Online Supplementary Material

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 $CX_3CR1^{GFP/+}$ mice (on C57BL/6 background) of 8 to 12 weeks old were used in this study. $CX_3CR1^{GFP/+}$ mice were generated by targeted gene disruption as previously detailed[1] 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 B_4 (LTB_4), 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[2] and the anti-mouse MRP14 mAb (used as a neutrophil marker) was a kind gift from Dr Nancy Hogg[3]. 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)[4]. The MMP-2/MMP-9 inhibitor III was purchased from Calbiochem (Merck, Nottingham, UK).

Neutrophil depletion:

Neutrophil depletion of the $CX_3CR1^{eGFP/+}$ 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 $CX_3CR1^{eGFP/+}GR1^+$ 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 LTB_4 (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 reported[5,6]. 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 ($50\ mg\ kg^{-1}\ 200\ \mu l^{-1}$ 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 4ºC 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 20-24ºC. 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 [8]. 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. LTB_4: 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 $CX_3CR1^{eGFP/+}$ 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 Ca^{2+}/Mg^{2+} and 5mM glucose). Leukocytes (1×10^5 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

studies[9,10]. The bottom wells contained medium, CCL2 (5×10^{-9} M) or LTB_4 (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. CX_3CR1^{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 LTB_4 (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.

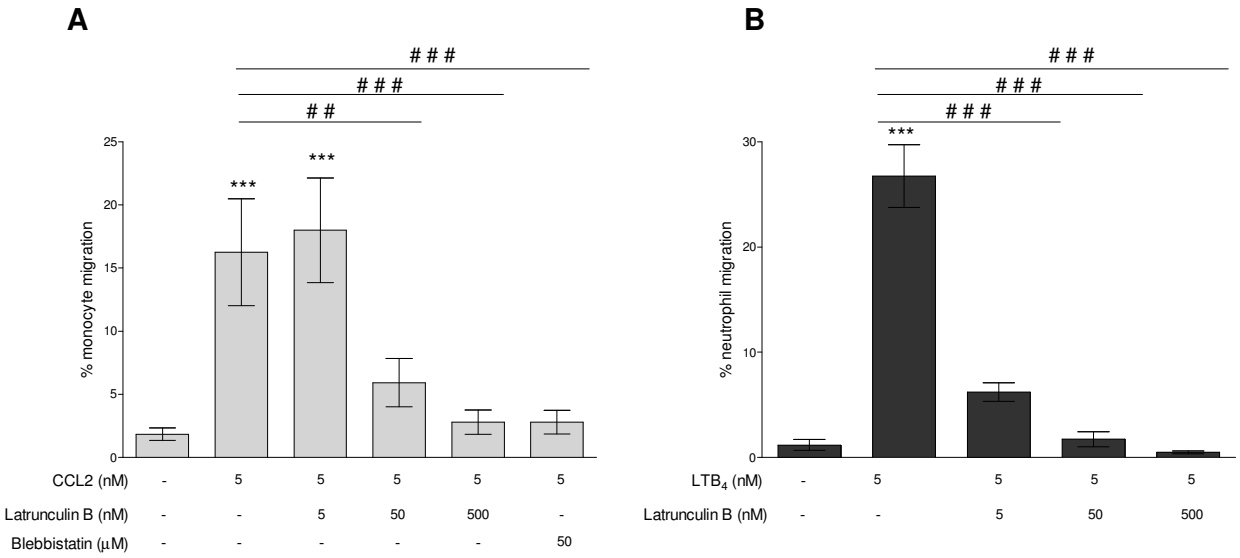
Online Video1 legend

The Supplementary Video 1 is a 3D reconstructed section of a blood vessel of a $CX_3CR1^{GFP/+}$ 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

