CCL2 Is a Vascular Permeability Factor Inducing CCR2-Dependent Endothelial Retraction during Lung Metastasis

Marko Roblek1, Darya Protsyuk1, Paul F. Becker2, Cristina Stefanescu1, Christian Gorzelanny3, Jesus F. Glaus Garzon1, Lucia Knopfova4,5, Mathias Heikenwalder2,6, Bruno Luckow7, Stefan W. Schneider3, and Lubor Borsig1

Abstract

Increased levels of the chemokine CCL2 in cancer patients are associated with poor prognosis. Experimental evidence suggests that CCL2 correlates with inflammatory monocyte recruitment and induction of vascular activation, but the functionality remains open. Here, we show that endothelial Ccr2 facilitates pulmonary metastasis using an endothelial-specific Ccr2-deficient mouse model (Ccr2fl/fl). Similar levels of circulating monocytes and equal leukocyte recruitment to metastatic lesions of Ccr2−/−KO and Ccr2−/−littermates were observed. The absence of endothelial Ccr2 strongly reduced pulmonary metastasis, while the primary tumor growth was unaffected. Despite a comparable cytokine milieu in Ccr2−/−KO and Ccr2−/− littermates the absence of vascular permeability induction was observed only in Ccr2−/−KO mice. CCL2 stimulation of pulmonary endothelial cells resulted in increased phosphorylation of MLC2, endothelial cell retraction, and vascular leakiness that was blocked by an addition of a CCR2 inhibitor. These data demonstrate that endothelial CCR2 expression is required for tumor cell extravasation and pulmonary metastasis.

Implications: The findings provide mechanistic insight into how CCL2–CCR2 signaling in endothelial cells promotes their activation through myosin light chain phosphorylation, resulting in endothelial retraction and enhanced tumor cell migration and metastasis.

Introduction

Metastasis is the main reason for cancer-related fatalities. Hematogenous metastasis is a multistep process depending on interactions of disseminated tumor cells with the microenvironment (e.g., platelets and leukocytes) and ultimately with the endothelium in distant organs (1, 2). Tumor cell migration through vascular barriers is promoted by the recruitment of monocytic cells that contribute to formation of a metastatic niche (3–5). Proinflammatory chemokines, particularly CCL2, were linked to the accumulation of CCR2-expressing, inflammatory monocytes in metastasis (6–9). High levels of proinflammatory chemokines, e.g., CCL2 and CCL5, in circulation are associated with poor prognosis for cancer patients (reviewed in ref. 10). Therefore, many studies explored the genetic and pharmacologic inhibition of the CCL2–CCR2 axis as a mean to impair monocyte recruitment to metastatic sites. Whereas CCL2 clearly potentiates monocyte recruitment in vivo (11), other tumor- or stromal-derived chemokines (e.g., CCL3, CCL5) may contribute to this process in vivo (4, 10). Similarly, inflammatory monocytes, defined as Ly6C+ and CCR2+ cells, express several chemokine receptors, e.g., CCR1, which can facilitate chemokine-driven monocyte recruitment to sites of inflammation or tumorigenesis (12). For instance, CCR2 was not required for inflammatory monocyte recruitment in an acute inflammation model (13, 14). The CCL2–CCR2 chemokine/chemokine receptor axis is required for the egress of inflammatory monocytes from the bone marrow to the circulation during homeostasis and inflammation (13, 14). Thus, systemic Ccr2 deficiency or a treatment with CCL2 neutralizing agents resulted in reduced circulating monocyte numbers (8, 13). In addition, increased circulating serum levels of CCL2 resulted in reduced responsiveness of blood cells to other chemokines (15). Similarly, anti-CCL2 antibody treatment of rheumatoid arthritis patients results in higher CCL2 serum levels and worsening of the disease symptoms (16).

