Monocytes and Neutrophils Exhibit Both Distinct and Common Mechanisms in Penetrating the Vascular Basement Membrane In Vivo

Mathieu-Benoît Voisin, Abigail Woodfin, Sussan Nourshargh

Objectives—Leukocyte migration through venular walls is a fundamental event during inflammation, but many aspects of this response, including the mechanisms associated with leukocyte migration through the vascular basement membrane (BM) in vivo, are poorly understood. Here we investigated and compared the means by which neutrophils and monocytes migrate through the venular BM. Specifically, as we have previously reported on the existence of neutrophil permissive sites (termed matrix protein low expression regions; LERs) within the venular BM, we have now investigated the role of these sites in monocyte transmigration in vivo.

Methods and Results—Analysis of CCL2-stimulated mouse cremaster muscles by immunofluorescent staining and confocal microscopy demonstrated that both neutrophils and monocytes use LERs for penetrating venular walls, but independent and distinct mechanisms are used by the 2 cell types. Collectively, (1) neutrophil but not monocyte transmigration led to enlargement of LERs, (2) monocytes showed a greater extent of deformability in migrating through the venular BM, and (3) only extravasated neutrophils were associated with the carriage of laminin fragments.

Conclusions—The findings provide novel insights into mechanisms of leukocyte transmigration by presenting the first in vivo evidence for distinct modes used by neutrophils and monocytes in penetrating the vascular BM. (Arterioscler Thromb Vasc Biol. 2009;29:1193-1199.)

Key Words: monocytes • leukocyte transmigration • inflammation • vascular basement membrane • leukocyte shape-change

Migration of leukocytes from the circulation into the surrounding tissue plays a key role in host defense but can also contribute to the pathogenesis of inflammatory disorders. This response is mediated by a cascade of complex molecular and cellular events that have collectively been termed and investigated as the leukocyte adhesion cascade. There is a growing understanding of the mechanisms that mediate leukocyte responses within the vascular compartment, but less is known about the subsequent steps that mediate and regulate leukocyte migration through venular walls. The principal reason for the relatively slow progress in this aspect of the field is the complex nature of the vessel wall that cannot be accurately modeled and studied in vitro. Specifically, the venular wall is composed of 2 cellular components (endothelial cells and pericytes) and a noncellular specialized extracellular matrix barrier that surrounds blood vessels, the vascular basement membrane (BM). Endothelial cells (ECs) act as the first barrier for emigrating leukocytes, and although there is evidence for leukocyte transendothelial cell migration to occur via paracellular and transcellular routes, the former is the principal mode of crossing this barrier. In contrast to ECs, pericytes present a discontinuous cellular layer within venular walls, and there is in vivo evidence for leukocyte penetration of the pericyte sheath through gaps between nearby cells but also via a transcellular route. Pericytes are embedded within the vascular BM and together with ECs contribute to the generation of this structure. The vascular BM is a tightly packed network of extracellular matrix proteins, primarily collagen IV and laminins (laminin-8/10), interconnected by other glycoproteins such as perlecain and nidogens. The vascular BM provides a distinct and formidable barrier to emigrating leukocytes, but the mechanisms by which it is breached at sites of inflammation remains poorly understood.

Leukocytes have the ability to degrade and to interact with components of the venular BM via their range of proteases and specific integrins (eg, \( \beta_1 \) integrins), and there is much in vitro evidence suggesting the involvement of such molecular interactions in leukocyte migration.

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From Barts and the London School of Medicine and Dentistry, Queen Mary University of London, William Harvey Research Institute, London, UK.
Correspondence to Professor Sussan Nourshargh, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London, EC1M 6BQ, UK. E-mail s.nourshargh@qmul.ac.uk
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through the BM. Although the role of leukocyte proteases in this response remains contentious, there are now a number of in vivo studies to support the idea of a role for proteases in leukocyte penetration of the BM. Despite these findings, there is no in vivo evidence for long-term damage or compromising of the vascular wall at sites of inflammation, suggesting that the potential role of a proteolytic event is to facilitate leukocyte transmigration as mediated by other mechanisms, possibly governed by the nature of the vascular wall itself. In this context we have previously reported on the existence of regions of low matrix protein deposition (termed low expression regions or LERs) in the vascular BM of postcapillary venules of unstimulated mouse cremaster muscle. These regions were found in the networks of certain venular BM components (laminins and collagen IV) where they exhibited $\approx 60\%$ deposition of protein as compared to the average level found in the venular wall. LERs were on average $\approx 9 \ \mu m^2$ in area and were directly aligned with gaps between adjacent pericytes. Importantly, these sites acted as "gates" for infiltrating neutrophils and were transiently remodeled during neutrophil transmigration. To extend these significant findings we have now investigated the ability of monocytes, as compared to neutrophils, in penetrating the vascular BM via LERs and have found that although both these leukocyte subtypes preferentially use LERs for breaching the vascular BM, they use different and independent mechanisms. Briefly, the results indicate that although neutrophil migration leads to enlargement of LERs, monocyte migration occurs in the absence of BM remodeling apparently through the greater ability of this cell type to exhibit shape-change and deformability. Collectively, the findings of this study provide the first in vivo evidence for distinct mechanisms used by different leukocyte subtypes in penetrating the vascular BM.

