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Different microvascular permeability responses elicited by the CXC chemokines MIP-2 and KC during leukocyte recruitment: Role of LSP1

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ABSTRACT

The CXC chemokines keratinocyte-derived chemokine (KC, CXCL1) and macrophage inflammatory protein-2 (MIP-2, CXCL2) activate G-protein coupled receptor CXCR2 and are believed to have similar inflammatory effects in mice. Their specific signaling mechanisms remain elusive. A wide variety of cellular events, mediators and signaling pathways are known to regulate microvascular permeability. Leukocyte-specific protein 1 (LSP1), a Ca^{2+}- and F-actin binding protein, is one of the major downstream substrates of p38 MAPK. LSP1 was previously shown to play a pivotal role in leukocyte transmigration and microvascular permeability. Using intravital microscopy visualizing microvasculature of murine cremaster muscle, we demonstrate that KC and MIP-2 triggered increased leukocyte recruitment which was significantly reduced in LSP1-deficient mice compared to the wild-type control mice. Fluorescence imaging revealed that KC induced more substantial increases of microvascular permeability to FITC-labeled albumin than MIP-2. We found that LSP1 had a more prominent role in microvascular hyperpermeability induced by KC than that triggered by MIP-2. Moreover, Western blotting showed enhanced phosphorylation of p38 MAPK in the cremasteric tissue after stimulation with KC but not with MIP-2 and KC-induced but not MIP-2-induced hyperpermeability was blunted by pharmacological inhibition of p38 MAPK. In conclusion, LSP1 plays an important role in leukocyte recruitment induced by both KC and MIP-2. KC elicits more profoundly increased microvascular permeability than MIP-2. KC is at least partially effective through LSP1 and the phosphorylation of p38 MAPK.

1. Introduction

Leukocyte recruitment and vascular permeability increases in the postcapillary venules are the hallmarks of acute inflammation. Leukocyte–endothelial cell interactions elicit a variety of intracellular signaling events that are critical for the increases of microvascular permeability [1] and the passage of leukocytes to the extravascular tissue [2]. However, the specific role of various chemoattractants in modulating microvascular permeability during leukocyte recruitment remains obscure.

The keratinocyte-derived chemokine (KC, CXCL1) and the macrophage inflammatory protein-2 (MIP-2, CXCL2) belong to the ELR-CXC chemokine family with a 3-amino acid sequence motif that immediately precedes the CXC motif. Both chemokines participate in leukocyte recruitment in various tissues during inflammation [3,4]. The biological effects of KC and MIP-2 on leukocyte recruitment vary in different animal models and tissues. Neutrophil emigration in cremaster muscle treated with KC and MIP-2 did not differ significantly [5,6], whereas MIP-2 was shown to be more potent than KC in leukocyte recruitment [7,8] and in endothelial cell chemotaxis [9]. KC and MIP-2 have also been shown to complement each other in their functions [10]. MIP-2 but not KC was shown to augment cyclophilin A-induced neutrophil migration [11]. Tissue-specific and time-dependent expression patterns of KC and MIP-2 also differ suggesting a possible difference in their roles in tissue-specific neutrophil recruitment [12,13].

A key regulator of transendothelial migration of neutrophils is the intracellular Ca^{2+}- and F-actin-binding leukocyte-specific protein 1 (LSP1) which is expressed in leukocytes and endothelial cells and modulates cell polarity and motility [14]. LSP1 serves as a substrate for protein kinase C and p38 mitogen-activated protein kinase (MAPK) [15–17] and these kinases also regulate cytoskeletal dynamics in endothelial cells [18,19]. LSP1-deficient ($Lsp1^{-/-}$) neutrophils exhibit impaired chemotaxis in response to KC [20]. Moreover, $Lsp1^{-/-}$ mice showed impaired neutrophil emigration paralleled by a disproportionate increase in vascular permeability after stimulation with TNFα and KC [21,22]. The formation of

Abbreviations: CXCR2, CXC chemokine receptor 2; KC, keratinocyte-derived chemokine; MIP-2, macrophage inflammatory protein-2; LSP1, leukocyte-specific protein 1; p38 MAPK, p38 mitogen-activated protein kinase; TNFα, tumor necrosis factor α; PI3K, phosphoinositide 3-kinase; IL-8, interleukin-8.

