including leukotrienes and PAF.

To further elucidate the mechanisms underlying Ccl2/JE/MCP-1- and Ccl3/MIP-1α-dependent neutrophil recruitment, we differentially analyzed effects of these CC chemokines on potential target cells. Previously, chemokine receptors Ccr2, a receptor of Ccl2/JE/MCP-1, and Ccr5, a

Neutrophil Depletion

To assure efficacy and specificity of neutrophil depletion by using the anti–Gr-1 antibody RB6–8C5 or the anti–Ly-6G antibody 1A8, differential blood leukocyte counts were analyzed. Treatment with RB6–8C5 as well as with 1A8 significantly diminished the number of circulating neutrophils without affecting systemic monocyte counts (supplemental Table I).

Systemic Leukocyte Counts and Microhemodynamic Parameters

To assure intergroup comparability, quantitative analysis of inner vessel diameters, blood flow velocities, and shear rates of analyzed postcapillary venules as well as of systemic leukocyte counts was performed. No significant differences were detected among all experimental groups (supplemental Table I).

Staining and confocal laser scanning microscopy were performed in tissue samples of the cremaster muscle. In unstimulated control animals, a discontinuous expression of collagen IV was detected in postcapillary venules (Figure 6A). Analysis of intensity profiles demonstrated regions of low fluorescence intensity (less than 60% of average fluorescence intensity/unit area of the entire vessel segment). These low expression (LE) sites were detected at a density of 7.3±0.6 µm² (Figure 6B). Interestingly, stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α did not significantly alter the average density of these collagen IV LE sites. In contrast, both CC chemokines significantly enlarged the average site size of collagen IV LE sites (13.6±0.6 µm²; 13.3±1.1 µm²). This increase was completely abolished in neutrophil-depleted mice, respectively (7.1±0.4 µm²; 6.9±0.3 µm²).

Figure 6. Cremasteric postcapillary venule immunostained for collagen IV. White rings show LE sites (A; scale bar 10 µm). Size (B) and density (C) of LE sites in mice treated with anti–Gr-1-antibody or control antibody after stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α (mean±SEM; n=4 per group; #P<0.05 vs untreated; *P<0.05 vs control mAB).
receptor of Ccl3/MIP-1α have been identified on the surface of native murine neutrophils isolated from the peripheral blood. Incubation of neutrophils with Ccl2/JE/MCP-1 and Ccl3/MIP-1α did not critically alter surface expression of CD11b/Mac-1 and CD62L/L-selectin, 2 adhesion molecules commonly used as activation markers of neutrophils. These data suggest that both Ccl2/JE/MCP-1 and Ccl3/MIP-1α are not significantly involved in the direct activation of murine neutrophils. Noteworthy, it cannot be excluded that interactions between Ccl2/JE/MCP-1 as well as Ccl3/MIP-1α and their respective chemokine receptors on neutrophils might lead to alterations in the affinity state of neutrophil integrins as it has been demonstrated for different leukocyte subsets. Interestingly, the chemokine receptors Ccr1, another receptor of Ccl3/MIP-1α, and Ccr2 have also been identified on endothelial cells. Stimulation of the cremaster muscle with both Ccl2/JE/MCP-1 and Ccl3/MIP-1α markedly increased the expression of CD62E/E-selectin indicating that these CC chemokines are able to activate endothelial cells in vivo. Moreover, tissue mast cells have recently been demonstrated to express chemokine receptors Ccr1, Ccr2, and Ccr5. Mast cells are able to generate a number of proinflammatory mediators, and it has been shown that this cell population plays a critical role in CC chemokine-dependent neutrophil migration. Here, we demonstrate that the Ccl2/JE/MCP-1– and Ccl3/MIP-1α–elicited activation of endothelial cells as well as the (subsequent) firm adherence and transmigration of neutrophils is strictly dependent on the preceding activation of mast cells. Consequently, Ccl2/JE/MCP-1 and Ccl3/MIP-1α might trigger extravasation of neutrophils in vivo indirectly via intermediary activation of mast cells, subsequent activation of endothelial cells (and possibly other cells), induction of protein synthesis, and generation of further proinflammatory mediators including leukotrienes and PAF.