The CCL2–CCR2 chemokine/chemokine receptor axis is required for the egress of inflammatory monocytes from the bone marrow to the circulation during homeostasis and inflammation (13, 14). Thus, systemic Ccr2 deficiency or a treatment with CCL2 neutralizing agents resulted in reduced circulating monocyte numbers (8, 13). In addition, increased circulating serum levels of CCL2 resulted in reduced responsiveness of blood cells to other chemokines (15). Similarly, anti-CCL2 antibody treatment of rheumatoid arthritis patients results in higher CCL2 serum levels and worsening of the disease symptoms (16).
CCL2 induced vascular leakiness (18) and regulated macrophage transendothelial migration (TEM; ref. 17). Furthermore, CCR2-mediated endothelial activation was linked to tumor-associated angiogenesis (19). Although tumor cell–derived CCL2 expression is associated with enhanced metastasis, the analysis of metastatic lungs revealed that recruited monocytes and endothelial cells significantly contribute to an increased pool of CCL2 at metastatic sites (5). Recently, endothelial activation by CCL2 was directly linked to tumor cell–induced lung vascular permeability (9, 20); however, the underlying mechanism remains unclear.

Endothelial retraction is an essential step in metastasis both during intravasation and extravasation. Phosphorylation of VE-cadherin has been shown to be essential for initiation of TEM of leukocytes (21) and also tumor cells (5). Recent data show that VE-cadherin rearrangement in endothelial cells is associated with enhanced phosphorylation of myosin light chain 2 (MLC2) during angiogenesis (22). In addition, inhibition of VE-cadherin rearrangement, which is associated with reduced MLC2 phosphorylation, in endothelial cells maintains endothelial barrier function during inflammation (23).

Tumor-derived chemokines actively shape the tumor microenvironment and directly affect various cell types both at primary and at metastatic sites (10, 24). Here we show that CCR2 signaling in lung endothelial cells induces cytoskeletal rearrangement resulting in enhanced vascular permeability required for monocyte-assisted tumor cell TEM and metastatic initiation.

Materials and Methods

Cell culture

Primary lung endothelial cells were isolated by immunomagnetic selection using anti-CD31 antibody and cultivated as described previously (9). Bone marrow monocytes were isolated from femur and tibia followed by magnetic anti-CD115 purification (all inhibitors were purchased from Tocris). The first 4 inhibitors were used according to the manufacturer’s recommendations (Tocris): for H-1152 and blebbistatin, we used concentrations as previously published (28). Upon removal of inhibitors from the endothelial cells MC-38GFP cells (2 × 10⁴) were added in the presence or absence of CD115⁺ monocytes (1 × 10⁵) in RPMI1640/3% FCS. Transmigration was induced with RPMI1640/10% FCS in the lower chamber and terminated after 16 hours. Alternatively, MC-38GFP cells (2 × 10⁴) were added to the upper insert with or without rhCCL2 (1 μg/mL; kindly provided by A. Kungl, University of Graz). The number of transmigrated MC-38GFP cells was counted using a Zeiss AxioVision microscope (n ≥ 3).

Transendothelial electrical resistance

Transendothelial electrical resistance (TEER) was measured using Electric Cell-Substrate Impedance Sensing (ECIS) as reported (29). When a constant impedance of the endothelial layer was detected (24 hours after seeding), rhCCL2 (10 μg/mL) was added to cells. Impedance was measured every 48 seconds (ECIS-zeta system; Applied BioPhysics Inc.) for 22 hours at a frequency of 4,000 Hz, while cells were continuously maintained in a humidified atmosphere at 37°C and 5% CO₂. Statistical significance was calculated by an unpaired t test.

Preparation of cell lysates

Primary lung microvascular cells or bEnd.3 cells were stimulated with 40 ng/mL IL1β (R&D Systems) for 2 hours in RPMI1640/10% FCS and washed with PBS prior to the addition of CCL2; 100 ng/mL (R&D Systems) in RPMI1640/10% FCS for indicated times. CCR2 was blocked with 50 μmol/L RS504393 inhibitor during IL1β and CCL2 stimulation. Cells were scraped-off the plates in ice-cold 1× PBS, centrifuged for 5 minutes and lysed in 100 μL cell lysis buffer (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1% Triton X-100) supplemented with phosphatase inhibitor cocktail 3 (Sigma) and complete protease inhibitor cocktail (Roche) for 20 minutes on ice. After centrifugation, cell lysates were stored at –80°C.