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endothelial transmigratory dome-like structures which regulates microvascular permeability during neutrophil emigration is compromised in *Lsp1*$^{-/-}$ mice [21]. However, the role of LSP1 in the comparative functional effects of MIP-2 and KC in endothelial cell permeability has never been reported.

The present study explores the role of LSP1-mediated mechanisms that contribute to the differential effects of the chemokines KC and MIP-2 on microvascular permeability during neutrophil recruitment.

2. Materials and methods

2.1. Animals

129/SvJ WT mice were purchased from the Jackson Laboratory. *Lsp1*$^{-/-}$ mice on the 129/SvJ background were generated by homologous recombination by Jongstra-Bilen and colleagues as described previously [23]. Mice of these two genotypes were bred to obtain age- and sex-matched controls. The animal protocols were approved by the local University Committee on Animal Care and Supply.

2.2. Measurement of leukocyte recruitment by intravital microscopy

Male mice (8–16 weeks old) were anesthetised with an i.p. injection of 10 mg/kg xylazine (Bayer, Toronto, ON, Canada) and 200 mg/kg ketamine hydrochloride (Rogar, Montreal, QC, Canada) and the left jugular vein was cannulated for intravenous access. The mouse cremaster muscle preparation was used to study the behavior of leukocytes in the microcirculation as described previously [22,24,25]. The cremaster muscle was superfused with 37 °C-warmed bicarbonate-buffered saline (pH 7.4; containing in mM 133.9 NaCl, 4.7 KCl, 1.2 $MgSO_4$ and 20 $NaHCO_3$). An upright microscope (model BX61WI, Olympus) with a LUCPLFLN 20× objective lens was connected to a 3CCD color video camera (DXC-900, Sony) for bright-field intravital microscopy. The number of rolling, adherent, and emigrated leukocytes was determined in the cremasteric postcapillary venule (25–40 μm diameter) as described previously [21,22,25]. Where indicated, neutrophil recruitment was induced in the venule of the exposed cremaster muscles by superfusion with KC or MIP-2 (both 5 nM; from R&D Systems, MN, USA) or control buffer for 1 h. Histology studies confirmed that after KC or MIP-2 addition, more than 90% of the emigrated leukocytes after stimulation were neutrophils as previously reported [5,22,26] and, therefore, leukocytes are referred to as neutrophils in this study.

2.3. Measurement of microvascular permeability

The same cremasteric postcapillary venule was used for the measurements of leukocyte recruitment and microvascular permeability. FITC-labeled bovine serum albumin (BSA) at 25 mg/kg (Sigma, St. Louis, MO, USA) was injected to the mice i.v. at the start of the experiment, and FITC-derived fluorescence (excitation at 495 nm, and emission at 525 nm) in the venule was detected using a monochrome deep-cooled CCD digital camera (Retiga SRV, QImaging). Images were recorded at different time points during superfusion with KC, MIP-2 or control buffer and the permeability index was determined using the METAMORPH software (MetaMorph®, Molecular Devices Inc., PA, USA) as described previously [21,22]. Where indicated, the cremaster muscle was superfused (30 min prior to and 1 h following addition of MIP-2 or KC) with p38 MAPK inhibitor SB203580 (100 nM; EMD, Billenca, MA, USA).

2.4. Western blotting

For the detection of phosphorylation of p38 MAPK, cremaster muscles were stimulated with KC and MIP-2, excised and snapped frozen in liquid nitrogen. The tissue was homogenized and lysed in lysis buffer (pH 8.0; containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease and phosphatase inhibitor cocktails purchased from Fisher Scientific, Toronto, ON, Canada). Proteins (40 μg) were solubilized in Laemmli sample buffer at 95 °C for 5 min and resolved by 10% SDS–PAGE. For immunoblotting, proteins were transferred onto a nitrocellulose membrane and blocked with 5% BSA in TBST at room temperature for 1 h. Then, the membrane was incubated with affinity purified rabbit anti-phospho-p38 MAPK antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. After incubation with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature, antibody binding was detected with the ECL detection reagent (GE Healtcare, Baie d'Urfe Quebec, Canada). Total p38 MAPK (rabbit anti-p38 antibody, 1:1000, Cell Signaling Technology) and β-actin (mouse anti-β-actin antibody, 1:1000, Santa Cruz Biotechnology) were detected after stripping with a buffer (pH 6.8; contaning 0.5 M Tris–HCl, 2% SDS and 0.7% 2-β-mercaptoethanol). Densitometric quantification of the detected bands was performed using Gene Snap Software (Syngene, Frederick, MD, USA).