Materials and Methods

To elucidate mechanisms of neutrophil and monocyte migration through the vascular BM, cremasteric muscles of CX3CR1-GFP mice expressing endogenously fluorescent monocytes were stimulated with CCL2, LTB4, or LPS. At the end of the in vivo test-period, tissues were collected, fixed and immunostained for components of the vessel wall (eg, the vascular BM) and neutrophils, as previously described. Tissues were subsequently analyzed by confocal microscopy and 3D reconstructions of vessels were performed using 3D imaging software to both localize the position of the leukocytes relative to the BM and to characterize the profile of venular BM (eg, the size of LERs). A full description of the methods used is available as part of the supplemental materials (available online at http://atvb.ahajournals.org).

Results

Monocyte Migration Induced by CCL2 Occurs Through the Vascular BM LERs but Does Not Induce Their Remodeling

We have previously reported on the ability of neutrophils to use permissive regions characterized by low levels of laminin-10 and collagen IV (termed LERs) within the BM of mouse cremasteric venules in penetrating the vascular wall. To extend these findings to other leukocyte subtypes we have now investigated the role and regulation of expression of LERs in monocyte transmigration. For this purpose we studied leukocyte transmigration as elicited by topically applied CCL2 using the mouse strain CX3CR1-GFP that exhibit endogenously eGFP-labeled monocytes. After 1 to 4 hours, tissues were removed and whole mount immunostained with antibodies against MRP14 (used as a neutrophil marker) and laminin-α5 chain (Lmα5, marker for the venular BM) that enabled analysis of neutrophil and monocyte interactions with the venular BM. Of interest, topical application of CCL2 induced the transmigration of both neutrophils and monocytes (Figure 1A) in a time-dependent manner with a greater number of neutrophils accumulating within the first 2 hour postapplication of CCL2 (Figure 1B). The number of transmigrated neutrophils and monocytes were however almost identical at the 4-hour time-point. This noted difference in the kinetics of neutrophil and monocyte migration at the earlier time-points appeared to be a reflection of the number of these leukocyte subtypes found in circulating blood (ie, 13.7±1.1% and 4.5±0.8% for neutrophils and inflammatory monocytes, respectively). When corrected for this monocyte/neutrophil ratio, transmigration of these cells were identical in the first 2 hours postapplication of CCL2,
with monocyte transmigration being greater at the 4-hour time-point (Figure 1B, inset). Furthermore, although neutrophils were found at the vessel wall in clusters (“hot spot”; indicated by circles in Figure 1A), transmigrating monocytes appeared to be randomly positioned. Analysis of the relative intensity of the eGFP signal showed that >92% of the transmigrated monocytes exhibited the characteristics of the inflammatory subpopulation (ie, Gr1⁺CCR2⁺CX₃CR1low).13

Investigation of venular cross-sections demonstrated that transmigrating neutrophils and monocytes can penetrate the venular BM by migrating through LERs (Figure 1C). CCL2 also induced a time-dependent remodeling of the Lmο5 LERs (Figure 1D), a response that was directly associated with the kinetics of neutrophil but not monocyte migration. Indeed, when animals were treated with an antineutrophil-depleting antibody before CCL2-stimulation, neutrophil transmigration was completely inhibited but the monocyte numbers in blood (not shown) or in the tissue postapplication of CCL2 (Figure 2A) were unaltered. In contrast, the remodeling of Lmο5 LERs in CCL2-stimulated tissues was completely abolished in neutrophil-depleted animals, but this effect did not alter the ability of monocytes to use LERs for penetrating the BM (Figure 2B). Collectively these results demonstrate that in the present CCL2-driven reaction, Lmο5 LER remodeling is strictly neutrophil-dependant and that monocytes can penetrate the vascular BM independently of neutrophils and without the need to remodel the BM.

Monocytes Transmigrate Through LERs via Formation of Membrane Protrusions and Deformation of Their Cell Body In Vivo
As monocyte transmigration through the BM did not lead to enlargement of LERs, we sought to further investigate the mechanism by which these cells penetrate the venular BM by studying the morphology of transmigrating monocytes. Using the methods detailed above, observation of 3D reconstructed images of venules from CCL2-stimulated tissues identified monocytes at multiple stages of their emigration through the vascular BM (Figure 3A and supplemental Video I), ie, flattened under/within the BM (position 1 in Figure 3A and 3C), showing small “investigating” protrusions within the BM and directed toward the extravascular space (position 2 in Figure 3A and 3C) and finally, exhibiting larger (“body”) protrusions at a more advanced stage of their migration through the BM (>1/3 of the cell body outside the BM; position 3 in Figure 3A and 3C). Analysis of the protrusions indicated that investigating protrusions were of an average diameter of 1.97±0.10 μm and height of 4.78±0.41 μm which increased to protrusions of an average diameter of 3.27±0.19 μm at a more advanced stage of their emigration, ie, when almost fully through the BM. Of importance, almost all monocytes observed migrating through the BM were associated with an LER, the size of these regions generally being smaller than LERs remodeled postneutrophil transmigration (Figure 3A, middle panels; results not shown). Our results also indicated the ability of monocyte nuclei to squeeze through LERs (Figure 3B) with a diameter of 1.79±0.19 μm. Together these findings indicate that the preferred mechanism for monocytes to penetrate the vascular BM involves “squeezing” through small vulnerable regions, such as LERs.