To broader characterize the inflammatory cascade initiated by Ccl2/JE/MCP-1 and Ccl3/MIP-1α, we also analyzed whether LTβ4, which is suggested to be the most potent neutrophil attractant among the leukotrienes, and PAF themselves mediate neutrophil recruitment via a further generation of proinflammatory mediators. In contrast to our previous results for Ccl2/JE/MCP-1 and Ccl3/MIP-1α, blockade of protein synthesis as well as antagonism of leukotrienes and PAF had no effect on neutrophil responses elicited by either PAF or LTβ4. Conversely, however, both PAF and LTβ4 were able to directly activate neutrophils as indicated by rapid shedding of CD62L/L-selectin and marked upregulation of CD11b/Mac-1. Collectively, these data suggest that PAF– as well as LTβ4–elicited firm adherence and (subsequent) transmigration of neutrophils are, at least partially, the result of a direct activation of neutrophils and do not require further protein synthesis or generation of proinflammatory mediators such as leukotrienes or PAF. Consequently, PAF– as well as LTβ4–dependent firm adherence and (subsequent) transmigration of neutrophils represent later steps in the inflammatory cascades elicited by Ccl2/JE/MCP-1 and Ccl3/MIP-1α.

From a different perspective, our in vitro and in vivo findings reveal key differences in the mechanisms of action of lipid mediators (PAF, LTβ4) and of CC chemokines (Ccl2/JE/MCP-1, Ccl3/MIP-1α). Interestingly, a similar divergence in the mechanisms of action has been described for the cytokines IL-1β and TNF-α. Furthermore, it has been demonstrated that several inflammatory mediators including IL-1β, TNF-α, Ccl3/MIP-1α, PAF, and LTβ4 differentially implement adhesion molecules as well as proteases for the recruitment of neutrophils. In this context, our findings might also contribute to a better understanding of the mechanisms underlying the stimulus-specific regulation of the leukocyte recruitment process by uncovering divergent molecular and cellular targets of lipid mediators (PAF, LTβ4) and CC chemokines (Ccl2/JE/MCP-1, Ccl3/MIP-1α).

In addition to neutrophil recruitment, Ccl2/JE/MCP-1 and Ccl3/MIP-1α have recently been implicated in the regulation of microvascular permeability. The underlying mechanisms are not fully understood. Previous in vitro data suggest that Ccl2/JE/MCP-1 exerts direct effects on endothelial cells by inducing redistribution of endothelial tight junctions. Our in vivo data clearly demonstrate that the Ccl2/JE/MCP-1– and Ccl3/MIP-1α–induced microvascular leakage strictly requires protein synthesis, endogenous generation of leukotrienes as well as PAF, and, above all, the presence of neutrophils. Moreover, microvascular leakage elicited by PAF and LTβ4 themselves has also been found to be neutrophil-dependent indicating that in Ccl2/JE/MCP-1– and Ccl3/MIP-1α–elicited inflammation secondarily generated lipid mediators induce extravasation of neutrophils which, in turn, promote the microvascular leakage. Thereby, transmigrating neutrophils are suggested to disrupt endothelial junctions as well as to degrade the perivascular basement membrane as it has been supposed for different inflammatory reactions.

In this context, regions within the venular basement membrane have been identified where the expression of distinct basement membrane components such as collagen IV is significantly lower than the average vascular level. Our confocal microscopic findings demonstrate that the average size of these collagen IV low-expression (LE) sites is significantly enlarged in response to both Ccl2/JE/MCP-1 and Ccl3/MIP-1α. These data are in line with previous findings under different inflammatory conditions suggesting a general inflammatory phenomenon that contributes to microvascular leakage in the acute inflammatory response. However, the mechanisms underlying Ccl2/JE/MCP-1– and Ccl3/MIP-1α–dependent remodeling processes within the venular basement membrane are poorly understood. While we were preparing this manuscript, a study has shown that neutrophils facilitate remodeling events within the perivascular basement membrane in Ccl2/JE/MCP-1–elicited inflammation. Here, we extend these observations by demonstrating that neutrophils are critically involved in the enlargement of perivascular collagen IV LE sites not only in Ccl2/JE/MCP-1– but also in Ccl3/MIP-1α–dependent inflammatory responses. Previously, neutrophils have been shown to mediate collagen IV remodeling in response to the cytokine IL-1β implicating neutrophil-derived proteases such as neutrophil elastase or gelatinases to facilitate remodeling events within the venular vessel wall.
In conclusion, our data suggest that the CC chemokines Ccl2/JE/MCP-1 and Ccl3/MIP-1α initiate an inflammatory cascade regulating firm adherence and (subsequent) transmigration of neutrophils through activation of mast cells, induction of de novo protein synthesis, and a sequential release of lipid mediators including leukotrienes and PAF. In this cascade, LTB4 and PAF are thought to directly activate neutrophils facilitating firm adherence and (subsequent) transmigration of neutrophils independently of further protein synthesis or endogenous generation of proinflammatory mediators such as leukotrienes or PAF. Thereby, neutrophils disrupt the perivenular basement membrane and provoke microvascular leakage. These data highlight the significance of CC chemokines for neutrophil recruitment in vivo and provide new insights into underlying mechanisms and resulting consequences.

