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Expression, Regulation, and Function of Atypical Chemerin Receptor CCRL2 on Endothelial Cells

Justin Monnier,*†‡ Susanna Lewén,§ Edward O’Hara,*† Kexin Huang,§ Hua Tu,§ Eugene C. Butcher,*†‡ and Brian A. Zabel‡

Chemokine (CC motif) receptor-like 2 (CCRL2) binds leukocyte chemoattractant chemerin and can regulate local levels of the attractant, but does not itself support cell migration. In this study, we show that CCRL2 and VCAM-1 are upregulated on cultured human and mouse vascular endothelial cells (EC) and cell lines by proinflammatory stimuli. CCRL2 induction is dependent on NF-κB and JAK/STAT signaling pathways, and activated endothelial cells specifically bind chemerin. In vivo, CCRL2 is constitutively expressed at high levels by lung endothelial cells and at lower levels by liver endothelium; and liver but not lung EC respond to systemic LPS injection by further upregulation of the receptor. Plasma levels of total chemerin are elevated in CCRL2−/− mice and are significantly enhanced after systemic LPS treatment in CCRL2−/− mice compared with wild-type mice. Following acute LPS-induced pulmonary inflammation in vivo, chemokine-like receptor 1 (CMKLR1)+ NK cell recruitment to the airways is significantly impaired in CCRL2−/− mice compared with wild-type mice. In vitro, chemerin binding to CCRL2 on endothelial cells triggers robust adhesion of CMKLR1+ lymphoid cells through an α5β1 integrin/VCAM-1-dependent mechanism. In conclusion, CCRL2 is expressed by EC in a tissue- and activation-dependent fashion, regulates circulating chemerin levels and its bioactivity, and enhances chemerin- and CMKLR1-dependent lymphocyte/EC adhesion in vitro and recruitment to inflamed airways in vivo. Its expression and/or induction on EC by proinflammatory stimuli provide a novel and specific mechanism for the local enrichment of chemerin at inflammatory sites, regulating the recruitment of CMKLR1+ cells. The Journal of Immunology, 2012, 189: 956–967.

Chemerin is a chemotactic protein for dendritic cell subsets, macrophages, and NK cells (1–3). Chemerin circulates in an inactive proform: activation of chemerin requires proteolytic processing of the carboxyl terminus and removal of inhibitory amino acids (4–7). We and others identified chemerin as a natural nonchemokine chemoattractant ligand for chemokine-like receptor 1 (CMKLR1), and in a recent publication, we “de-orphaned” an additional second receptor for chemerin, serpentine chemokine (CC motif) receptor-like 2 (CCRL2) (3, 5, 8–10). Interestingly, although both CCRL2 and CMKLR1 bind chemerin with high affinity, the downstream functional consequences of ligand binding are quite different. Chemerin binding to CMKLR1 triggers calcium mobilization, receptor and ligand internalization, and cell migration. Alternatively, chemerin binding to CCRL2 does not induce intracellular calcium flux or ligand internalization, but can regulate chemerin bioavailability (3, 10). A third high-affinity chemerin receptor, G protein-coupled receptor 1 (GPR1), has also been recently reported, although it also does not itself support chemerin-dependent cell migration (8).

Chemokine-like receptors recruit leukocytes to inflamed tissues in part by triggering integrin-dependent adhesion to activated vascular endothelium. Several teams reported the colocalization of chemerin with vascular endothelial cells (EC) in multiple inflammatory disorders, such as multiple sclerosis, lupus, and psoriasis, and in endothelial venules of secondary lymphoid tissues (11–14). Although several human EC lines express CMKLR1 and can respond to chemerin in an angiogenesis assay, CCRL2 has not yet been fully investigated in EC biology (15). Given the reported association of chemerin with vascular EC and the potential role of nonclassical chemoattractant receptor CCRL2 in augmenting local chemerin levels, we characterized the expression, regulation, and function of CCRL2 on human and murine vascular EC (10).

In this study, we report that proinflammatory stimuli upregulate atypical chemerin receptor CCRL2 and VCAM-1 on EC via NF-κB and JAK/STAT intracellular signaling pathways. Plasma chemerin levels are significantly elevated in CCRL2−/− mice following systemic LPS injection compared with wild-type (WT) mice and untreated controls, implicating CCRL2 in the regulation of circulating chemerin during inflammation. In an in vivo pulmonary inflammation model, recruitment of CMKLR1+ NK cells into the airways is impaired in CCRL2−/− mice. In vitro, chemerin binding to CCRL2+ EC triggers robust adhesion of CMKLR1+ lymphoid cells via α5β1 integrin/VCAM-1–mediated sticking. Thus, CCRL2+ EC acts in concert with CMKLR1 to coordinate chemerin-dependent leukocyte adhesion in vitro and recruitment in vivo.