Neutrophil Migration Through Venular BM In Vivo Is Also Associated With Formation of Protrusions but to a Lesser Extent to That Observed With Monocytes
To directly compare the morphological changes exhibited by monocytes and neutrophils, neutrophil shape-change was analyzed in multiple inflammatory reactions, ie, as induced by CCL2, LTB₄, and LPS. As found with monocytes (and using the techniques detailed above), analysis of CCL2-stimulated tissues by confocal microscopy showed neutrophils in different stages of their emigration through the vascular BM. Specifically, neutrophils were seen flattened under/within the BM (arrow in Figure 4A) and also exhibiting protrusions (circle, Figure 4A). The profile of protrusions exhibited by neutrophils penetrating the vascular BM was however different from that seen with monocytes. Specifically, the early “investigating” protrusions exhibited by neutrophils had a diameter of 2.93±0.27 μm (32.7% bigger than that observed with monocytes, P<0.01), whereas later-stage cell “body” protrusions showed a diameter of 3.97±0.34 μm (17.6% bigger than that observed with monocytes, P<0.05). Furthermore, a significantly greater percentage of monocytes within the vascular BM showed protrusions (53.7±7.2% and 19.2±10.3%, monocytes and neutrophils, respectively, P<0.01).

Remarkably similar results were obtained when leukocyte migration through cremasteric venules was analyzed...
Figure 3. Monocyte protrusion formation whilst penetrating LERs. A, Monocytes embedded within the BM and exhibiting at least 3 distinct morphological shapes after CCL2-stimulation. B, Venular cross-section showing a monocyte squeezing both its body and nucleus. C, Schematic diagram of the different stages of monocyte migration. (Please see the supplemental materials for details).
in tissues stimulated by 2 other stimuli, LTB₄ and LPS. LTB₄, a highly potent neutrophil chemoattractant, induced a large neutrophil migration response (Figure 4B), but within the in vivo time period investigated (2 hours) did not cause monocyte infiltration (not shown). In LPS-stimulated tissues (6 hours) both neutrophil and monocyte migration were noted (Figure 4C). These reactions were both associated with enlargement of LERs (not shown) and also formation of cellular protrusions during leukocyte migration through the BM (Figure 4B and 4C, bottom panels), in line with the findings in CCL2-stimulated tissues. Detailed analysis of the characteristics of cell protrusions in these 3 different inflammatory reactions demonstrated that although both leukocyte subtypes exhibited “investigative” and “body” protrusions, neutrophil investigative protrusions were consistently larger in diameter than that observed for monocytes (Figure 4D). At the more advanced stage of their migration through the BM, neutrophils also showed a trend toward forming larger body protrusions than that noted for monocytes (data not shown). Finally, similarly to the CCL2-induced reaction, in LPS-stimulated tissues a significantly greater percentage of monocytes penetrating the vascular BM showed protrusions (76.6±6.0% and 47.7±8.1%, for monocytes and neutrophils, respectively, *P<0.01*) and in the LTB₄-induced reaction, only 32.4±7.3% of neutrophils exhibited protrusions. The formation of protrusions by both leukocyte subtypes during their emigration through the BM in vivo was confirmed in an in vitro transwell assay using inhibitors of actin G polymerization (latrunculin B) and of myosin-II contraction (blebbistatin) (supplemental Result I). Collectively the results indicate that although neutrophils can also exhibit protrusions at the level of the vascular BM in vivo, their shape and frequency is significantly different from that observed with monocytes.

**Transmigrated Neutrophils but not Monocytes Are Laminin-Positive In Vivo**

Because in CCL2-stimulated tissues, the remodeling of LERs in the vascular BM is entirely neutrophil-dependent (Figure 2A), we sought to investigate the potential mechanisms for this response. As previously described with IL-1β, analysis of CCL2-stimulated cremaster muscles indicated that a large percentage of transmigrated neutrophils were Lmα5-positive (87.0±3.4% at 2 hours post-stimulation). In contrast, no laminin-positive transmigrated monocytes were detected in tissues stimulated with CCL2 (Figure 5A). These results suggested the involvement of a neutrophil-specific proteolytic event, and based on our previous findings, the potential role of neutrophil elastase in this reaction was investigated. Intravenous pretreatment of animals with a specific inhibitor of neutrophil elastase (NE), ONO-5046, led to a significant inhibition of CCL2-induced neutrophil migration and LER remodeling (71% and 54% reduction, respectively) but had no effect
on monocyte infiltration (Figure 5B). Furthermore, in ONO-5046–treated mice, a greater number of neutrophils were detected at the level of the vascular BM as compared to vehicle-treated controls (5.2 ± 1.1 cells versus 1.8 ± 0.4 cells/200 μm vessel segment, respectively; P < 0.01). The inhibitory effect of ONO-5046 on neutrophil migration (but not monocyte migration) through small permissive regions in vivo was confirmed in an in vitro transwell assay (supplemental Result II). Of interest, an inhibitor of MMP-2/MMP-9 had no effect on CCL2-induced neutrophil or monocyte transmigration in vivo but significantly suppressed neutrophil migration in TNFα-stimulated tissues (supplemental Result III), suggesting that the role of specific proteases in neutrophil transmigration in vivo is governed by the inflammatory stimulus. Collectively the present findings suggest that neutrophil, but not monocyte, migration through the vascular BM involves a protease-dependent carriage of BM components.