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Disclosures
None.

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Supplement material

Supplementary methods

Animals

Male BALB/c mice were purchased from Charles River (Sulzfeld, Germany). All experiments were performed with male mice at the age of 10 – 12 weeks. Animals were housed under conventional conditions with free access to food and water. All experiments were performed according to German legislation for the protection of animals and approved by the local government authorities.

M. cremaster assay

Surgical procedure

The surgical preparation was performed as originally described by Baez with minor modifications \(^1,^2\). Mice were anesthetized using a ketamine/xylazine mixture (100 mg/kg ketamine and 10 mg/kg xylazine), administered by intra-peritoneal injection. The right carotid and the left femoral artery were cannulated in a retrograde manner for continuous blood pressure monitoring and for administration of microspheres and drugs (see below). The right cremaster muscle was exposed through a ventral incision of the scrotum. The muscle was opened ventrally in a relatively avascular zone, using careful electrocautery to stop any bleeding, and spread over the transparent pedestal of a custom-made microscopic stage. Epididymis and testicle were detached from the cremaster muscle and placed into the abdominal cavity. Throughout the procedure as well as after surgical preparation during \textit{in vivo} microscopy, the muscle was superfused with warm-buffered saline.
In vivo microscopy

The setup for in vivo microscopy was centered around an Olympus BX 50 upright microscope (Olympus Microscopy, Hamburg, Germany), equipped for stroboscopic fluorescence epifluorescence microscopy. Light from a 75-W xenon source was narrowed to a near-monochromatic beam of a wavelength of 700 nm by a galvanometric scanner (Polychrome II, TILL Photonics, Graefelfing, Germany) and directed onto the specimen via a FITC filter cube equipped with dichroic and emission filters (DCLP 500, LP515, Olympus). Microscopic images were obtained with Olympus water immersion lenses [20x/numerical aperture (NA) 0.5 and 10x/NA 0.3] and recorded with an analog black-and-white charge-coupled device (CCD) video camera (Cohu 4920, Cohu, San Diego, CA, USA) and an analog video recorder (AG-7350-E, Panasonic, Tokyo, Japan). Oblique illumination was obtained by positioning a mirroring surface (reflector) directly below the specimen and tilting its angle relative to the horizontal plane. The reflector consisted of a round cover glass (thickness, 0.19–0.22 mm; diameter, 11.8 mm), which was coated with aluminum vapor (Freichel, Kaufbeuren, Germany) and brought into direct contact with the overlying specimen as described previously ². For measurement of centerline blood flow velocity, green fluorescent microspheres (2.0 µm diameter, Molecular Probes, Leiden, The Netherlands) were injected via the femoral artery catheter, and their passage through the vessels of interest was recorded using the FITC filter cube under appropriate stroboscopic illumination (exposure, 1 ms; cycle time, 10 ms; \( \lambda = 488 \) nm), integrating video images for sufficient time (>80 ms) to allow for the recording of several images of the same bead on one frame. Beads that were flowing freely along the centerline of the vessels were used to determine blood flow velocity (see below).
Experimental protocols

Leukocyte recruitment to the cremaster muscle was induced by intra-scrotal injection of Ccl2/JE/MCP-1, Ccl3/MIP-1α [300 ng in 0.4 ml PBS, R&D Systems Europe Ltd., Wiesbaden, Germany], PAF, or LTB₄ (0.4 ml of 10⁻⁶ M solutions; Sigma Aldrich, Deisenhofen, Germany). After 180 min, five vessel segments were randomly chosen in a central area of the spread-out cremaster muscle among those that were at least 150 µm away from neighboring postcapillary venules and did not branch over a distance of at least 150 µm. After having obtained recordings of migration parameters, blood flow velocity was determined as described above. Subsequently, FITC dextran was infused intra-arterially (i.a.) for the analysis of microvascular permeability (see below). After in vivo microscopy, tissue samples of the cremaster muscle were taken for immunohistochemistry (see below). Blood samples were collected by cardiac puncture for the determination of systemic leukocyte counts using a Coulter ACT Counter (Coulter Corp., Miami, FL, USA). Anesthetized animals were then killed by bleeding to death.