2.5. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-hoc test or Student *t* test, as appropriate. *n* denotes the number of mice studied in each group. Values of $p < 0.05$ were considered statistically significant.

3. Results

By using intravital microscopy, the effects of the same molar concentration of MIP-2 and KC (5 nM) on leukocyte recruitment to cremasteric postcapillary venules were measured in WT and *Lsp1*$^{-/-}$ mice. Either MIP-2 or KC superfusion on the cremaster muscle similarly reduced leukocyte rolling flux and rolling velocity, an observation that was not significantly different in the two genotypes (data not shown). MIP-2 and KC similarly increased neutrophil adhesion in WT mice, whereas neutrophil adhesion in *Lsp1*$^{-/-}$ mice was slighltly but significantly higher in response to MIP-2 than to KC (Fig. 1A). A further series of experiments explored the role of LSP1 in neutrophil emigration from postcapillary venules upon treatment with MIP-2 or KC. Treatment with either MIP-2 or KC significantly increased emigration of neutrophils which was higher in WT mice than in *Lsp1*$^{-/-}$ mice, an observation pointing to the participation of LSP1 in emigration upon stimulation with both CXC chemokines (Fig. 1B).

Next, we explored the role of LSP1 in vascular permeability changes during neutrophil recruitment by quantifying the fluorescence dynamics of FITC-labeled albumin leakage after intravenous infusion. As illustrated in Fig. 2, the permeability index in cremasteric postcapillary venules in WT and *Lsp1*$^{-/-}$ mice was slightly increased at 30 min and significantly enhanced on 60 min superfusion with KC in comparison to equal molar concentrations of MIP-2. The permeability index did not significantly differ in the two genotypes of mice on treatment with either MIP-2 or KC. As was already demonstrated in Fig. 1A, LSP1 played an important role in neutrophil adhesion triggered by KC but not MIP-2. It is