**Discussion**

The difficulties associated with creating physiologically relevant in vitro models of venular walls have been instrumental in the relatively slow progress in our understanding of the mechanisms of leukocyte migration through this complex structure. This is particularly relevant with respect to the mechanisms by which leukocytes migrate through the vascular basement membrane, a specialized and vital matrix protein component of vessel wall. To enable us to study the process of leukocyte migration through the vascular BM in vivo, we have developed an experimental approach that allows 3D analysis of the vascular BM in intact whole mounted tissues as investigated by immunofluorescent staining and confocal microscopy. Using this approach we identified for the first time regions within the BM of mouse cremasteric venules where the expression of certain matrix proteins was lower than the average venular level, sites that have been termed low expression regions (LERs). Of importance, these sites appeared to act as “gates” for emigrating neutrophils and were remodeled by transmigrating neutrophils in response to IL-1β. To extend these significant findings we have now investigated the involvement of these regions in transmigration of monocytes as compared to neutrophils.

To investigate the potential role of LERs in monocyte transmigration, leukocyte migration in CCL2-stimulated mouse cremasteric venules was analyzed. CCL2 is a potent chemoattractant for inflammatory monocytes, cells that express high levels of the principal CCL2 receptor CCR2 on their cell surface. Of interest, in the murine system, topically applied CCL2 induced a time-dependent transmigration of both monocytes and neutrophils, a finding that is in line with the reported expression of CCR2 on murine neutrophils (data not shown). Analysis of the vascular BM in CCL2-stimulated cremaster muscles indicated a time-dependent remodeling of LERs. Furthermore, as found with neutrophils, monocytes used LERs for emigrating through venular BM. As a number of studies have suggested that neutrophils can pave the way for subsequent monocyte migration, the ability of CCL2 to induce both neutrophil and monocyte migration enabled us to investigate whether neutrophil-mediated remodeling of the BM facilitated monocyte infiltration. Although depleting the mice of their circulating neutrophils had no effect on monocyte infiltration, it totally inhibited the remodeling of the BM noted in CCL2-stimulated tissues. Under these conditions the percentage of monocytes that transmigrated through LERs was unaltered, suggesting that monocyte migration through permissive regions within the vascular BM does not involve remodeling of these sites and can occur independently of neutrophils, a finding which is in accordance with other reports. The neutrophil-independent migration of monocytes was also supported by the fact that transmigrating monocytes were found randomly positioned within the vessel wall and often associated with small LERs (ie, similar size to LERs that had not been remodeled). In contrast, neutrophils were often found at the vessel wall in clusters, transmigrating at specific sites (“hot-spots”) that were distinct from regions of monocyte emigration. Collectively the profile and kinetics of the present results demonstrate that monocyte migration may occur independently of neutrophils and vice versa, but they do not rule out the possibility that under certain inflammatory conditions monocyte migration may be facilitated by neutrophils or that neutrophil migration may be aided through monocyte emigration.

To gain further insight into the mechanism by which monocytes penetrate the vascular BM, detailed analysis of monocyte morphology during their emigration through LERs was performed. CCL2-elicited transmigrating monocytes were seen to exhibit small protrusions within the BM at an early stage of their emigration through the vascular BM. Similar results were noted in LPS-stimulated tissues. Because of similarities in their shape/size and localization, these protrusions could be related to the membrane invaginations and invasive protrusions described previously during leukocyte migration through endothelial cells but also during cell motility at the interface with matrix proteins. The observed early expression of membrane protrusions at the level of the BM could therefore be a mechanism by which monocytes detect permissive regions within the BM such as LERs. Whether these protrusions act as a means of presenting adhesive or proteolytic molecules to the BM requires further investigations. However, the fact that monocyte migration is not associated with remodeling of the BM and was not suppressed by an MMP-2/MMP-9 inhibitor argues against a proteolytic event in this response.

Neutrophils are well known for their ability to squeeze through narrow regions (eg, capillaries) and in our studies exhibited significant protrusions and shape-change at the level of the vascular BM in vivo in multiple inflammatory reactions (CCL2, LPS, and LTB4). The frequency and profile of neutrophil protrusions was however significantly different to that observed for monocytes. Specifically, in all reactions investigated, percentage of neutrophils showing protrusions was on average ∼2-fold less than that
noted for monocytes and neutrophils characteristically showed investigative protrusions with significantly bigger diameters. The apparently less invasive profile of neutrophil shape change at the level of the vascular BM suggests that neutrophils may use complementary mechanisms to facilitate their migration through this barrier. In this context, transmigrated neutrophils but not monocytes were found to be laminin-positive in the extravascular tissue, indicating that neutrophil but not monocyte transmigration involves proteolytic cleavage or carriage of BM-derived laminin fragments. Indeed in line with our previous findings where neutrophil migration in response to IL-1β and LTB₄ was suppressed by a specific neutrophil elastase (NE) inhibitor, CCL2-induced neutrophil emigration also appeared to be NE-dependent. In addition, pretreatment of mice with an NE inhibitor suppressed CCL2-induced remodeling of LERs without affecting monocyte infiltration. Taken together our data suggest that although monocyte migration through the vascular BM can occur through the ability of these cells to squeeze through small permissive sites (ie, LERs), neutrophil migration through the BM is facilitated by neutrophil-derived NE, thus overriding a need for neutrophils to develop multiple or smaller invasive protrusions (Figure 5C).