Quantification of leukocyte kinetics and microhemodynamic parameters

For off-line analysis of parameters describing the sequential steps of leukocyte extravasation, we used the Cap-Image image analysis software (Dr. Zeintl, Heidelberg, Germany). Rolling leukocytes were defined as those moving slower than the associated blood flow and quantified for 30 s. Firmly adherent cells were determined as those resting in the associated blood flow for more than 30 s and related to the luminal surface per 100 µm vessel length. Transmigrated cells were counted in regions of interest (ROI), covering 75 µm on both sides of a vessel over 100 µm vessel length. By measuring the distance between several images of one fluorescent bead under stroboscopic illumination, centerline blood flow velocity was determined. From measured vessel diameters and centerline blood flow velocity, apparent wall shear stress was calculated, assuming a parabolic flow velocity profile over the vessel cross section.
Microvascular permeability

For the analysis of microvascular permeability, the macromolecule FITC dextran (5 mg in 0.1 ml saline, Mr 150,000, Sigma-Aldrich) was infused intra-arterially after determination of centerline blood flow velocity (see above). Five postcapillary vessel segments as well as the surrounding perivascular tissue were excited at 488 nm, and emission >515 nm was recorded by a CCD camera (Sensicam, PCO, Kelheim, Germany) 30 min after injection of FITC dextran using an appropriate emission filter (LP 515). Mean gray values of fluorescence intensity were measured by digital image analysis (TILLvisION 4.0, TILL Photonics) in six randomly selected ROIs (50x50 µm²), localized 50 µm distant from the postcapillary venule under investigation.

Peritonitis assay

Under inhalation anesthesia with isoflurane-N₂O, leukocyte recruitment to the peritoneal cavity was induced by intra-peritoneal injection of Ccl2/JE/MCP-1, Ccl3/MIP-1α (300 µg in 1.0 ml PBS; R&D Systems Europe Ltd.), PAF, or LTB₄, (1.0 ml of 10⁻⁶ M solutions; Sigma Aldrich). Three hours after induction of peritoneal inflammation, mice were sacrificed, and their peritoneal cavity was washed with 15 ml of ice-cold PBS. The total number of leukocytes recovered from the peritoneal lavage fluid was analyzed by using a Coulter A C T counter (Coulter Corp.) and differential leukocyte counts were determined as indicated below. As a measure of microvascular permeability, leakage of Evans blue into the peritoneal cavity was analyzed in a separate set of experiments. For this purpose, Evans blue (6.25 mg ml⁻¹ in 200 µl of PBS) was administered intra-arterially 10 min prior to administration of Ccl2/JE/MCP-1 or Ccl3/MIP-1α. Three hours after induction of inflammation, mice were sacrificed, and their peritoneal cavity was washed with 7.5 ml of ice-cold PBS. The cells were spun down and as an indicator of Evans blue leakage into the peritoneal cavity, the optical
density (OD) of the supernatant at 620 nm was measured using a multiplate reader (Infinite F200, Tecan Group Ltd., Männedorf, Switzerland)

**Experimental groups**

**M. cremaster assay:** Control mice with an intra-scrotal (i.s.) injection of PBS as well as mice receiving either actinomycin D, BN 52021, MK-886, AA-861, indomethacin, cromolyn or respective drug vehicle undergoing stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α (see below; n=6 each group). Additional experiments were performed in mice receiving actinomycin D, MK-886, or respective drug vehicle undergoing stimulation with PAF as well as in mice receiving actinomycin D, BN 52021, or respective drug vehicle undergoing stimulation with LTB₄ (see below; n=6 each group). Finally, experiments were carried out in mice pretreated with the monoclonal anti-Gr-1 antibody RB6-8C5 or isotype control antibody undergoing stimulation with Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, or LTB₄ (n=4 each group).