Western blot

Cell lysate (20 μg) was separated on a 15% SDS-PAGE gel, transferred to a nitrocellulose membrane Protran 0.45 NC (GE Healthcare) and blocked with 5% BSA/TBS-T (1% Tween 20) for 1 hour at RT. Primary antibodies: rabbit antiphosphoMLC2 (Ser19; Cell Signaling Technology), anti–β-catenin (Cell Signaling Technology), anti–VE-cadherin (Abcam), and mouse anti–β-actin (Sigma) were incubated overnight at 4°C. After washing with TBS-T (3 ×), the membrane was incubated with a secondary antibody: HRP-linked-anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG, HRP-linked (Cell Signaling Technology) for 1 hour at RT. After washing with TBS-T (3 ×), the membrane was developed with ECL West Dura solution (Thermo Fisher Scientific), and chemiluminescent signal was detected using Hyperfilm ECL (GE Healthcare).

Immunoprecipitation

The endothelial cell line bEnd.3 was stimulated with 40 ng/mL IL1β (R&D Systems) for 2 hours in RPMI1640/10% FCS. After

Downloaded from mcr.aacrjournals.org on March 22, 2021. © 2019 American Association for Cancer Research.
washing with PBS, cells were incubated with 1 μg/mL rhCCL2 in RPMI/10% FCS for 1 hour. Cell lysates were prepared as mentioned above, and β-catenin was immunoprecipitated with anti–β-catenin antibody overnight at 4°C in the presence of protein G beads (GE Healthcare). Beads were washed (3×) with the lysis buffer, boiled in Laemmli buffer and separated on a 7.5% SDS-PAGE gel, followed by Western blotting.

Cell sorting of endothelial cells
Lungs of mice were perfused with PBS followed by digestion with Collagenase A and Collagenase D (2 mg/mL each, Roche) in RPMI1640/2% FCS for 1 hour at 37°C. The digested tissue was filtered through a 100-μm cell strainer, RBC lysed, and filtered again through a 40-μm cell strainer. Resuspended cells were incubated with an F, block (eBioscience) for 10 minutes in FACS buffer (PBS/10 mmol/L EDTA, 2% FCS), followed by incubation with antibodies: CD45-PB, CD11b-APC-Cy7, CD31-PE-Cy7, Ly6C-FITC, and Ly6G-PerCP-Cy5.5 (all from BD) for 30 minutes on ice. After washing, endothelial cells (CD45–CD11b–CD31+) were sorted on a FACSAria III 5L (BD).

RNA preparation, cDNA generation, and qPCR
RNA extraction was used for RNA isolation from cells in vitro, and TRI Reagent (Sigma) was used for RNA isolation from perfused lungs. cDNA was prepared from 250 ng RNA using the Omniscript RT Kit (Qiagen) according to the manufacturer’s instructions. Real-time PCR was performed using SYBR Green JumpStart Taq ReadyMix kit (Sigma) with gene-specific primer pairs. cDNA was prepared from 250 ng RNA using the Omniscript RT Kit (Qiagen) according to the manufacturer’s instructions. Real-time PCR was performed using SYBR Green JumpStart Taq ReadyMix kit (Sigma) with gene-specific primer pairs.

Flow cytometry of peripheral blood and lungs
Blood was mixed with PBS/5 mmol/L EDTA and spun down. Lung tissue was digested as described above. After RBC lysis with PharmLyse (BD), resuspended leukocytes were incubated with F, block (eBioscience) in FACS buffer for 10 minutes and followed by staining with the following antibodies: CD45-APC-Cy7, CD11b-PE-Cy7, Ly6C-FITC, Ly6G-PerCP-Cy5.5, CD45-PerCP-Cy5.5, CD3-APC-Cy7, CD4-FITC, CD8-PE-Cy7, CD11b-BV510, CD19-APC, NK1.1-PerCP-Cy5.5, CD45-APC-Cy7, CD11b-PE-Cy7, Ly6C-FITC, Ly6G-PerCP-Cy5.5 (all from BD) for 30 minutes on ice. For staining purposes, the samples were fixed again with 4% paraformaldehyde. The digested tissue was filtered through a 100-μm cell strainer. Resuspended cells (CD45–CD11b–CD31+) were sorted on a FACSAria III 5L (BD).

Cytokine analysis
Cytokines in lung tissue lysates (200 μg) were measured with the ProcartaPlex Mouse Panel 1 (26-plex) kit and VEGF (10 ng) with the Mouse VEGF-A Platinum ELISA kit (both eBioscience). Statistical analysis was performed with the GraphPad Prism software (version 6.03). All data are presented as mean ± SEM and were analyzed by ANOVA with the post hoc Bonferroni multiple comparison test. Analysis of 2 groups was performed with the Mann–Whitney test unless stated otherwise.