In conclusion, the present study demonstrates small permissive regions within the vascular BM termed LERs are used as the preferred sites by both emigrating neutrophils and monocytes in penetrating the venular wall. However, although neutrophil migration leads to remodeling of these sites, monocyte migration is strongly governed by the deformability of these cells. Indeed, monocytes exhibited significantly more invasive morphological changes in vivo suggesting that the principal mechanism by which they migrate through the vascular BM is by “squeezing” through small permissive sites. These findings shed much light on the mechanisms of leukocyte migration through the venular BM and provide the first line of in vivo evidence for distinct modes used by different leukocyte subtypes in penetrating this critical vascular structure.

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Disclosures
None.

References


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Abbreviations

BM, Basement Membrane; Coll IV, Collagen IV; EC, Endothelial Cell; LER, Low Expression Region; Lmα5, Laminin-α5 chain; NE, Neutrophil Elastase.
Online Supplementary Methods

Animals:
Male CX3CR1<sup>GFP/+</sup> mice (on C57BL/6 background) of 8 to 12 weeks old were used in this study. CX3CR1<sup>GFP/+</sup> mice were generated by targeted gene disruption as previously detailed<sup>1</sup> and obtained from the European Mutant Mouse Archive (EMMA, Orleans, France). All experiments were carried out under UK legislation for the protection of animals.

Reagents:
Recombinant murine collagen IV was purchased from R&D Systems (Abingdon, United Kingdom), leukotriene B4 (LTB4), latrunculin B and blebbistatin were purchased from Calbiochem (La Jolla, CA). Polyconal murine laminins derived from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma and lipopolysaccharides (LPS) were from Sigma-Aldrich (Poole, UK). Purified or APC-conjugated anti-mouse GR1 (RB6-8C5) mAbs were from Becton Dickinson (Cowley, UK) and rabbit anti–mouse collagen IV polyclonal Ab was from Abcam (Cambridge, UK). The rabbit anti-mouse laminin α5 chain Ab (specific to the laminin-10/11 isoforms) was a gift from Dr Takako Sasaki<sup>2</sup> and the anti-mouse MRP14 mAb (used as a neutrophil marker) was a kind gift from Dr Nancy Hogg<sup>3</sup>. Secondary antibodies conjugated to Alexa Fluor 488, 568, or 633 were from Molecular Probes (Invitrogen, UK). The neutrophil elastase inhibitor ONO-5046 was a gift from ONO Pharmaceutical (Osaka, Japan)<sup>4</sup>. The MMP-2/MMP-9 inhibitor III was purchased from Calbiochem (Merck, Nottingham, UK).
Neutrophil depletion:

Neutrophil depletion of the CX₃CR1ₑGFP/ᵐice was induced by intraperitoneal injection of anti-GR1 (or an isotype matched control antibody, both at 25µg/day for 3 days). Blood neutrophil numbers were quantified in treated mice by flow cytometry (Gr1⁺ cells) and found to be reduced by 99.5%. Of importance this pre-treatment had no effect on the proportion of blood CX₃CR1ₑGFP⁺GR1⁺ monocytes (n=6 mice/group).

Induction of inflammation in the mouse cremaster muscle:

The cremaster muscle was selected as the principal tissue for analysis of venules by immunofluorescent staining and confocal microscopy due to its thin nature enabling the investigator to obtain images of high resolution suitable for accurate quantifications. Surgically exteriorised tissues were stimulated by topical application of the chemokine CCL2 (5×10⁻⁹ M, up to 4h in vivo test period) or of the chemoattractant LTB₄ (10⁻⁷ M, up to 2h in vivo test period), or by intrascrotal (i.s.) injection of LPS (300ng, 6h in vivo test period) as previously reported⁵,⁶. Control experiments consisted of superfusion of exteriorised cremaster muscles with Tyrode’s solution or i.s. injection of saline, as appropriate. In some experiments, mice were treated i.v. with the elastase inhibitor ONO-5046 (50 mg kg⁻¹ 200 µl⁻¹ bolus followed by a continuous infusion of 50 mg kg⁻¹ h⁻¹ until the end of the experiment) or its vehicle (saline). In another set of experiments, mice were co-injected intrascrotally with CCL2 (500ng) or TNFα (300ng) and the MMP-2/MMP-9 inhibitor III (300 µg) or its vehicle using a 2-4h reaction time. At the end of the in vivo test period, mice were killed by an anaesthetic overdose and cremaster muscles were dissected away for analysis of venules by immunofluorescent staining and confocal microscopy.
Analysis of tissues by Immunofluorescence labelling and confocal microscopy:

Mice were humanly sacrificed and the cremasteric muscle was removed and directly fixed into 100% ice-cold methanol for 1h at 4°C prior to being subjected to immunostaining procedures. Briefly, fixed whole mounted tissues were blocked and permeabilised in PBS containing 10% normal goat serum, 10% of FCS, 5% of mouse serum and 0.5% Triton X-100 for 2 h at room temperature. The tissues were then immunostained for BM markers collagen IV or laminin 10/11 (anti-laminin α5 chain), and for neutrophils (anti-MRP14) in PBS + 10% FCS overnight at room temperature. In some studies the nuclear stain, Draq5 (Biostatus Limited, Shepshed, UK), was also used to assist in the study of leukocyte shape change during transmigration. Following 3 washes in PBS, tissues were subsequently incubated with specific 488-, 555-, or 633-conjugated anti-rat or anti-rabbit secondary antibodies, as determined by the combination of primary antibodies used in the relevant experiment, for 2-4 h at RT in PBS + 10% FCS. Samples were then viewed using a Zeiss LSM 5 Pascal laser-scanning confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) incorporating a × 40 water-dipping objective lens (0.75 numerical aperture) or with a Leica TCS SP5 confocal (Leica Microsystems, Milton Keynes, UK) with a × 20 water dipping objective lens (1 numerical aperture) at 20-24°C. Acquired Z-stack images (<1μm optical section) were used for 3D-reconstruction of whole vessels (200 μm length; 4-6 vessels per tissue). The size (area), of venular matrix protein low expression regions (LER) was measured as detailed previously 7 using Image J software (NIH, USA). In addition, the position and morphology of transmigrating leukocytes relative to the vascular basement membrane was analysed with the image processing software IMARIS (Bitplane, Switzerland). The imaged tissues were also
quantified for transmigrated leukocytes, defined as the number of leukocytes in the extravascular tissue across a 200 µm vessel segment and within 100 µm of tissue to the vessel of interest. For these studies, neutrophil transmigration was quantified by measuring MRP14 positive cells and monocyte transmigration was quantified by counting the number of eGFP labelled cells as previously described. To quantify diameter of leukocyte protrusions in inflamed tissues the following number of mice, vessels and cells were quantified. CCL2: 126 monocytes and 240 neutrophils within the BM of 3-5 vessels/cremaster muscle tissue using N=6-13 mice were analysed. In this reaction, the quantification of the diameter of monocyte nucleus involved analysing a total of 62 cells from 3-4 venules/tissue obtained from N=6 mice. LTB₄: 133 neutrophils within the BM of 3-5 vessels/cremaster muscle tissue using N=5 mice were analysed. LPS: 70 monocytes and 106 neutrophils within the BM of 4-6 vessels/cremaster muscle tissue using N=4 mice were analysed.

In vitro leukocyte transmigration assay:
Blood from CX₃CR1eGFP/+ mice was collected into citrate (1/10) and leukocytes were isolated by dextran sedimentation (1 part of blood for 4 parts of 1.25% dextran solution) at room temperature for 45 minutes. Leukocyte rich supernatants were harvested and washed in PBS twice then resuspended in modified PBS (containing 0.25% heat inactivated FCS, 1mM Ca²⁺/Mg²⁺ and 5mM glucose). Leukocytes (1×10⁵ cells/well) were then added to the top well of transwell chambers incorporating 5 diameter pore filters (NeuroProbe, Gaithersburg, MD, USA) pre-coated overnight with a combination of 15 µg/ml of murine collagen IV and a mixture of murine laminins, as detailed in Reagents. Cells were pre-treated with multiple concentrations of inhibitors such as blebbistatin, latrunculin B and ONO-5046 based on previous
The bottom wells contained medium, CCL2 (5×10^{-9} M) or LTB4 (5×10^{-9} M) and the chambers were incubated at 37ºC for 3h. The total number of cells that had migrated into the bottom wells was measured microscopically and the differential leukocyte quantification was analysed by flow cytometry (Dako-Cyan, Dako, Ely, UK) after labelling of cells with an APC-conjugated anti-GR1 antibody (30min incubation at 4ºC). Neutrophil and monocyte populations were identified based on their characteristic scatter profiles and Gr1 vs. CX3CR1^{eGFP} expressions as previously detailed^{11}.

**Statistical Analysis:**

All data were processed and analysed with Prism 4 GraphPad software (San Diego, USA). Statistical significance was assessed by one way ANOVA followed by Student-Newman-Keuls multiple comparison test. Where two variables were analyzed, an unpaired t test was used. P<0.05 was considered significant. The results are given as mean values ± SEM.
Online Supplementary Figure legends

Figure 1:
CCL2-induced monocyte and neutrophil transmigration through LERs and BM remodelling. (A) 3D images of control and CCL2-stimulated post capillary venules at 4h. (B) Time course of neutrophil and monocyte transmigration in CCL2-stimulated tissues. Insert: Leukocyte migration response when normalized for the neutrophil:monocyte ratio in blood. (C) Latitudinal cross-sections (1.2µm thick) of a CCL2-stimulated post capillary venule showing a monocyte (green) and a neutrophil (blue) migrating through Lmα5 LERs (arrows). (D) Remodelling of the Lmα5 LER area following stimulation with CCL2 (1620 LERs counted). Mean±S.E.M from N=3-6 animals/group, >4 vessels/animal. Significant CCL2-induced responses, * P<0.05 and *** P<0.001. Other indicated comparisons # P<0.05 and ## P<0.01. Bars=10µm.
Figure 2:

CCL2-induced neutrophil-dependent LER remodelling. (A) Quantification of neutrophil and monocyte transmigration and the corresponding size of Lmα5 LERs in CCL2-stimulated cremaster muscles (2h) in control and neutrophil-depleted animals. (B) Percentage of transmigrating monocytes associated with a BM LER in control and neutrophil-depleted mice. Mean±S.E.M from N=3-6 animals/group, >4 vessels/animal (1135 LERs analysed). CCL2 vs. Tyrodes, * P<0.05 and *** P<0.001. Other indicated comparisons ### P<0.001.
Figure 3:

Monocyte protrusion formation whilst penetrating LERs. (A) 3D image of a CCL2-stimulated cremasteric venule (2h) showing monocytes embedded within the BM (top panel) and exhibiting at least three distinct morphologic shapes (position 1, 2 and 3). Greater magnification of the regions of interest (bottom panels) viewed at different angle positions shows “flat” monocytes embedded in/under the BM (position 1) and monocytes exhibiting small (position 2) or bigger (position 3) protrusions (shown in circles) toward the extravascular space. Transmigrating monocytes are associated with LERs within the BM as indicated by the intensity plot of a latitudinal section of the BM within the collagen IV network (dashed arrow). Examples of LERs are identified in the intensity plots by arrows (N=3-6 animals, >4 vessels/animal). Bar=10µm. (B) Representative longitudinal cross section (1µm) of a venule showing a transmigrating monocyte through a collagen IV LER (single headed arrow) exhibiting a “squeezing” of both the body and the nucleus (double headed arrow) (N=4 vessels/mice, 3 mice). (C) Schematic diagram of the different stages monocyte migration through LERs of the venular BM.
Figure 4:

Neutrophil protrusion formation during venular BM penetration. (A) Two neutrophils migrating through the BM of a CCL2-stimulated cremaster (2h) either flattened below/within the BM (arrow) or exhibiting a protrusion towards the extracellular space (circle). The protrusion diameter is indicated on the image (N=16 tissues). (B-C) 3D images of venules illustrating the neutrophil infiltration in response to LTB₄ (2h; B) and the infiltration of both monocytes and neutrophils in LPS-stimulated tissues (6h; C). The small lower inserts: examples of transmigrating leukocytes at the level of the BM exhibiting protrusions (arrow). (D) Diameter of “invasive” protrusions as detected under different inflammatory reactions in the cremaster muscle. Mean±SEM of 4-6 vessels/cremaster, >3 mice/group. Neutrophils vs. monocytes, ** P<0.01. Bar=10µm.
Figure 5:
CCL2-induced neutrophil but not monocyte transmigration is associated with the carriage of Lmα5 on the emigrated cells and is suppressed by a neutrophil elastase inhibitor. (A) 3D reconstructions of a venule post CCL2-stimulation (2h) and immunostained for MRP14 and Lmα5 demonstrating the carriage of Lmα5 on the surface of transmigrated neutrophils (filled arrows) but not monocytes (open arrows). Right panel: Leukocytes are made semi-transparent (opacity filter used for the fluorescence intensity), highlighting the existence of neutrophils decorated with Lmα5 from the venular basement membrane. (B) Mice were treated either with i.v. saline or a specific NE inhibitor, ONO-5046, during the course of cremaster muscle stimulation with CCL2. Leukocyte transmigration and the size of Lmα5 BM LERs were quantified. Mean±S.E.M of N=3-6 animals/group, >4 vessels/animal. Significant CCL2-induced responses, ** P<0.01 and *** P<0.001. Other indicated comparisons, ## P<0.01. Bar=10µm. (C) Schematic diagram of potential stages of monocyte (left) and neutrophil (right) migration through low expression regions of the venular BM, showing that both cells use shape changes and LERs as “gates” to cross the BM. Both cell types exhibit distinct cellular morphologies: 1) Flattened under/within the BM, 2) formation of “investigating” protrusions toward the extravascular space, and finally, 3) formation of large “body” protrusions at a more advanced stage of their emigration. Neutrophil penetration of the venular BM is also associated with enlargement of LERs, possibly via a proteolytic event involving neutrophil elastase (NE) and with the expression of fragments of BM laminin on their cell surface.
Online Video1 legend

The Supplementary Video 1 is a 3D reconstructed section of a blood vessel of a CX₃CR1^{GFP/+} mice cremaster as depicted in Figure 3. The video illustrates 3 distinct morphological shapes of transmigrating monocytes within the BM: 1) flattened and embedded under/within the basement membrane, 2) monocyte exhibiting a small investigative protrusion toward the extracellular space and 3) monocyte exhibiting a larger protrusion at a more advanced stage of its emigration.
Online supplementary Results

Supplementary result I

To further investigate the deformability properties of neutrophils and monocytes, the effects of an inhibitor of actin G polymerisation, latrunculin B, and an inhibitor of myosin II contraction, blebbistatin⁹, were tested on leukocyte transmigration in vitro using a transwell assay. Briefly, the migration of monocytes and neutrophils (from a mixed leukocyte preparation isolated from CX₃CR₁<sup>eGFP/+</sup> mice added to the top chamber through filters of 5µm pores) was assayed in response to CCL2 or LTB₄ (added to the bottom chambers) in the presence or absence of inhibitors (Supplementary Figure IA and IB, respectively). Both inhibitors suppressed CCL2-induced monocyte and neutrophil migration (Supplementary Figure IA and data not shown, respectively). With LTB₄, only significant neutrophil migration was noted, a response that was inhibited when actin polymerisation was blocked by latrunculin B (Supplementary Figure IB). Collectively these data confirmed the ability of both monocytes and neutrophils to squeeze through small pores in vitro in a manner that involves formation of actin-dependent protrusions and myosin II dependent-contraction movements.
**Supplementary Figure I**: Profile of leukocyte migration through small permissive regions *in vitro*. Total peripheral blood leukocytes from CX3CR1 eGFP/+ mice were pre-treated with latrunculin B or blebbistatin at the indicated concentrations before being added to the top chambers of laminin/collagen IV coated filters of chemotaxis chambers (pore diameter size of 5µm). Cells were incubated at 37°C for 3h in the absence or presence of CCL2 (A) or LTB₄ (B), both at a concentration of 5×10⁻⁹M in the bottom wells. Migrated cells (bottom well) were then harvested, counted and analysed by flow cytometry to quantify the percentage of both transmigrated neutrophils and monocytes as discriminated by GR1 staining and CX3CR1 eGFP. The results show the migratory response of monocytes (A) and neutrophils (B) in response to CCL2 and LTB₄, respectively. Results are the mean±S.E.M. of experiments performed in triplicates (cells obtained from 3-4 mice/experiment). Significant differences in migratory responses between PBS and CCL2/LTB₄-containing chambers are indicated by asterisks, *** P<0.001. Further comparisons are shown with lines and hash symbols, ## P<0.01; ###P<0.001.
Supplementary Result II

