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-B_4 (LTB_4) themselves were not affected by actinomycin D, BN 52021, MK-886, or AA-861. Conversely, PAF and LTB_4, 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

Leukocyte 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 CX_3C 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-B_4 (LTB_4).[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/

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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.[11,12] 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.[11] 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 LTB_4, 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 peritoneal lavage fluid 3 hours after intraperitoneal application of the respective inflammatory stimuli. Activation of native murine neutrophils isolated from the peripheral blood was measured by using flow cytometry.

Expanded methods are provided in the supplemental material (available online at http://atvb.ahajournals.org).

Results

Effect of Actinomycin D, BN 52021, MK-886, AA-861, and Indomethacin on Leukocyte Recruitment Elicited by Ccl2/JE/MCP-1 or Ccl3/MIP-1α

In a first set of experiments, effects of the inhibitor of RNA transcription actinomycin D, the PAF receptor antagonist BN 52021, the 5-lipoxygenase activating protein (FLAP) antagonist MK-886, the 5-lipoxygenase (5-LO) inhibitor AA-861, and the cyclooxygenase (COX) inhibitor indomethacin on Ccl2/JE/MCP-1- or Ccl3/MIP-1α-elicited leukocyte recruitment to the peritoneal cavity was analyzed. Stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α induced a significant increase in numbers of extravasated 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 I).

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

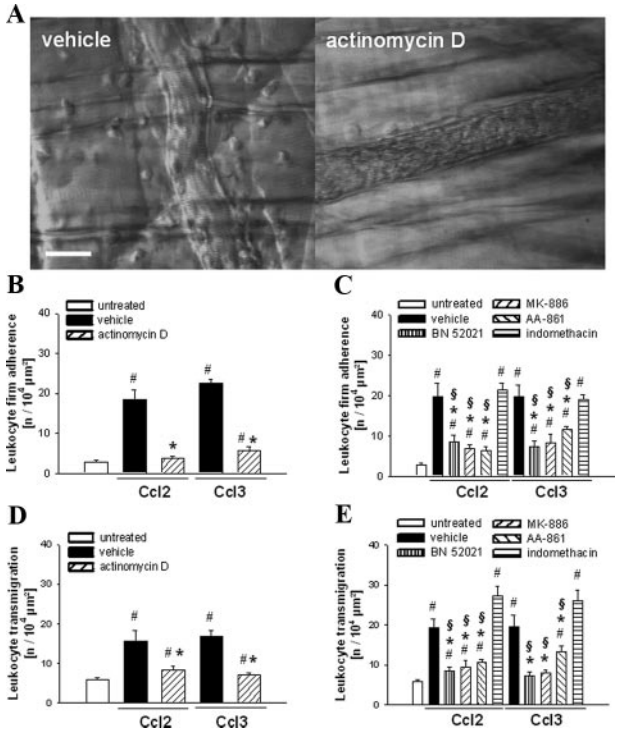

Figure 1. Ccl2/JE/MCP-1–stimulated postcapillary venules in the cremaster muscle treated with vehicle or actinomycin D (A; scale bar 20 μm). Leukocyte responses in mice treated with actinomycin D (B and D), BN 52021, MK-886, AA-861, indomethacin (C and E), or vehicle undergoing stimulation with Ccl2/JE/MCP-1 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).

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. Similar to our findings for leukocyte firm adherence, this elevation was almost completely abolished in animals treated with actinomycin D as well as significantly reduced in animals receiving BN 52021, MK-886, or AA-861 but remained unchanged in mice receiving indomethacin (Figure 1D and 1E).