Results
Characterization of mice with endothelial-specific deletion of Ccr2
To define the function of endothelial CCR2 in controlling tumor cell extravasation, we made a mouse with an endothelial cell-specific deletion of Ccr2. For this purpose, the VE-cadherin-Cre mouse (26) was bred with the Ccr2fl/fl mouse (25) to generate the VE-cad-Cre/Cr2fl mice (hereafter Ccr2KO mice). The cell-specific deletion of Ccr2 was confirmed by qPCR of sorted pulmonary endothelial cells (Supplementary Fig. S1A). We observed the same levels of circulating inflammatory (Ly6Cint) and patrolling (Ly6Chi) monocytes in Ccr2fl/fl and Ccr2fl/fl mice (Supplementary Fig. S1B). In contrast, reduced numbers of Ly6Cint and Ly6Chi monocytes were detected in Ccr2fl/fl mice as reported previously (9, 13). Other circulating leukocyte subsets were not altered between Ccr2fl/fl and Ccr2fl/fl littermates. Unchanged amount of CCR2 expression was detected on inflammatory monocytes (Ly6Cint) in the peripheral blood of Ccr2fl/fl and Ccr2fl/fl mice (Supplementary Fig. S1C, left). Because VE-cadherin expression has also been detected in myeloid CD11b+ cells (26), we analyzed the expression of CCR2 in knock-in mice expressing...
VE-cadherin-GFP from the VE-cadherin genetic locus Cdh5-EGFP (27). We observed no GFP expression in circulating Ly6C\(^{hi}\) cells (Supplementary Fig. S1C, right). Next, we analyzed the endothelial progenitor cells (CD45\(^{-}\) CD31\(^{+}\) CD146\(^{+}\)) of the lungs (31). A reduced CCR2 expression was detected on endothelial progenitor cells from Ccr2\(^{-/-}\)KO mice when compared with Ccr2\(^{+/+}\) mice (Fig. 1A). Importantly, the level of CCR2 expression on inflammatory monocytes in Ccr2\(^{-/-}\)KO mice remained unaffected (Supplementary Fig. S1C). Overall, these data indicate that endothelial Ccr2 deletion does not interfere with the homeostasis of inflammatory monocytes.

Primary tumor growth is not altered by the absence of endothelial Ccr2
To assess the role of endothelial Ccr2 during tumorigenesis, mice were s.c. injected with Lewis lung carcinoma (LLC1.1) cells and spontaneous metastases to the lungs were determined. The tumor weight of dissected primary tumors was similar in all mouse genotypes after 14 days (Fig. 1B). Histologic analysis of primary tumors revealed no difference in the number of recruited F4/80\(^{+}\) macrophages, Ly6G\(^{+}\) neutrophils, CD3\(^{+}\) T cells, and B220\(^{+}\) B cells between Ccr2\(^{+/+}\) and Ccr2\(^{-/-}\)KO mice (Fig. 1C; Supplementary Fig. S1D). The number of proliferating cells...
(Ki67+ cells), apoptotic cells (cleaved-caspase3+ cells) in primary tumors was identical between both mouse genotypes. To assess the effect of endothelial CCR2 deficiency on tumor angiogenesis, we analyzed tumor vessel density, vessel area and vessel permeability (Fig. 1D). We observed no differences in any of the endothelial parameters in primary tumors irrespective of endothelial CCR2 expression, suggesting that overall endothelial physiology, including tumor angiogenesis, is not affected by endothelial CCR2.

The absence of endothelial Ccr2 attenuates lung metastases

After the primary tumor removal, mice were terminated at 28 days after tumor cell injection (p.i.) and spontaneous lung metastases analyzed (Fig. 2A and B). We observed a significant reduction of metastases in Ccr2 ecKO mice, when compared with Ccr2fl/fl littermates. Although the number of metastases in Ccr2fl/fl mice was comparable with wild-type (C57BL/6) mice, the reduced metastases detected in Ccr2ecKO mice were comparable with Ccr2−/− mice. Histologic analysis of lung metastases revealed no difference in the number of infiltrating macrophages (F4/80+ cells), neutrophils (Ly6G+), T cells (CD3+), and B cells (B220+) between lungs from Ccr2−/− or Ccr2fl/fl mice (Fig. 2C; Supplementary Fig. S2A). Importantly, we found similar vascular density (CD31+ area) within the metastatic foci in both mouse genotypes, indicating no difference in angiogenesis (Fig. 2C). The number of proliferating cells (Ki67+ cells) and apoptotic cells (cleaved caspase-3+ cells) was equal in the metastatic foci of Ccr2ecKO and Ccr2fl/fl mice.