**Peritonitis assay:** Control mice with an intra-peritoneal (i.p.) injection of PBS as well as mice receiving either actinomycin D, cromolyn, BN 52021, MK-886, AA-861, indomethacin, or respective drug vehicle undergoing stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α (see below; n=4 each group). Additional experiments were performed in mice receiving actinomycin D, AA-861, or respective drug vehicle undergoing stimulation with PAF as well as in mice receiving actinomycin D, BN 52021, or respective drug vehicle undergoing stimulation with LTB₄ (see below; n=5 each group). Finally, experiments were carried out in mice pretreated with the neutrophil-depleting monoclonal anti-Gr-1 antibody RB6-8C5, the neutrophil-depleting monoclonal anti-Ly-6G antibody A18, or isotype control antibody undergoing stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α (n=4 each group).
Reagents

1A8 (Neutrophil-depleting anti-Ly-6G mAB; 100 µg i.p.; 24 h prior to induction of inflammation; BD Biosciences, San Jose, CA, USA). AA-861 (5-LO antagonist; 10 mg/kg i.a.; 15 min prior to induction of inflammation; Sigma-Aldrich). Actinomycin D [Inhibitor of RNA transcription; 0.2 mg/kg intra-scrotally (i.s.) or intra-peritoneally (i.p.); co-administered with inflammatory mediators; Sigma-Aldrich]. BN 52021 (PAF receptor antagonist; 20 mg/kg i.a.; 15 min prior to induction of inflammation; Sigma-Aldrich). Indomethacin (COX inhibitor; 4 mg/kg i.a.; 15 min prior to induction of inflammation; Sigma-Aldrich). MK-886 (FLAP/PPARα antagonist; 1 mg/kg i.a.; 15 min prior to induction of inflammation; Sigma-Aldrich). RB6-8C5 (Neutrophil-depleting ant-Gr-1 mAB; 150 µg i.p.; 24 h prior to induction of inflammation; BD Biosciences). Sodium cromoglycate (cromolyn; inhibitor of mast cell degranulation; 200 mg/kg i.a.; 15 min prior to induction of inflammation; Sigma-Aldrich). Control animals received corresponding drug vehicle/isotype-matched control antibody.

Phenotyping transmigrated leukocytes

To determine the phenotype of transmigrated leukocytes in the cremaster muscle, immunostaining of paraffin-embedded serial tissue sections of the cremaster muscle was performed. Sections were incubated with primary rat anti-mouse anti-Gr-1, anti-CD45 (BD Biosciences, San Jose, CA, USA), or anti-F4/80 (Serotec, Oxford, UK) IgG antibodies. Afterwards, the paraffin sections were stained with commercially available immunohistochemistry kits (Gr-1, CD45, Super Sensitive Link-Label IHC detection system, BioGenex, San Ramon, CA, USA; F4/80, Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA), obtaining an easily detectable reddish or brownish endproduct, respectively. Finally, the sections were counterstained with Mayer’s hemalaun. The number of extravascularly localized Gr-1-, CD45-, or F4/80-positive cells was quantified by light microscopy (magnification 400x) on three sections (10 observation fields per section) from six
individual animals per experimental group in a blinded manner, respectively. The number of transmigrated Gr-1-positive cells (neutrophils and some monocyte subsets) and F4/80-positive cells (monocytes/macrophages) is expressed as the percentage of total CD45-positive leukocytes.

To determine the phenotype of leukocytes extravasated to the peritoneal cavity, slide preparations were made in a cytocentrifuge (Cytospin, Frankfurt, Germany) using 200 µl of the peritoneal lavage fluid by spinning at 500 rpm for 5 min. Slides were stained (May-Gruenwald-Giemsa) and cell differentiation was made using an oil immersion microscope.

**Histochemical staining**

To assess the localization of activated mast cells in the mouse cremaster muscle, ruthenium red staining was performed. Briefly, Ccl3/MIP-1α-stimulated cremaster muscles were superfused for 60 min with a 0.001 % solution of ruthenium red (Sigma Aldrich) and mounted in PermaFluor (Beckman Coulter).