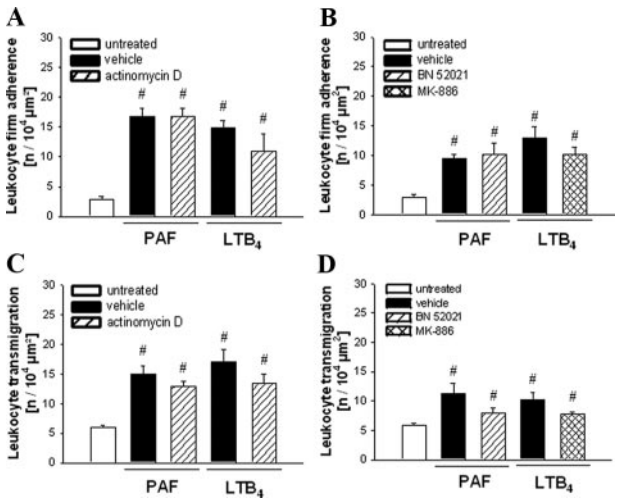

Figure 2. Leukocyte responses in the cremaster muscle of mice treated with actinomycin D (A and C), BN 52021, MK-886 (B and D), or vehicle undergoing stimulation with PAF and LTB_4 (mean±SEM; n=6 per group; #$P<0.05$ vs untreated).

Effect of Actinomycin D, BN 52021, MK-886, and AA-861 on Leukocyte Recruitment Elicited by PAF or LTB_4

Using in vivo microscopy in the mouse cremaster muscle, we further investigated the effect of actinomycin D, BN 52021, MK-886, and AA-861 on the single steps of the leukocyte recruitment process elicited by PAF or LTB_4, respectively. No significant differences were detected in numbers of rolling leukocytes among all experimental groups (data not shown). In the control group, only few leukocytes were found attached to the inner vessel wall of postcapillary venules (Figure 2A and 2B) as well as transmigrated into the perivascular tissue (Figure 2C and 2D). After 3 hours of stimulation with PAF or LTB_4, there was a significant elevation in numbers of firmly adherent and transmigrated leukocytes as compared to unstimulated controls. In contrast to our previous results for Ccl2/JE/MCP-1– and Ccl3/MIP-1α–dependent leukocyte responses, blockade of RNA synthesis had no effect on leukocyte firm adherence and transmigration on stimulation with PAF or LTB_4, respectively. Moreover, antagonism of the PAF receptor or of leukotriene synthesis did not significantly alter leukocyte firm adherence and (subsequent) transmigration in response to PAF or LTB_4, respectively.

In a separate set of experiments, intraperitoneal injection of PAF or LTB_4 induced a significant elevation in numbers of extravasated leukocytes to the peritoneal cavity as compared to unstimulated controls. This elevation of leukocyte extravasation was not altered in animals treated with actinomycin D, BN 52021, or AA-861, respectively (supplemental Figure II).

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 LTB_4, more than 80% of transmigrated leukocytes were positive for Gr-1 and $\approx$ 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-Gruenwald-Giemsa staining of cell preparations from the peritoneal lavage. Three hours after stimulation with Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, or LTB_4 (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 Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, and LTB_4 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 LTB_4 (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 LTB_4 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 LTB_4.

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 4C and 4D). Moreover, stimulation with Ccl2/JE/MCP-1 as well as Ccl3/MIP-1α induced a strong increase in RNA expression of CD62E/E-selectin which was inhibited in the cremaster muscle of 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