We further tested the endothelial CCR2 requirement for metastases using intravenous injection of LLC1.1 and colon carcinoma cells MC-38GFP. Experimental metastases of both cell types were significantly reduced in Ccr2ecKO mice when compared with Ccr2fl/fl littermates (Supplementary Fig. S2B and S2C). These results show that the absence of endothelial Ccr2 strongly attenuates the generation of pulmonary metastases.
Figure 3.
Initiation of the premetastatic niche is not impaired in Ccr2ecKO mice. A, Flow cytometry analysis of inflammatory monocytes (CD45+CD11b+Ly6G-Ly6C+) recruited to the lungs at 12 and 24 hours after i.v. MC-38GFP injection of Ccr2fl/fl and Ccr2ecKO mice. Untreated naive mice (n) were used as controls. B, Endothelial cells (CD45-CD10-Th-CD31+) sorted from lungs of Ccr2fl/fl and Ccr2ecKO mice 12 hours after i.v. MC-38GFP injection or untreated (naive) mice were analyzed for the expression levels of E-selectin, VCAM-1, SAA1+2, and SAA3 (n = 3–6). C, Amounts of cytokines in perfused lung homogenates of Ccr2fl/fl and Ccr2ecKO mice at 12 and 24 hours after i.v. MC-38GFP injection. Untreated mice (control) were used as controls. D, Flow cytometry analysis of myeloid cells (CD45+CD11b+), granulocytes (CD45+CD11b+Ly6G+), Ly6Cint monocytes (CD45+CD11b+LyG-C0 Ly6Cint), and inflammatory monocytes (CD45+CD11b+Ly6G-Ly6C+) recruited to lungs of mice s.c. injected with LLC1.1 cells after 7 and 14 days. E, Amounts of cytokines detected in lung homogenates of mice s.c. injected with LLC1.1 cells after 7 and 14 days. F, Lung vascular permeability assay of Ccr2fl/fl and Ccr2ecKO mice 14 days after s.c. injection of LLC cells, including representative macroscopic images. Statistical significance in B, D, E, and F was assessed using an unpaired t test; *, P < 0.05; **, P < 0.01; ††, P < 0.001.
Formation of the metastatic niche in lungs remains unaltered in Ccr2−/−KO mice.

To test whether the endothelial Ccr2-deficiency influences the metastatic niche formation, we analyzed lungs from mice that were intravenously injected with MC-38GFP cells. Previous analysis has shown that LLC1.1 and MC-38GFP cells induce similar responses during lung metastasis (5). We observed increased recruitment of inflammatory monocytes (Ly6G−/C0 cells) at 12 hours p.i. in both Ccr2−/−fl and Ccr2−/−KO littermates (Fig. 3A), which returned to normal levels 24 hours p.i., as reported previously for wt mice (9). The numbers and the recruitment kinetics of myeloid cells (CD11b+/C0), granulocytes (Ly6G−), and Ly6Cint monocytes were similar between the 2 genotypes (Supplementary Fig. S3A).

The analysis of tumor cell–induced endothelial activation revealed an increase in E-selectin and VCAM-1 mRNA expression 12 hours p.i. that was similar in both Ccr2−/−fl and Ccr2−/−KO littermates (Fig. 3B). Serum amyloid A3 (SAA3) and S100A8 amyloid A expressions (SAA1, SAA2, and SAA3) in sorted endothelial cells from lungs of C57BL/6 mice (EC) were pretreated with RS-504393 (50 μmol/L), and blebbistatin (2.5 μmol/L) for 2 hours and washed out of the inhibitor prior to the addition of tumor cells (Supplementary Fig. S3B). Similarly, increased amounts of myeloid cell subpopulations were detected in the peripheral blood at day 14, which correlated with the primary tumor growth progression (Supplementary Fig. S3C).