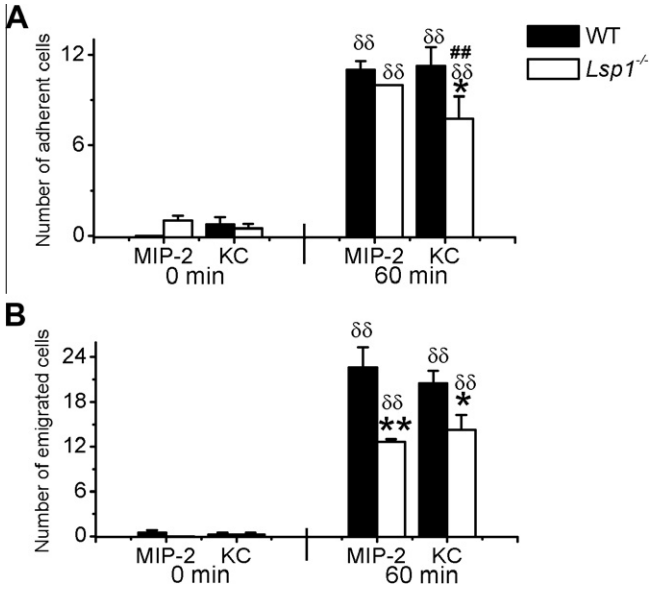

Fig. 1. The role of LSP1 in MIP-2- and KC-triggered neutrophil adhesion and emigration. (A) The number of adherent neutrophils (cells/100-μm vessel) at the start of superfusion (0 min, left bars) and 60 min after superfusion (right bars) with 5 nM of MIP-2 or KC in wild type (WT; black bars) and in LSP1-deficient mice ($Lsp1^{-/-}$; white bars). Data are means ± SEM ($n = 3$). * indicates significant difference ($p < 0.05$) between the genotypes. ## indicates significant difference ($p < 0.01$) between treatment with different CXC chemokines. δδ indicates significant difference ($p < 0.01$) between time points (t test). (B) The number of emigrated neutrophils (cells/220x210 μm) at the start of superfusion (0 min, left bars) and 60 min after superfusion (right bars) with 5 nM of MIP-2 or KC in wild type (WT; black bars) and in LSP1-deficient mice ($Lsp1^{-/-}$; white bars). Data are means ± SEM (n = 3). *, ** indicate significant difference ($p < 0.05$ and $p < 0.01$, respectively) between the genotypes. δδ indicates significant difference ($p < 0.01$) between time points (t test).

interesting to note that superfusion of KC elicited a significantly increased vascular permeability in comparison to MIP-2 (Fig. 2).

To characterize the role of LSP1 in microvascular permeability changes during neutrophil recruitment, we addressed the potential cross-talk of vascular permeability with neutrophil adhesion and emigration by evaluating the ratio of the permeability indexes over the numbers of adherent and emigrated neutrophils 60 min after superfusion with either MIP-2 or KC. In response to KC, on average, each adherent neutrophil from $Lsp1^{-/-}$ mice significantly contributed more to the permeability increase than each adherent neutrophil from WT mice (Fig. 3A). On treatment with MIP-2, the ratio between permeability index and number of adherent cells did not differ between WT and $Lsp1^{-/-}$ mice (Fig. 3A). However, in $Lsp1^{-/-}$ mice, each adherent neutrophil contributed more than 2.5-fold to microvascular permeability in response to KC than that in response to MIP-2 ($p < 0.01$). In response to KC or MIP-2, each emigrated neutrophil from $Lsp1^{-/-}$ mice increased significantly more microvascular permeability than each emigrated neutrophil in WT mice (Fig. 3B). It is worth noting that in $Lsp1^{-/-}$ mice, each emigrated neutrophil contributed about twice as much as microvascular permeability in response to KC as compared with the contribution of each emigrated neutrophil in response to MIP-2, demonsrating the additional difference in the role of LSP1 in permeability changes in response to KC and MIP-2.

In view of LSP1 being one of the major substrates downstream of the p38 MAPK signaling pathway, we performed further experiments to elucidate the signaling mechanisms in the modulation of p38 MAPK in KC- and MIP-2-induced microvascular permeability during neutrophil recruitment in vivo. To this end, WT cremaster tissue was superfused with equal molar concentration of KC or MIP-2 or with bicarbonate-buffered saline as the control for 1 h. After 1 h chemokine superfusion, phosphorylated p38 MAPK was significantly higher in KC-stimulated cremaster tissue than in MIP-2- or saline-treated cremaster muscles, implying the activation of p38 MAPK-mediated signaling mechanisms after KC but not MIP-2 stimulation (Fig. 4A and 4B). Additional experiments explored whether enhanced p38 MAPK phosphorylation was critical in KC-induced microvascular permeability. To this end, pharmacological inhibition of p38 MAPK by superfusion with SB203580 (100 nM) in WT mice, significantly blunted the hyperpermeability induced by KC but not by MIP-2 (Fig. 4C) suggesting the participation of p38 MAPK in KC- but not MIP-2-elicited microvascular permeability increases.

4. Discussion

Microvascular hyperpermeability during inflammation is regulated by various signaling events initiated during neutrophil–endothelial cell interactions [27]. Inflammatory mediators including chemokines foster endothelial cell contractility and microvascular permeability increases [1]. The endothelial CXC chemokine receptor 2 (CXCR2) is critical in the regulation of microvascular permeability by inflammatory stimuli [28] and pharmacological inhibition of CXCR2 attenuated both neutrophil recruitment and vascular permeability [29]. A recent study reported that endothelial LSP1 deficiency resulted in disproportionate microvascular permeability despite profoundly lower numbers of transmigrating neutrophils on stimulation with KC and TNFα [21,22]. Our present observations extend this recent work by elucidating the effect of the CXC chemokine MIP-2 and comparing it to that of KC on neutrophil adhesion, transmigration and microvascular permeability by defining the LSP1-mediated molecular mechanisms that contribute to the differences between the two CXC chemokines. Similarly to KC, MIP-2 triggered an increase in the number of adherent and transmigrated neutrophils in the cremasteric microvasculature. LSP1 deficiency, however, led to decreased neutrophil transmigration but not adhesion upon stimulation with MIP-2. Previous observations reported diminished leukocyte adhesion in $Lsp1^{-/-}$ mice upon TNFα and IL-1β but the decrease was not as substantial as in transendothelial migration [14,21,22]. It is possible that LSP1 may be involved in regulating signaling mechanisms and adhesion molecules in response to KC but not MIP-2 during neutrophil adhesion to the endothelium.