**Confocal microscopy**

For the analysis of collagen IV expression, cremaster muscles were fixed in 4% paraformaldehyde. Tissues were then blocked and permeabilized in PBS, supplemented with 10% goat serum (Sigma-Aldrich) and 0.5% Triton X-100 (Sigma-Aldrich). After incubation with the primary rabbit anti-mouse collagen IV polyclonal antibody (Abcam, Cambridge, UK) at room temperature for 12 h, tissues were incubated with the secondary Alexa Fluor 488-linked goat anti-rabbit (Invitrogen, Carlsbad, CA, USA) antibody for 3 h at room temperature. Immunostained tissues were mounted in PermaFluor (Beckman Coulter, Fullerton, CA, USA) on glass slides and observed using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with an oil immersion lens (Leica; 40x/NA 1.25–0.75). Optical sections of tissue samples through the whole depth of the tissue were obtained using,
as far as possible, the same settings for all samples analyzed. Z-stack digital images were collected optically at every 0.5 µm depth and applied to three-dimensional (3D) reconstruction analysis using Leica Application Suite software. Three-dimensional images of vessels were split in the middle along the longitudinal axis and images of “semi-vessels” were analyzed for fluorescence intensity measurements. To investigate the expression profile of collagen IV within immunostained samples, intensity profiles of regions of interest were measured and compared with the average intensity of the entire vessel within the same field of view. For this purpose, regions of interest (immunoreactive and non-immunoreactive) within 3D images of semi-vessels were identified manually in a blinded manner and their area and density were measured using the Leica Application Suite software. Collagen IV LE sites were defined as those regions in which the average fluorescence intensity/unit area was less than 60% of the average fluorescence intensity in the whole vessel segment under investigation. LE sites from three vessel segments/tissue (n=4 mice per group) were analyzed. LE site size was determined using Leica Application Suite software, and LE site density was calculated for the total surface area of the semi-vessels.

Flow cytometry

To analyze the effect of CC chemokines and lipid mediators on activation of native murine neutrophils, anticoagulated whole blood samples were incubated (20 min; room temperature) with different concentrations (1 and 100 ng ml\(^{-1}\)) of Ccl2/JE/MCP-1, Ccl3/MIP-1\(\alpha\), PAF, and LTB\(_4\) as well as PMA (1 ng ml\(^{-1}\)) as a positive control and with PBS used as vehicle control. After washing, samples were incubated with primary monoclonal antibodies directed against either CD11b/Mac-1 or CD62L/L-selectin (BD Biosciences) on ice. Isotype-matched controls (BD Biosciences) were also used in all experiments. After lysis of erythrocytes, stained cells were analyzed on a flow cytometer (FACSort, Becton Dickinson, San Jose, CA). Neutrophils,
monocytes, and lymphocytes were identified by their light-scatter properties and expression of CD11b/Mac-1. Approximately 10,000 gated events were collected in each analysis.

**RT-PCR**

Cremaster muscles were divided into two tissue samples and total RNA contents were extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturers’ instruction manual. Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Hamburg, Germany). Real-time PCR was conducted with the TaqMan® Universal Master Mix Kit in a 7300 Real-Time PCR System (all Applied Biosystems, Hamburg, Germany). The following primers and probes (Biomers, Ulm, Germany) were used: E-selectin forward: 5’-caac gct caa ggt tca aaa caa tca g-3’. E-selectin reverse: 5’-tta agc agg caa gag gaa cca -3’ (bp 1-2912; gene bank accession number NM_011345); E-selectin TaqMan probe: 5’- FAM –cac aaa tgc atc gtg gga - BHQ-3’; GAPDH forward: 5’-tgc agt ggc aaa gtg gag at-3’. GAPDH reverse: 5’- tgc cgt gag tgg agt cat act-3’ (bp 1-1254; gene bank accession number NM_008084); GAPDH TaqMan probe: 5’- FAM –cca tca aeg acc cct tca ttg acc tc- BHQ-3’. GAPDH was used as internal housekeeping gene. Content of mRNA is expressed as x-fold increase versus control.