Materials and Methods

Animals

CCRL2−/− mice were obtained from Lexicon (The Woodlands, TX) and backcrossed nine generations on the BALB/c background. WT BALB/c mice were obtained from The Jackson Laboratory (Sacramento, CA).

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Abbreviations used in this article: BAL, bronchoalveolar lavage; CCRL2, chemokine (CC motif) receptor-like 2; CMKLR1, chemokine-like receptor 1; EC, endothelial cell; GAG, glycosaminoglycan; GPR1, G protein-coupled receptor 1; h, human; HDMEC, human dermal microvascular endothelial cell; m, mouse; poly(I:C), polyinosinic-polycytidylic acid; RT-qPCR, reverse transcription quantitative PCR; WT, wild-type.

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Reagents

**Soluble mediators.** IL-1, IL-2, IL-4, IFN-γ, IL-10, IL-12, IFN-α, IFN-β, TNF-α, and chemerin were purchased from R&D Systems (Minneapolis, MN). Lipo-tein biochrome (TLR2), fagelisin (TLR3), poly(I:C) (TLR3), CpgA (TLR9), LPS (TLR4), polynucleotides, and glycine-sodium polyacrylate (poly(LC)) [TLR3] were purchased from InvivoGen (San Diego, CA). Vitamin D3, Vitamin D2, and deoxymeth- ansone were obtained from Sigma-Aldrich (Saint Louis, MO, USA). IFN-α, IFN-β were obtained from PBL InterferonSource (Piscataway, NJ, USA).

**Primary Abs.** Mouse (m) Abs included anti-mCCRL2 (clone B2Z3E, generated in-house), rat IgG2a isotype control (clone 9B5, generated in-house), anti-mCMKL1 (clone BZ194, generated in-house, rat IgG2a; clone BZ186, generated in-house, mg1G1), anti-mGPR1 (clone BZ248, generated in-house, rat IgG2a), and anti-mVCAM-1 (clone MK2.7, generated in-house, rat IgG1). Anti-mCD31-PE-Cy7, anti-mCD146-FTTC, anti-mVCAM-1-allophycoerythrin (clone 429) were purchased from Bio-Legend (San Diego, CA, USA), and anti-CD3-PE-Cy7, anti-Ly6G-FITC, and anti-DX5-PE were purchased from eBiosciences (San Diego, CA, USA). Anti-human (h) Abs included anti-hCMKL1 (clone BZ232, generated in-house), anti-hGPR1 (clone BZ1274, generated in-house, rat IgG2b), anti-hVCAM-1-FTTC (clone E110), mfgG2b-FTTC isotype control, and mouse anti-hCCRL2 (clone 152211) were purchased from R&D Systems.

**Secondary Abs.** Secondary Abs include goat anti-rat IgG-PE (R&D Systems), goat anti-flgG-PE (Invirotech, Carlsbad, CA), and goat anti-mouse IgG-Alexa 488 ( Molecular Probes, Carlsbad, CA).

**Primers.** Primers specific for mCCM1-A, hVCAM-1, and hCCRL1 were purchased from SABiosciences (Valencia, CA, USA). Primer sequences for mGPR1, hGPR1, and hCCRL2 are as follows: mGPR1 (forward, 5′-GAGCGCTCAGCATTCATCACA-3′, reverse, 5′-CAACTGGGCAGTGAAGGAAT-3′), hGPR1 (forward, 5′-AATGCACTGTGATTTGTGTTT-3′, reverse, 5′-CAACTGGCGGAGTAGAATG-3′), and hCCRL2 (forward, 5′-GAGCGACTGGAAGTGAAGTA-3′, reverse, 5′-ATTTGCCAGGTGGTGAAGGT-3′). Primers for mCCRL2, mCMKL1, mouse chemerin, human chemerin, and human β-actin were as previously described (10, 16, 17).