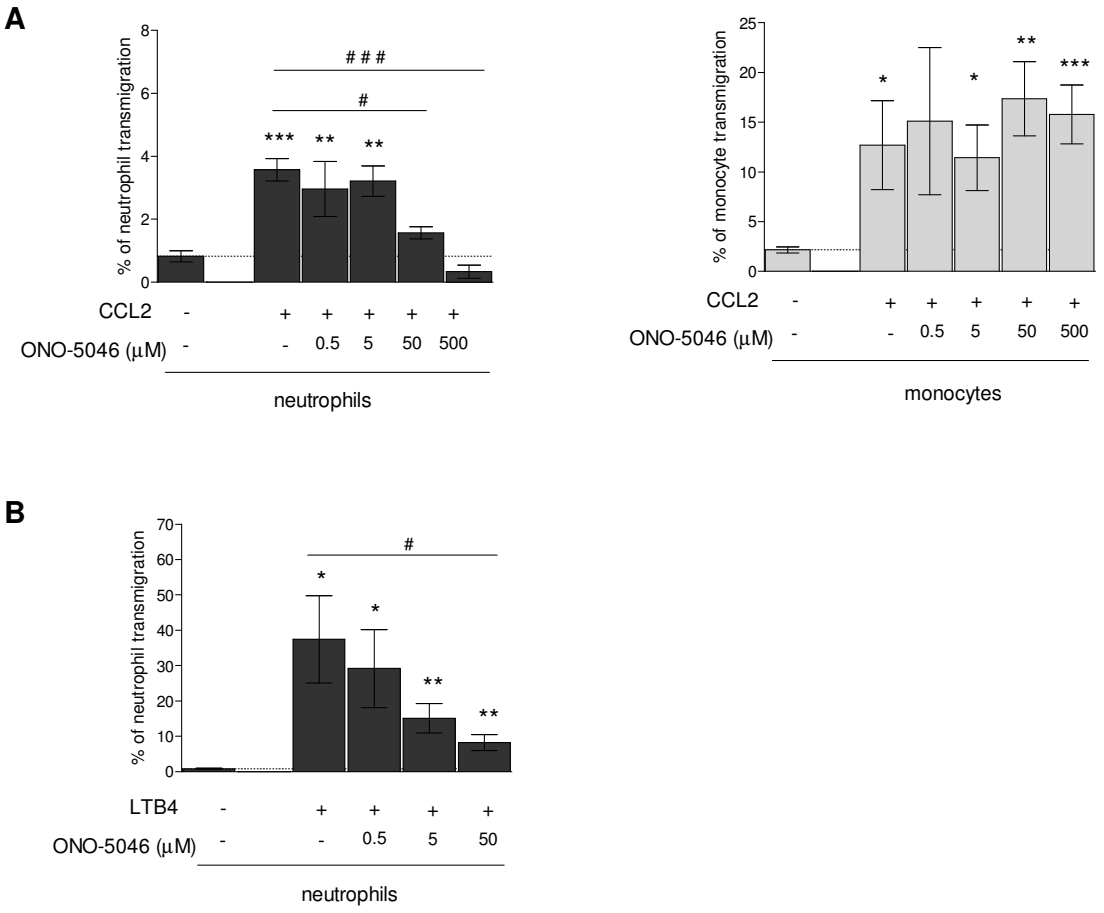
To further investigate the deformability properties of neutrophils and monocytes, the effects of an inhibitor of actin G polymerisation, latrunculin B, and an inhibitor of myosin II contraction, blebbistatin[9], were tested on leukocyte transmigration *in vitro* using a transwell assay. Briefly, the migration of monocytes and neutrophils (from a mixed leukocyte preparation isolated from $CX_3CR1^{eGFP/+}$ mice added to the top chamber through filters of 5μm pores) was assayed in response to CCL2 or LTB_4 (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 LTB_4, 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 37ºC for 3h in the absence or presence of CCL2 (A) or LTB_4 (B), both at a concentration of 5×10^{-9}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 and monocytes as discriminated by GR1 staining and CX_3CR1^{eGFP}. The results show the migratory response of monocytes (A) and neutrophils (B) in response to CCL2 and LTB_4, 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/LTB_4-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 $CX_3CR1^{eGFP/+}$ 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_4 (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_4 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_4 did not induce the migration of murine monocytes in the present *in vitro* assay (not shown).