We show that microvascular permeability upon KC stimulation is significantly higher compared to stimulation with MIP-2 and LSP1 deficiency did not abrogate the increased microvascular permeability after stimulation with both chemokines. Various steps of leukocyte-endothelial interactions critically govern the transendothelial passage of intravascular solutes [30,31]. Neutrophil adhesion to the endothelium is essential to stimulate microvascular permeability via ligation of endothelial ICAM-1 which further stimulates the generation of reactive oxygen species, enhanced endothelial cytosolic Ca^{2+} activity and cytoskeletal reorganization [32]. In view of this phenomenon, the microvascular permeability per adherent neutrophil was similar in $Lsp1^{-/-}$ mice and WT mice upon stimulation with MIP-2 but was much higher in $Lsp1^{-/-}$ mice after stimulation with KC than with MIP-2. Assessing the proportionality between the number of emigrated neutrophils and permeability, our data reveal significantly higher permeability index per emigrated cell on KC treatment compared to MIP-2 in $Lsp1^{-/-}$ mice. These results clearly indicate that LSP1 is a key player in regulating microvascular permeability upon stimulation with KC but its role is minimal upon stimulation with MIP-2.

p38 MAPK activates LSP1 which promotes actin polymerization and remodeling [33]. Remarkably, stimulation with KC but not MIP-2 enhanced the phosphorylation of p38 MAPK in the cremasteric tissue. By using specific p38 MAPK inhibitor, we confirmed