**Inhibitors.** Inhibitors included I KKβ phosphorylation inhibitor BAY-11-7082 (Calbiochem, Gibbstown, NJ) and JAK-1 inhibitor sc-204021 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Primary EC isolation**

Mouse liver and lung EC were isolated from BALB/c WT and CCRL2−/− mice. Briefly, livers and lungs were isolated from 8- to 10-week-old mice and digested in 5 mg/ml PBS/collagenase IV (Worthington, Lakewood, NJ) for 45 min at 37°C. Digested tissue was passed over cell striainers of decreasing size (100, 40, and 20 μM) and then centrifuged for 10 min at 300 × g at 4°C. Endothelial cells were enriched using 30% HistoDenz/RPMI 1640 solution (Sigma-Aldrich) after centrifugation at 1500 × g at 4°C. Endothelial cells were enriched using 30% HistoDenz/RPMI 1640 solution (Sigma-Aldrich) after centrifugation at 1500 × g at 4°C. Endothelial cells were enriched using 30% HistoDenz/RPMI 1640 solution (Sigma-Aldrich) after centrifugation at 1500 × g at 4°C. Endothelial cells were enriched using 30% HistoDenz/RPMI 1640 solution (Sigma-Aldrich) after centrifugation at 1500 × g at 4°C.

**Cell culture**

Mouse EC line culture. bEND.3 cells were grown in DMEM media (Life Technologies, Carlsbad, CA, USA) supplemented with pyruvate, nonessential amino acids, l-glutamine, penicillin-streptomycin, and 10% FBS. For in-hibitor experiments, bEND.3 cells were preincubated with the indicated concentration of inhibitor for 1 h, after which fresh media with or without inhibitor with the indicated cytokines were added to the cells and incubated for an additional 24 h. HEK293 and L1.2 cells (pre-B cell mouse lymphoma) were grown in RPMI 1640 supplemented with pyruvate, nonessential amino acids, l-glutamine, penicillin-streptomycin, and 10% FBS. (Life Technologies). Human EC culture. HUVEC and human dermal microvascular EC (HDMEC) were purchased from Lonza (Basel, Switzerland), and a novel human brain microvascular endothelial cell line, hCMEC/D3, was a gift from Prof. P.O. Couraud (Université René Descartes, Paris, France) (18).

**RNA isolation and reverse transcription quantitative PCR**

Total RNA was extracted from cells using an RNAeasy kit (Qiagen, Valencia, CA), after which the total RNA concentration was measured using the Nanodrop spectrophotometer ND-100. Reverse transcription quantitative PCR (RT-qPCR) was performed using RT/Taq SYBR Green qPCR reagents (Invitrogen) using a Stratagene MX3000P thermocycler (Agilent Technolo-gies, Santa Clara, CA). Primers were validated using stringent criteria by verifying that the dissociation curve showed only one peak, and “no reverse transcriptase” controls were used to confirm that qPCR results reflected RNA expression and not genomic DNA contamination. Gene expression was normalized to CD34 for mouse samples and β-actin for human samples. The relative induction value of our genes of interest was calculated using the 2−DDCt method (19). All PCR reactions were done in duplicate.

**Flow cytometry**

A total of 0.5 million cells were used for each staining. For unconjugated Abs, cells were incubated with the indicated primary Abs at 4°C for 30 min in 100 μl PBS/2% FBS/2% mouse serum. Cells were then washed with PBS and centrifuged for 3 min at 2000 rpm. Following the washing step, cells were incubated with secondary goat anti-rat PE (R&D Systems) in 50 μl PBS/2% FBS/2% goat serum. For directly conjugated Abs, cells are incubated with labeled Ab at 4°C for 30 min in 100 μl PBS/2% FBS/2% mouse serum. Cells were washed and centrifuged for 3 min at 2000 rpm, resuspended, and fixed in 200 μl PBS/1% paraformaldehyde and then analyzed by a FACS Calibur (BD Biosciences, Franklin Lake, NJ).

**[125I]checmerin binding assay**

For radioligand binding assays, radiolabeled chemerin (residues 21–148; R&D Systems; custom radiolabeling performed by PerkinElmer) was provided as a gift from J. Jaen (ChemoCentryx, Mountain View, CA). To assess the ability of chemerin to bind to bEND.3 cells treated with cytokines, 5 × 104 cells per well were mixed with 4-fold dilutions of unlabeled chemerin competitor and ~1 nM (0.025 μCi) [125I]checmerin tracer per well in a total volume of 200 μl and agitated at 4°C for 3 h. Levels of cell-bound radioactivity were determined by harvesting the cells on polyethyleneimine-treated 96-well glass filters (PerkinElmer, Waltham, MA) using a cell harvester (PerkinElmer), washing the filters twice with buffer (25 mM HEPES, 500 mM NaCl, 1 mM CaCl2, and 5 mM MgCl2 adjusted to pH 7.1) and measuring the amount of [125I]checmerin bound to each filter (cpm) with a TopCount scintillation counter (PerkinElmer).