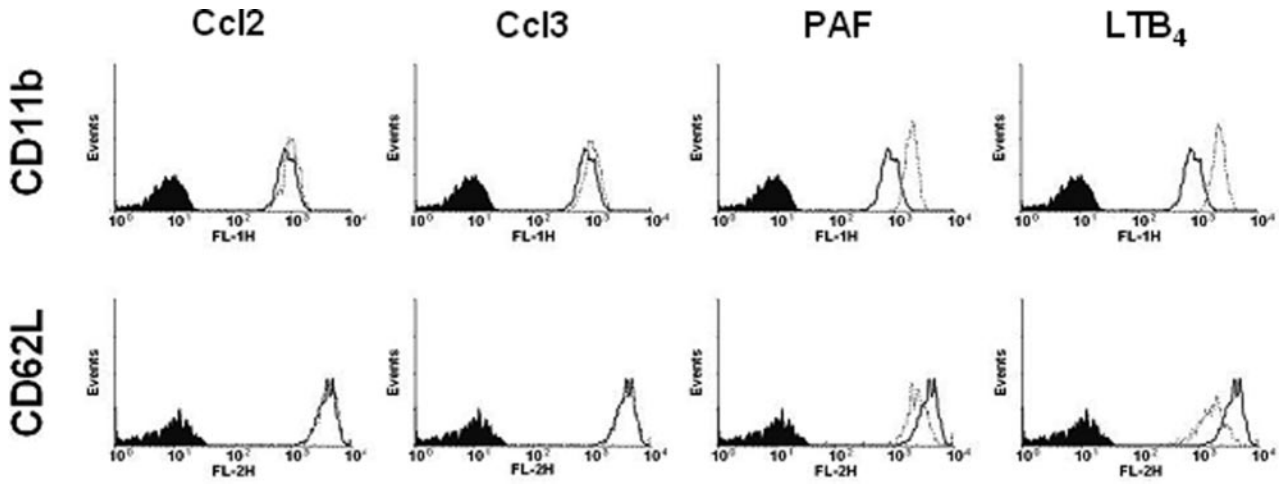

Figure 3. Representative fluorescence histograms for expression of CD11b/Mac-1 and CD62L/L-selectin on murine neutrophils undergoing stimulation with either Ccl2/JE/MCP-1, Ccl3/MIP-1α, PAF, LTB_4 (open histograms with broken lines), or vehicle (open histograms with solid lines) as compared to isotype-matched control IgG (solid histograms).

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 LTB_4, 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, there was a significant elevation in the leakage of Evans blue as compared to unstimulated controls. This elevation of 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

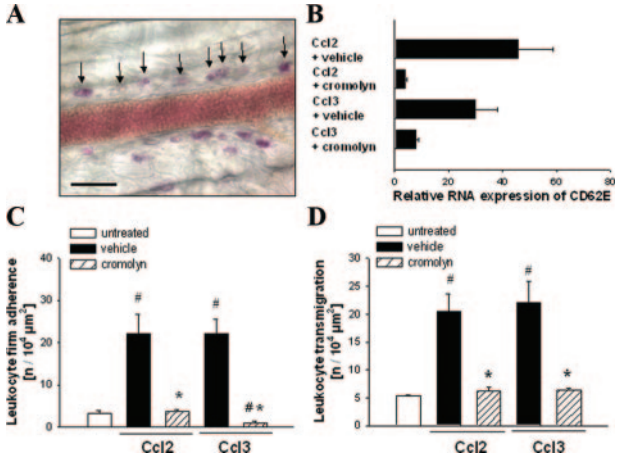

Figure 4. Cremasteric ruthenium red-positive mast cells (A; arrows; scale bar 25 μm). Relative RNA expression of CD62E/E-selectin (B) and leukocyte responses (C and D) in the cremaster muscle of mice treated with cromolyn or vehicle undergoing stimulation with Ccl2/JE/MCP-1 or Ccl3/MIP-1α (mean±SEM; n=4 per group; #P<0.05 vs untreated; *P<0.05 vs vehicle).

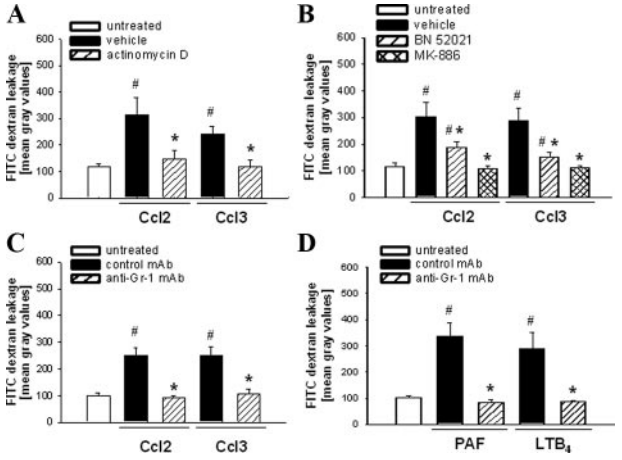

Figure 5. FITC dextran leakage in the cremaster muscle of mice treated with actinomycin D (A), BN 52021, MK-886 (B), and a neutrophil-depleting anti–Gr1-antibody (C) or vehicle/control antibody undergoing stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α or PAF and LTB_4 (D; mean±SEM; n=4 to 6 per group; #P<0.05 vs untreated; *P<0.05 vs vehicle/control mAB).