An increase in chemokines (e.g., CXCL1, CCL7, and CCL2) in premetastatic lungs was observed with tumor growth progression (Fig. 3C). Chemokines (CXCL1, CCL2, CCL3, and CCL7) and cytokines (GM-CSF and IL6) associated with endothelial activation and monocyte recruitment/activation increased at 12 hours p.i. and returned to normal levels at 24 hours p.i. irrespective of a mouse genotype. Thus, the absence of endothelial Ccr2 has no apparent effect on leukocyte recruitment, cytokine milieu, or endothelial activation in naive or metastatic lungs.

Increased vascular permeability is dependent on endothelial Ccr2 expression

To test whether the absence of endothelial Ccr2 affects the formation of a premetastatic niche, we analyzed lungs of mice s.c. injected with LLC1.1 cells at 7 and 14 days p.i. by flow cytometry. Increased recruitment of myeloid cells (CD11b+), granulocytes (Ly6G−), Ly6Cint monocytes, and inflammatory monocytes (Ly6C+ to the premetastatic lungs was detected at day 14 in both Ccr2−/−fl and Ccr2−/−KO littermates (Fig. 3D). Lymphoid cell recruitment remained the same irrespective of a mouse genotype (Supplementary Fig. S3B). Similarly, increased amounts of myeloid cell subpopulations were detected in the peripheral blood at day 14, which correlated with the primary tumor growth progression (Supplementary Fig. S3C).

Figure 4.

CCL2 stimulation induces endothelial retraction and TEM of tumor cells. A. TEM of MC-38GFP cells through lung endothelial cells isolated from C57BL/6 wild-type (BL6), Ccr2−/−fl and Ccr2−/−KO mice, either in the absence (−) or in the presence of CD115+ monocytes (+). The number of migrated tumor cells was counted per view field (pvf) and is normalized to the control (−monocytes); ns; not significant; *P < 0.05. B. TEM of MC-38GFP cells in the absence (−) or in the presence of CD115+ monocytes (+). Endothelial cells from C57BL/6 mice (EC) were pretreated with RS-504393 (50 μmol/L), 2-APB (50 μmol/L), BAPTA-AM (10 μmol/L), ML-7 (10 μmol/L), H-1152 (5 μmol/L), and blebbistatin (2.5 μmol/L) for 2 hours and washed out of the inhibitor prior to the addition of tumor cells (+monocytes). DMSO as a diluent of inhibitors was used as a control. Tumor cell migration was analyzed as described in A. *P < 0.001. C. TEM of MC-38GFP cells (without monocytes) only in the presence of rhCCL2 (1 μg/mL) compared with a control. **P < 0.01. D. TEER measurement of primary lung endothelial cells upon stimulation with rhCCL2 (10 μg/mL). TEER values for rhCCL2-treated and control samples were normalized to 1 at the start of stimulation (0 hours). Black bars, control; open bars, CCL2 treated; n = 4; *P < 0.05; other time points showed no significant differences.
but did not differ between Ccr2<sup>fl/fl</sup> and Ccr2<sup>ecKO</sup> mice (Fig. 3E). Despite reduced metastasis in the absence of endothelial Ccr2, no changes in the metastatic niche of the lungs were observed. Thus, we tested whether the lung vasculature is altered in tumor-bearing mice at 14 days. We detected small, but significant, reduction in the vascular permeability of Ccr2<sup>ecKO</sup> mice when compared with Ccr2<sup>fl/fl</sup> littermates (Fig. 3F). These findings indicate that the endothelial Ccr2 deficiency prevents induction of vascular permeability, which is required for lung metastasis.