**Statistics**

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Unless otherwise stated, the ANOVA on ranks test followed by the Student-Newman-Keuls test was used for the estimation of stochastic probability in intergroup comparisons. Mean values and SEM are given. *P* values <0.05 were considered significant.
### Supplementary data

#### Table I

<table>
<thead>
<tr>
<th></th>
<th>Systemic leukocyte counts [10^6 µl⁻¹]</th>
<th>Inner diameter [µm]</th>
<th>V_b [mm s⁻¹]</th>
<th>shear rate [s⁻¹]</th>
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<tbody>
<tr>
<td><strong>control</strong></td>
<td>4.2 ± 0.6</td>
<td>22.4 ± 0.5</td>
<td>1.3 ± 0.1</td>
<td>2365.7 ± 117.4</td>
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<td><strong>Ccl2/JE/MCP-1 vehicle</strong></td>
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<td>1.3 ± 0.1</td>
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<td>Act. D vehicle</td>
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<td><strong>MK-886</strong></td>
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<td><strong>PAF</strong></td>
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Table II

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<th>CD11b</th>
<th>CD62L</th>
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<td>1383.0 ± 29.9 *</td>
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Table III

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<tr>
<th></th>
<th>Systemic leukocyte counts [10^6 µl⁻¹]</th>
<th>Neutrophils [%]</th>
<th>Monocytes [%]</th>
<th>Lymphocytes [%]</th>
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<td>1A8</td>
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<td>0.9 ± 0.4</td>
<td>5.4 ± 0.5</td>
<td>93.7 ± 0.8</td>
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</table>
Figure I

A

Leukocytes per cavity [n x 10^6]

B

Neutrophils per cavity [n x 10^6]

C

MN per cavity [n x 10^6]
Figure II

A

[Graph showing Leukocytes per cavity with bars for unstimulated, vehicle, actinomycin D, AA-861, and BN 52021 under PAF and LTB₄ stimulation.]

B

[Graph showing Neutrophils per cavity with bars for PAF and LTB₄ stimulation.]

C

[Graph showing MN per cavity with bars for PAF and LTB₄ stimulation.]
Figure III

![Graph showing OD at 620 nm for Ccl2 and Ccl3 under different conditions: unstimulated, control mAb, RB6-8C5, and 1A8. The graph includes error bars and symbols indicating statistical significance.]

- OD at 620 nm values for Ccl2 and Ccl3
- Control mAb, RB6-8C5, and 1A8 treatments
- Unstimulated control
Supplementary table and figure legends

**Table I.** Systemic blood leukocyte counts as well as microhemodynamic parameters including inner vessel diameter, blood flow velocity, and wall shear rate were obtained as detailed in *Supplementary Methods* (mean ± SEM for *n* = 4 – 6 per group).

**Table II.** Flow cytometric analysis of expression of adhesion molecules CD11b/Mac-1 and CD62L/L-selectin on native murine neutrophils isolated from the peripheral blood undergoing stimulation with either PMA, Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, LTB₄, or vehicle (mean±SEM for *n*=4 per group; ANOVA on ranks followed by Dunnett test; * p<0.05, vs. control).

**Table III.** Differential blood leukocyte counts from mice treated with RB6-8C5, 1A8, or isotype control antibody were obtained by flow cytometry as detailed in *Supplementary Methods* (mean±SEM for *n*=3 – 4 per group).

**Figure I.** Recruitment of total leukocytes (A), neutrophils (B), and mononuclear cells (MN; C) to the peritoneal cavity of unstimulated control mice as well as of mice treated with actinomycin D, cromolyn, BN 52021, MK-886, AA-861, indomethacin, or drug vehicle undergoing stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α (mean±SEM for *n*=4 per group; ANOVA on ranks followed by Dunnett test; # p<0.05, vs. vehicle).

**Figure II.** Recruitment of total leukocytes (A), neutrophils (B), and mononuclear cells (MN; C) to the peritoneal cavity of unstimulated control mice as well as of mice treated with BN 52021, AA-861, or drug vehicle undergoing stimulation with PAF or LTB₄ (mean±SEM for *n*=5 per group; ANOVA on ranks followed by Dunnett test; # p<0.05, vs. vehicle).

**Figure III.** As a measure of microvascular permeability, leakage of *Evans blue* into the peritoneal cavity was quantified. Panels show results for unstimulated control mice as well as for mice treated with a neutrophil-depleting anti-Gr-1 antibody, aneutrophil-depleting anti-Ly-6G antibody 1A8, or isotype control antibody undergoing stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α (mean±SEM for *n*=4 per group; # p<0.05, vs. unstimulated; * p<0.05, vs. vehicle).
Supplementary references