The inhibitory effect of the neutrophil elastase inhibitor ONO-5046 on neutrophil but not monocyte migration following CCL2 stimulation of mouse cremasteric muscle in vivo (Figure 5B) was further investigated using an in vitro transmigration assay. For this purpose, mixed leukocyte preparations isolated from CX3CR1<sup>GFP/+</sup> mice were added to the top chambers of 5µm porous filter membranes pre-coated with murine laminin and recombinant collagen IV (15µg/each) and monocyte and neutrophil migratory responses as elicited by CCL2 or LTB<sub>4</sub> (added to the bottom chambers) were quantified. Using this assay, the neutrophil elastase inhibitor ONO-5046 was found to suppress neutrophil migration as induced by CCL2 and LTB<sub>4</sub> in a concentration dependent manner (Supplementary Figure IIA left panel and Figure IIB, respectively). Pre-treatment of cells with ONO-5046 had no effect however on CCL2-induced monocyte migration through the filters (Supplementary Figure IIA, right panel). Of interest, in contrast to CCL2, LTB<sub>4</sub> did not induce the migration of murine monocytes in the present in vitro assay (not shown).
Supplementary Figure II: Effect of ONO-5046 on leukocyte migration through protein-coated filters in vitro. Total peripheral blood leukocytes from CX3CR1<sup>eGFP/+</sup> mice were pre-treated with different concentrations of ONO-5046 (0.5-500 µM) before being added to the top chambers of laminin/collagen IV coated filters of chemotaxis chambers (pore diameter size of 5µm). Cells were incubated at 37°C for 3h in the absence or presence of CCL2 (A) or LTB<sub>4</sub> (B), both at a concentration of 5×10<sup>-9</sup>M in the bottom wells. Migrated cells (bottom well) were then harvested, counted and analysed by flow cytometry to quantify the percentage of both transmigrated neutrophils (A, left panel, and B) and monocytes (A, right panel) as discriminated by GR1 staining and expression of CX3CR1<sup>eGFP</sup>. The results show the migratory response of monocytes and neutrophils in response to CCL2 or LTB<sub>4</sub> under
control or post- pre-treatment with ONO-5046. Results are the mean±S.E.M. of experiments performed in triplicates (cells obtained from 6 mice/experiment). Significant differences in migratory responses between PBS and CCL2/LTB₄-containing chambers are indicated by asterisks, * P< 0.05, ** P< 0.01 and *** P<0.001. Further comparisons are shown with lines and hash symbols, # P<0.05 and ###P< 0.001.
**Supplementary result III**

The role of MMPs in CCL2-induced monocyte migration through cremasteric venules *in vivo* was investigated using the specific MMP-2/MMP-9 inhibitor III. Briefly, CX₃CR1⁺GFP/⁺ mice received an intrascrotal injection of CCL2 (500ng) plus saline (control mice) or the inhibitor (300µg) and responses were quantified after 2 h. Total leukocyte adhesion and transmigration responses in cremasteric venules were quantified by intravital microscopy as previously described⁶ demonstrating an increase in both leukocyte adhesion and transmigration (not shown). At the end of this real-time quantification, animals were sacrificed and tissues were removed, fixed and immunostained for laminin-α5 and MRP14 and subsequently viewed and analysed by confocal microscopy, as described in Material and Methods. The results showed that CCL2-induced neutrophil and monocyte transmigration was unaffected by the MMP-2/MMP-9 inhibitor (Supplementary Figure IIIA and IIIB). Using the same experimental approach, the MMP-2/MMP-9 inhibitor III significantly suppressed neutrophil transmigration in response to locally administered TNFα (Supplementary Figure IIIC).
Supplementary Figure III: Effect of MMP-2/MMP-9 inhibitor III on monocyte and neutrophil migration in CCL2 stimulated cremaster muscles. CX3CR1<sup>eGFP</sup>/mice received an intrascrotal injection of CCL2 (500ng/cremaster) plus saline (control mice) or CCL2 plus the inhibitor (300µg). After a 2h reaction time, tissues were removed and whole mount immunostained for laminin-α5 and MRP14 to label the venular BM and neutrophils, respectively. Monocytes were identified by the GFP
signal. The images in panel A are 3D reconstructed vessels from control CCL2-stimulated tissues (top) and CCL-2 + MMP-2/MMP-9 treated cremaster muscles (bottom). Panel B shows the quantification of monocyte (top) and neutrophil (bottom) transmigration. (C) The effect of the MMP-2/MMP-9 inhibitor was also tested on neutrophil migration through cremasteric venules as induced by TNFα (300ng) using a 4h reaction time. Results shown are from n = 5-6 mice per group with at least 4 vessels per animal being quantified. Values are expressed as mean±S.E.M. Significant differences in migratory responses between control and inhibitor-treated animals are indicated by an asterisk, * P<0.05.
References