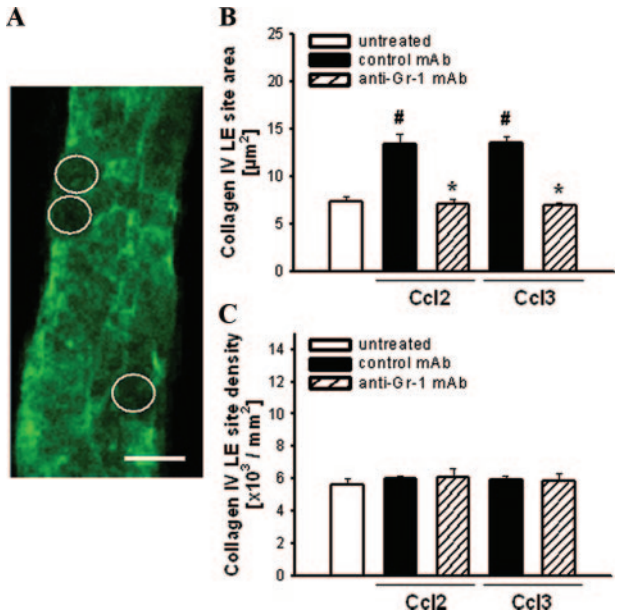

Figure 6. Cremasteric postcapillary venule immunostained for collagen IV. White rings show LE sites (A; scale bar 10 μm). Size (B) and density (C) of LE sites in mice treated with anti–Gr-1-antibody or control antibody after stimulation with Ccl2/JE/MCP-1 and Ccl3/MIP-1α (mean±SEM; n=4 per group; #$P<0.05$ vs untreated; *$P<0.05$ vs control mAB).

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 $5.6\pm0.4\times10^3$ / mm^2 (Figure 6C) and had an average size of 7.3 ± 0.6 μm^2 (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 μm^2; 13.3 ± 1.1 μm^2). This increase was completely abolished in neutrophil-depleted mice, respectively (7.1 ± 0.4 μm^2; 6.9 ± 0.3 μm^2).

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 III).

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).

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 A_2, cyclooxygenase, 5-lipoxygenase, or lyso-PAF 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 LTB_4 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

receptor of Ccl3/MIP-1α have been identified on the surface of native murine neutrophils isolated from the peripheral blood.[17] 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.[4] Interestingly, the chemokine receptors Ccr1, another receptor of Ccl3/MIP-1α, and Ccr2 have also been identified on endothelial cells.[18,19] 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.[20] 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.[21,22] 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 LTB_4, 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 LTB_4. Conversely, however, both PAF and LTB_4 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 LTB_4-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 LTB_4-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, LTB_4) 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.[23] Furthermore, it has been demonstrated that several inflammatory mediators including IL-1β, TNF-α, Ccl3/MIP-1α, PAF, and LTB_4 differentially implement adhesion molecules as well as proteases for the recruitment of neutrophils.[11,24] 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, LTB_4) 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.[11,12] 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.[12] 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 LTB_4 themselves has also been found to be neutrophil-dependent[25,26] 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.[27]

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.[28] 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.[11,28] 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 perivenular basement membrane in Ccl2/JE/MCP-1-elicited inflammation.[29] Here, we extend these observations by demonstrating that neutrophils are critically involved in the enlargement of perivenular 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.[11,28]

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, LTB_4 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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