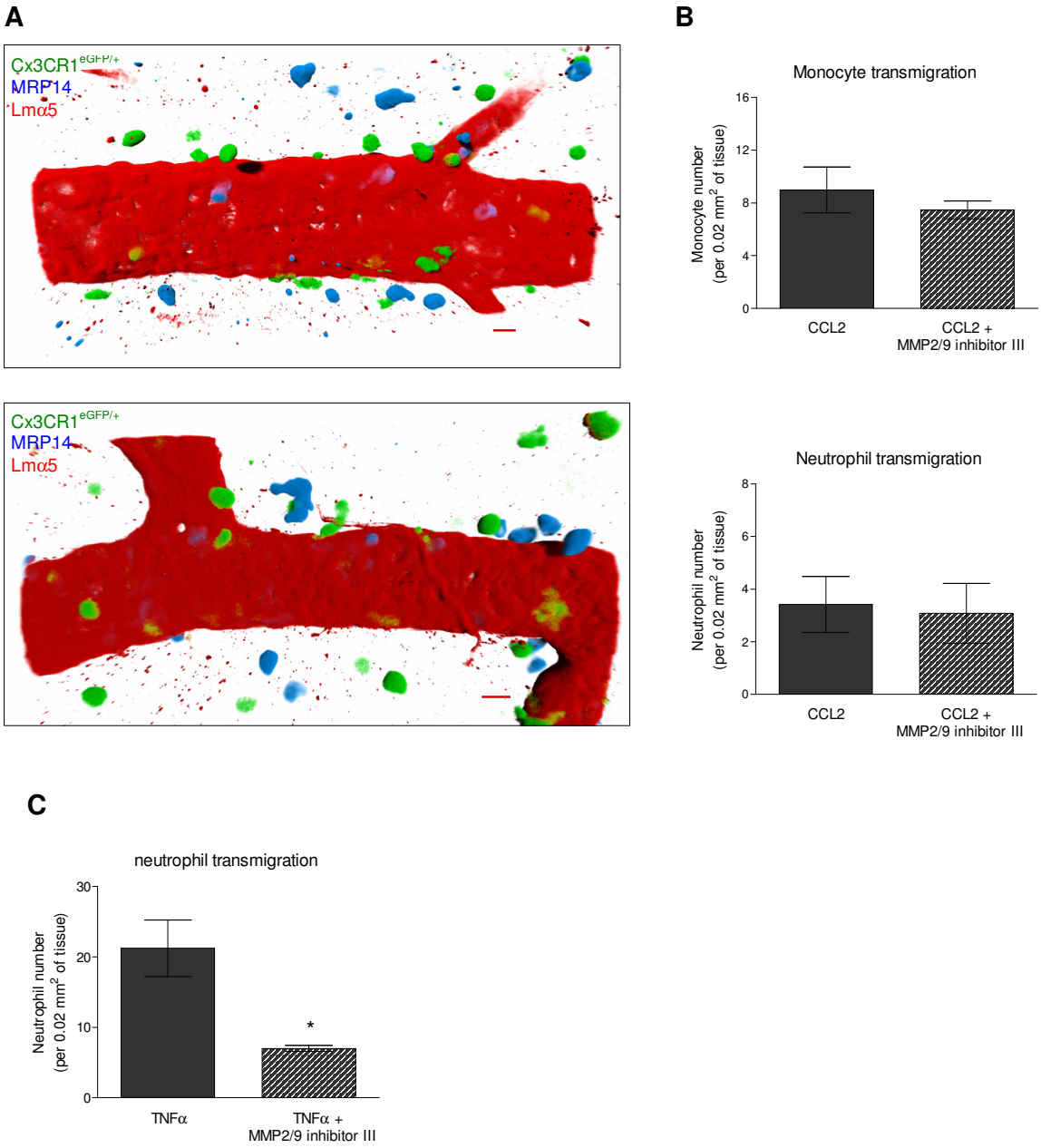


Supplementary Figure II: Effect of ONO-5046 on leukocyte migration through protein-coated filters *in vitro*. Total peripheral blood leukocytes from CX3CR1$^{eGFP/+}$ mice were pre-treated with different concentrations of ONO-5046 (0.5-500 μM) before being added to the top chambers of laminin/collagen IV coated filters of chemotaxis chambers (pore diameter size of 5μm). Cells were incubated at 37ºC for 3h in the absence or presence of CCL2 (A) or LTB_4 (B), both at a concentration of 5×10^{-9}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 CX_3CR1^{eGFP}. The results show the migratory response of monocytes and neutrophils in response to CCL2 or LTB_4 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/LTB_4-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, $CX_3CR1^{eGFP/+}$ 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 described[6] 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$^{eGFP/+}$ 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, * $P<0.05$.

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