**CCL2 induces loosening of endothelial junctions and the contraction of endothelial cells**

Reduced vascular permeability has been previously linked to attenuated tumor cell extravasation and metastasis (5, 9). Thus, we tested the capacity of tumor cells to migrate through pulmonary endothelial cells derived from Ccr2<sup>ecKO</sup> and Ccr2<sup>fl/fl</sup> mice. TEM of tumor cells through wild-type endothelial cells was significantly potentiated by the addition of monocytes (Fig. 4A), as described previously (9). However, TEM of tumor cells was potentiated by monocytes only through endothelial cells derived from Ccr2<sup>fl/fl</sup> but not from Ccr2<sup>ecKO</sup> mice (Fig. 4A). To identify the signaling cascade facilitating the CCL2-induced vascular permeability, we targeted the G-protein–coupled receptor pathway that is required for the actin–myosin complex activation (Fig. 4B). Interestingly, the preincubation of endothelial cells with inhibitors of CCR2 (RS504393), IP<sub>3</sub>-receptor (2-APB), intracellular Ca<sup>2+</sup> (BAPTA-AM), myosin light chain kinase MLCK (ML-7), Rho-associated kinase ROCK (H-1152), and myosin II ATPase (Blebbistatin) significantly reduced TEM of tumor cells. Of note, endothelial cells pretreated with RS504393, ML-7, or H-1152 also showed impaired TEM of 4T1 breast cancer cells [Supplementary Fig. S4A]. These data indicate that inhibition of endothelial retraction attenuates monocyte-assisted TEM of tumor cells. Importantly, an addition of recombinant CCL2 also increased TEM of MC-38GFP cells in the absence of monocytes (Fig. 4C). Because MC-38GFP cells do not express Ccr2 (32), we tested the hypothesis that CCL2 activates endothelial CCR2, which results in endothelial retraction. We measured TEER of primary pulmonary endothelial cells after stimulation with CCL2. TEER of the endothelial monolayer decreased upon CCL2 stimulation compared with the control (Fig. 4D; Supplementary Fig. S4B), confirming that CCL2 stimulation triggers endothelial retraction.

Activation of actin–myosin contraction is required for cell retraction and depends on phosphorylation of myosin light chain 2 (pMLC2; ref. 33). Stimulation of endothelial cells with CCL2 resulted in accumulation of pMLC2 (Fig. 5A). The analysis of an endothelial cell line bEnd.3 showed that CCL2 activation induced the accumulation of pMLC2 1 to 2 hours after stimulation (Fig. 5B). We observed no changes in total MLC2 expression but only an increase in MLC2 phosphorylation (Supplementary Fig. S5).
Fig. S5A). To prove that endothelial pMLC2 accumulation is driven by CCL2 stimulation, we treated bEnd.3 cells with a CCR2 inhibitor that resulted in diminished phosphorylation of MLC2 (Fig. S5C). Loosening of the endothelial barrier is caused by the dissociation of VE-cadherin/β-catenin complex (34). CCL2 stimulation of endothelial cells resulted in dissociation of the VE-cadherin/β-catenin complex (Fig. 5D), hence confirming a direct effect of CCL2 on endothelial barrier function. CCL2-activated bEnd.3 cells also showed reduced VE-cadherin colocalization with β-catenin (Supplementary Fig. S5B–S5C), thereby complementing the immunoprecipitation data (Fig. 5D). These data provide evidence that CCL2-driven activation of endothelial CCR2 results in the cytoskeletal rearrangement and loosening of the endothelial cell junctions and thereby facilitates an efficient TEM of tumor cells.

Discussion

Dissemination of tumor cells during metastasis is promoted by platelet- and monocyte-assisted extravasation from the circulation (3, 5, 9, 35). Particularly, the chemokine/chemokine receptor axis CCL2–CCR2 appears to be involved in several steps during metastasis, including leukocyte recruitment, angiogenesis, immune suppression, and cancer cell extravasation (6–9, 35). Because the systemic inhibition of CCR2/CCL2 interferes with the egress of inflammatory monocytes from the bone marrow (13, 36), the observed reduced recruitment of monocytes to metastatic sites is rather a consequence of reduced numbers of circulating Ly6C+ cells. To dissect the contribution of endothelial Ccr2 from the Ccr2-dependent monocyte recruitment to metastasis, we generated an endothelial cell–specific Ccr2 deletion mouse model (Ccr2flflKO mice). In this model, we observed no alteration in the numbers of circulating cells or in the recruitment of inflammatory monocytes to metastatic lungs. Nevertheless, a significant attenuation of metastasis was observed in Ccr2flflKO mice when compared with control littermates. Taken together, these findings suggest that the local activation of endothelial Ccr2 is essential for tumor cell extravasation during lung metastasis.