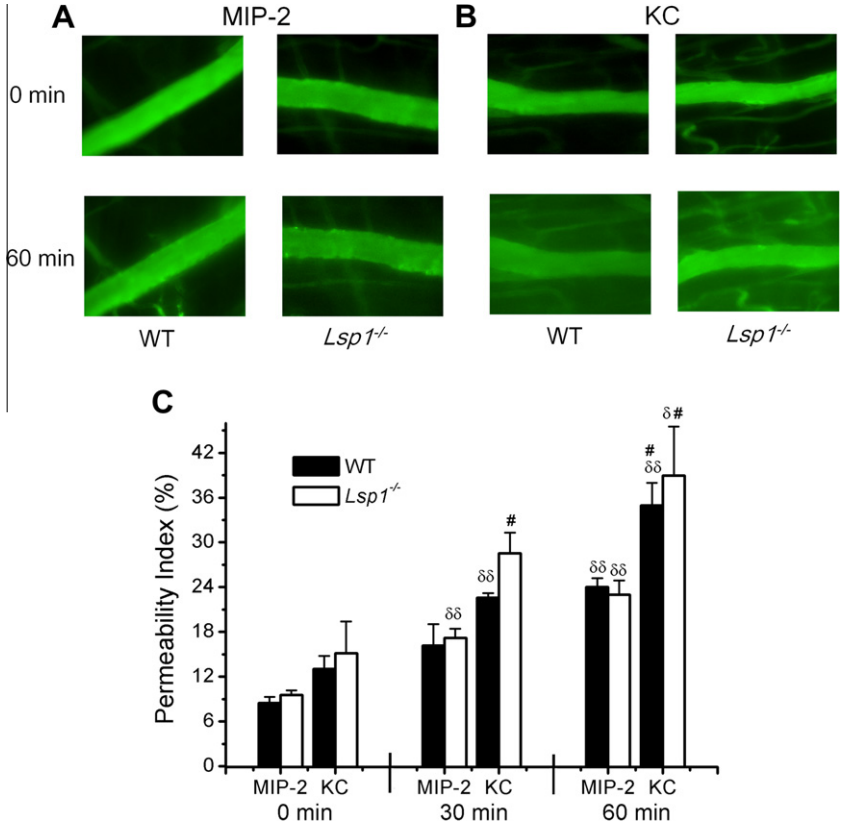

Fig. 2. The role of LSP1 in MIP-2- and KC-triggered microvascular permeability. (A, B) Representative fluorescence microscopy images of cremasteric postcapillary venules immediately after i.v. of FITC-labeled BSA in wild type (WT, left panels) and LSP1-deficient mice (*Lsp1*$^{-/-}$; right panels) before (0 min, upper panels) and 60 min after superfusion (lower panels) with MIP-2 (A) and KC (B). (C) The permeability index (PI%) in the cremasteric postcapillary venules at the start of superfusion (0 min, left bars), 30 min (middle bars) and 60 min after superfusion (right bars) with 5 nM of MIP-2 or KC in wild type (WT; black bars) and in LSP1-deficient mice (*Lsp1*$^{-/-}$; white bars). Data are means ± SEM ($n = 3$). # indicates significant difference ($p < 0.01$) between treatments with CXC chemokines. δ and δδ indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) when comparing to 0 min (*t* test).

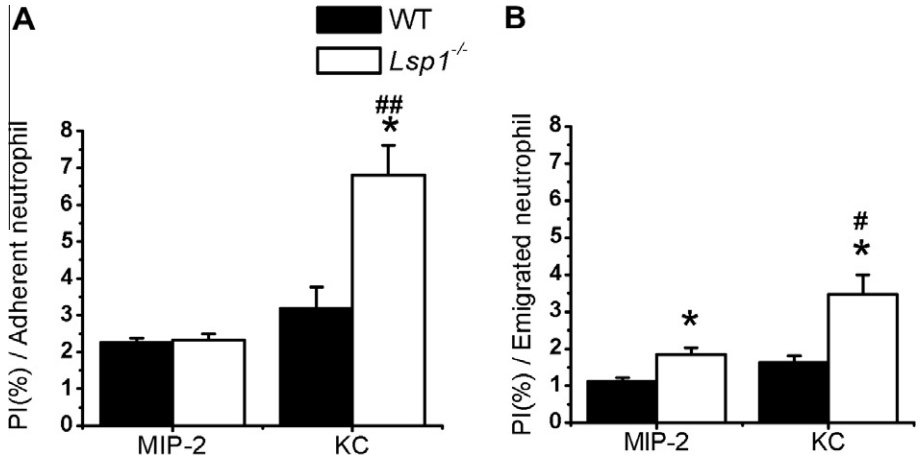

Fig. 3. The role of LSP1 in the adhesion- and emigration-mediated microvascular permeability elicited by MIP-2 and KC. (A) The permeability index/adherent neutrophil ratio after 60 min superfusion with 5 nM of MIP-2 (left bars) and KC (right bars) in wild type (WT; black bars) and in LSP1-deficient mice (*Lsp1*$^{-/-}$; white bars). Data are means ± SEM ($n = 3$). * indicates significant difference ($p < 0.05$) between the two genotypes (*t* test). ## indicates significant difference ($p < 0.01$) between the treatment groups (*t* test). (B) The permeability index/emigrated neutrophil ratio after 60 min superfusion with 5 nM MIP-2 (left bars) and KC (right bars) in wild type (WT; black bars) and in LSP1-deficient mice (*Lsp1*$^{-/-}$; white bars). Data are means ± SEM ($n = 3$). * indicates significant difference ($p < 0.05$) between the two genotypes (*t* test). # indicates significant difference ($p < 0.05$) between the treatment groups (*t* test).

that p38 MAPK signaling is a key regulator in KC- but not in MIP-2-triggered microvascular permeability increases. Specific chemokine-induced signaling mechanisms are not completely understood. There is a growing body of evidence that the signaling mechanisms evoked during neutrophil recruitment may vary depending on the chemoattractant [34,35]. Previously it has been shown that MIP-2-mediated neutrophil recruitment may function in a p38 MAPK-independent [36] and PI3K-dependent [37] manner during inflammation. Pharmacological inhibition of p38 MAPK did not significantly modify leukocyte rolling, adhesion and emigration