**Fc-chemerin**

Recombinant Fc-chemerin protein (residues 23–156) was produced and purified from Chinese hamster ovary cells transient transfection and protein A purification. A DNA fragment corresponding to bioactive mouse chemerin isoform ending in residue 156 (serine) was amplified by PCR and cloned in-frame downstream of human or mouse IgG1 Fc domain, which is downstream of a secretion signal peptide in mammalian expression vector pLEV113 (LakePharma, Belmont, CA). There is a 9-aa glycine-rich linker between the Fc and chemerin domain. Plasmid DNA was transfected into CHO cells using Lipofectamine 2000 (Life Technologies), and cell culture supernatant was collected 3–5 d after transfection. Fc fusion proteins were purified with protein A resin (MabSelect SuRe; GE Healthcare) and final proteins were formulated in 100 mM Tris (pH 7.5), 150 mM NaCl, and 0.45% NaOAc.

**EC adhesion assay**

To assess the ability of CCRL2 on bEND.3 cells to induce adhesion, bEND.3 cells were grown to confluence in 96-well petri dishes. After 24 h treatment with TNF-α/LPS/IFN-γ (20 ng/ml, 1 μg/ml, and 50 ng/ml, respectively), bEND.3 cells were loaded with 50 μl 200 nM chemerin in PBS/0.1% BSA and incubated at 37°C for 30 min. This step serves to load CCRL2 with chemerin. The cells are then washed with PBS to remove unbound chemerin. L1.2-CMKLR1−/− cells (100 μl) at a concentration of 5 × 105 cells/ml, prelabelled with calcein AM (BD Biosciences), were placed on top of the bEND.3 cells and coincubated for 30 min at 37°C. The cells were washed twice with PBS without calcium and magnesium. The number of cells that adhered to the monolayer was then measured by a plate reader at an emission/excitation of 494/517 nm. Images of adherent cells were taken using a fluorescent microscope. Blocking Abs against VCAM-1 (MK27) and α5β1 integrin (P52) were used at a concentration of 10 μg/ml.

**ELISA**

Mice were injected i.p. with LPS (12 mg/kg), euthanized 12 h later, and blood was collected by cardiac puncture. Plasma chemerin concentrations were measured by ELISA (R&D Systems).

**Chemerin internalization assay**

HEK293 cells transfected with hCMKL1 or hCCRL2, bEND.3 cells, and HUVEC were used for chemerin internalization assays. One hundred
thousand cells per well were incubated with mFc-human chemerin for 30 min at 4°C and then washed with cold PBS to remove unbound chemerin. For the microscopy studies, HEK293 transfectants and bEND.3 cells were incubated with secondary Ab goat anti-mouse IgG Alexa 488 (Molecular Probes). After 20 min incubation at 4°C the cells were washed in cold PBS. Subsequently, cells were either placed back at 4°C or incubated at 37°C to allow for labeled Fc-chemerin to internalize. After a final wash in cold PBS, cells were fixed in PBS/1% paraformaldehyde and spun down on microscope slides by cytospin. Fc-chemerin internalization was analyzed by epifluorescence microscopy. For the flow cytometry studies, Fc-chemerin–loaded HUVEC were incubated at 4°C or 37°C for 30 min, washed, and then stained with secondary Ab goat anti-mouse PE. Fc-chemerin internalization was analyzed by flow cytometry.

Acute LPS-induced lung inflammation

WT and CCR2L knockout mice were anesthetized and dosed with 1 μg LPS in 50 μl saline by intranasal injection. Twelve hours after LPS injection the mice were euthanized and the leukocytes that accumulated in the airways were collected by bronchoalveolar lavage (BAL).

BAL fluid leukocyte isolation

After mice were euthanized, a blunt needle was inserted in the exposed trachea. The airway of the mice was washed three times with 1 ml PBS. The recovered fluid was centrifuged, and the recovered leukocytes in the BAL fluid were directly stained with surface markers for T cells (CD3, neutrophils (Ly6G), and NK cells (DX5).

Blood leukocyte isolation

Blood was collected by cardiac puncture after euthanasia and directly mixed with 5 ml PBS without Ca2+/Mg2+ supplemented with 4 mM EDTA to prevent clotting. An equal volume of 2% dextran T-500 in PBS was added, so that the supernatant was collected and centrifuged and incubated with 2 ml RBC lysis buffer (Sigma-Aldrich). The pelletted WBCs were then stained and analyzed by flow cytometry.