Endothelial CCR2 has been linked to regulation of the blood–brain barrier vascular permeability during inflammation (18, 37) and to the regulation of angiogenesis (38, 39). Recently, we have shown that endothelial CCR2 promotes metastasis by facilitating tumor cell extravasation (9). In this report, we show that neither the primary tumor growth nor the tumor angiogenesis were affected by the absence of endothelial CCR2, which is in agreement with previous findings that endothelial CCR2 expression has been observed only in the brain and the lungs (9, 38). Notably, the absence of endothelial Ccr2 did not alter the endothelial activation upon tumor cell challenge.

TEM of cells is a tightly regulated process that controls distribution of leukocytes throughout the organism (40). Induction of vascular permeability is essential for an efficient TEM of tumor cells (9, 20, 41). Enhanced vascular permeability in lungs was previously associated with a Ccr2-dependent increase of inflammatory permeability factors SAA1+2 and SAA3 (20). We observed similar cytokine and chemokine levels in the lungs of tumor-challenged mice of Ccr2flflKO and Ccr2fl+/+ genotypes. Although similar levels of permeability factors such as VEGF-A, SAA3, and CCL2 were detected in the lungs of Ccr2flflKO and Ccr2fl+/+ mice, we observed impaired induction of vascular permeability only in Ccr2flflKO mice. These results demonstrate that endothelial CCR2 is the regulator of tumor cell–induced lung vascular permeability. Furthermore, TEER was decreased by CCL2 stimulation of endothelial cells, indicating the loosening and disassembly of endothelial intercellular junctions. Inhibition of CCR2 or the proteins regulating the endothelial actin–myosin cytoskeleton (MLCK, ROCKII, and myosin II) severely impaired tumor cell TEM in vitro. In endothelial cells, CCL2 phosphorylation represents the sole regulation of myosin II ATPase activity during inflammation or angiogenesis (22, 23, 42–44). Indeed, CCL2 stimulation of endothelial cells induced phosphorylation of MLCK, which could be blocked by a CCR2 inhibitor. Presented data provide evidence that CCR2-mediated endothelial activation induces disassembly of adherens junctions, thereby facilitating TEM of tumor cells.

In conclusion, we describe a mechanism how endothelial CCR2 regulates lung metastasis through the activation of the actin–myosin cytoskeleton using by an endothelial cell–specific Ccr2-deficient (Ccr2flflKO) mouse model. Translation of CCR2 inhibition into clinical setting will require timely and spatially defined approach, due to the homeostatic function of CCL2–CCR2 signaling mediating the release of monocytes from the bone marrow (36). Systemic inhibition of CCL2 in mouse models led to reduction of breast cancer metastasis, however; cessation of a treatment resulted in accelerated tumor growth due to an enhanced release of monocytes from the bone marrow (8). Nevertheless, targeting of CCR2 inhibitors to the metastatic niche attenuated metastasis without any side effects (32, 45). Further progress in cell-specific targeting will show whether CCR2 targeting will be of therapeutic value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Roblek, M. Heikenwalder, L. Borsig Development of methodology: M. Roblek, D. Protsyuk Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Roblek, D. Protsyuk, C. Stefanescu, C. Gorzelanny, J.F. Glau Gaszoon, L. Knoplova, B. Luckow, S.W. Schneider, L. Borsig Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Roblek, D. Protsyuk, P.F. Becker, C. Stefanescu, J.F. Glau Gaszoon, M. Heikenwalder, S.W. Schneider, L. Borsig Writing, review, and/or revision of the manuscript: M. Roblek, P.F. Becker, M. Heikenwalder, B. Luckow, L. Borsig Study supervision: L. Borsig

Acknowledgments

This study was supported by the SNF grant #310030-173076 (L. Borsig). The authors acknowledge the assistance of the Center for Microscopy and Image Analysis, University of Zurich, for confocal microscopy experiments. M. Heikenwalder was supported by an ERC Consolidator grant (HepatoMeta- boPath). We thank the group of Dr. D. Vestweber for providing the VE-Cad-GFP mouse.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 23, 2018; revised August 30, 2018; accepted December 4, 2018; published first December 14, 2018.
References


23. Burg N, Swendeman S, Worgall S, Hla T, Salmon JE. Sphingosine-1-Phosphate Receptor-1 signaling mediates leukocyte extravasation and cell barri-}


Molecular Cancer Research

CCL2 Is a Vascular Permeability Factor Inducing CCR2-Dependent Endothelial Retraction during Lung Metastasis

Marko Roblek, Darya Protsyuk, Paul F. Becker, et al.