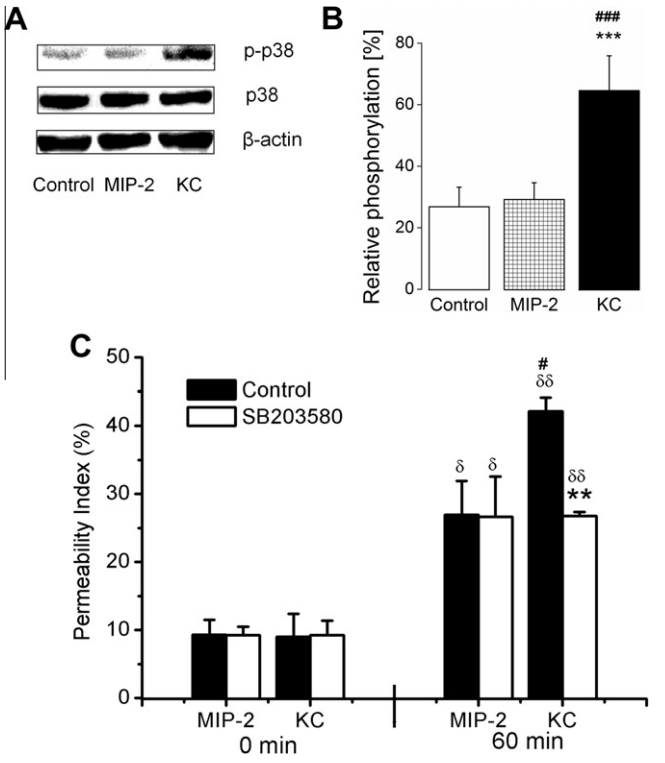

Fig. 4. Phosphorylation of p38 MAPK and its role in microvascular permeability increases induced by MIP-2 and KC. (A) Original Western blots (representative for four similar experiments) demonstrating the expression of phosphorylated p38 MAPK (p-p38, upper bands), total p38 MAPK (p38, middle bands) and the respective β-actin protein abundance (β-actin, lower bands) determined in cremasteric tissue after superfusion with saline (Control) or 5 nM MIP-2 or KC for 1 h. (B) The relative abundance of phosphorylated p38 MAPK over total p38 MAPK in cremaster muscles determined by densitometric analysis after 1 h superfusion with saline (Control; white), MIP-2 (check) and KC (black). Data are means ± SEM ($n = 4$). *** indicates significant difference ($p < 0.001$) in comparison to saline superfusion. ### indicates significant difference ($p < 0.001$) between the two chemokine superfusions (ANOVA). (C) The permeability index (PI%) in the cremasteric postcapillary venules at the start of superfusion (0 min, left bars) and 60 min after superfusion (right bars) with 5 nM of MIP-2 or KC (Control; black bars) and on superfusion (30 min prior to and 60 min following addition of MIP-2 or KC) with SB203580 (100 nM; white bars). Data are means ± SEM ($n = 3$). # indicates significant difference ($p < 0.01$) between treatments with CXC chemokines. * indicates significant difference ($p < 0.05$) in comparison to saline superfusion. δ and δδ indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) when comparing to 0 min (t test).

stimulated by MIP-2 [36]. p38 MAPK signaling regulates endothelial cell hyperpermeability induced by different mediators such as thrombin and platelet-activating factor [38,39]. KC has previously been shown to stimulate p38 MAPK phosphorylation and modulates leukocyte emigration but not rolling and adhesion [40]. Interestingly, IL-8-mediated neutrophil recruitment was shown to be independent of p38 MAPK signaling [41]. These reports strongly suggest the signaling and functional variations between closely related CXC chemokines.

In conclusion, the present observations provide a novel insight into the mechanistic differences in CXC chemokine-induced endothelial hyperpermeability. Our observations demonstrate that KC has a more potent effect on permeability in cremasteric microvasculature than MIP-2, an effect at least partially regulated by p38 MAPK in KC-mediated hyperpermeability. Endothelial LSP1 is critical in neutrophil adhesion and permeability elicited by KC but not MIP-2. Stimulation with KC but not MIP-2 induces phosphorylation of p38 MAPK which affects the function of the downstream molecule LSP1 and this may explain the divergent effects of the two CXC chemokines.

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