In vitro transwell chemotaxis

mCMKLR1/L1.2 cells were used to assess chemerin bioactivity by in vitro transwell migration as previously described (3). For migration experiments, 2.5 × 105 mCMKLR1/L1.2 cells in 100 μl chemotaxis media (either RPMI 1640 plus 10% FCS for prochemerin activation experiments, or RPMI 1640 plus 0.5% BSA for detection of bioactive chemerin in the absence of proteases) were added to the top wells of 5-mm porous transwell plates for 2 h at 37°C, the bottom well cells were harvested and flow cytometry was used to assess migration. To test the amount of prochemerin in plasma samples, 25 μl plasma samples in 600 μl PBS without Ca2+/Mg2+ supplemented with 4 mM EDTA to prevent clotting. An equal volume of 2% dextran T-500 in PBS was added, so that the supernatant was collected and centrifuged and incubated with 2 ml RBC lysis buffer (Sigma-Aldrich). The pelletted WBCs were then stained and analyzed by flow cytometry.

Statistical analysis

Evaluation of significance was performed using a Student t test or ANOVA followed by a Bonferroni posttest. Statistical tests were calculated using the Instat statistical program (GraphPad Software, La Jolla, CA), and graphs were plotted using Prism graphing software (GraphPad Software). Data are expressed as means ± SD or SEM as indicated, and a p value of <0.05 was considered to be significant.

Results

CCR2L and VCAM-1 are upregulated on mouse brain vascular endothelioma cells by proinflammatory cytokines and certain TLR ligands

Given the reported colocalization of chemerin with activated EC in multiple inflammatory diseases, we tested a panel of cytokines and TLR ligands for CCR2L induction in bEND.3 endothelioma cells, a model cell line of mouse brain vascular EC (11–14, 20). A subset of proinflammatory cytokines and TLR ligands [TNF-α, LPS, IFN-γ, IL-1β, IFN-β, and poly(I:C)] induced CCR2L protein expression (Fig. 1A, 1B). The cytokines and factors that upregulated CCR2L were similar to those that induced VCAM-1, although optimal upregulation of CCR2L required synergistic activity of TNF-α with other stimuli (e.g., IFN-γ and LPS), whereas VCAM-1 was highly induced by TNF-α alone (Fig. 1C, 1D); the latter observation is consistent with previous reports (20). Chemerin receptors CMKLR1 and GPR1 were not expressed under any condition, whether assessed by Ab staining or RNA analysis (data not shown).

Kinetics of CCR2L and VCAM-1 RNA and protein induction in LPS-, IFN-γ-, and TNF-α-treated bEND.3 cells

Consistent with the protein expression analysis, CCR2L and VCAM-1 RNA were upregulated by proinflammatory stimuli (Fig. 2A, 2B). We next examined the RNA and protein induction kinetics of CCR2L and VCAM-1 following treatment with TNF-α, LPS, and IFN-γ (Fig. 2B, 2D). RNA expression for both CCR2L and VCAM-1 occurred rapidly and peaked after just 2 h for each. Despite similar RNA induction kinetics, CCR2L protein expression peaked at 24 h after treatment, whereas VCAM-1 surface expression peaked at 8 h (Fig. 2C, 2D).

CCR2L induction is controlled by NF-κB and JAK/STAT signaling pathways

The robust and synergistic induction of CCR2L by the combination of TNF-α, LPS, and IFN-γ in bEND.3 cells prompted us to investigate the intracellular pathways involved. Previously, it was shown that TNF-α and LPS trigger the intracellular NF-κB pathway in EC, whereas IFN-γ activates the JAK-1/STAT-1 pathway; however, more recent evidence indicates that TNF-α and LPS can also activate the STAT-1 pathway, and, conversely, that IFN-γ can indirectly stimulate the NF-κB pathway (21–23). To test the role of NF-κB and JAK-1/STAT-1 in the upregulation of CCR2L, we used pharmacoinhibitors of the NF-κB pathway (BAY 11-7082) and of the JAK-1/STAT-1 pathway (sodium 204021). The NF-κB inhibitor almost completely prevented the induction of CCR2L in bEND.3 cells by TNF-α, LPS, and poly(I:C), but only partially inhibited IFN-γ- or IFN-β–dependent CCR2L induction (Fig. 3). Alternatively, the JAK-1 inhibitor marginally inhibited TNF-α–, LPS–, IL-1β–, or poly(I:C)–induced induction of CCR2L, but almost completely blocked IFN-γ– or IFN-β–dependent CCR2L induction (Fig. 3). Cells treated with NF-κB or JAK inhibitors only partially blocked CCR2L induction when TNF-α, LPS, and IFN-γ were combined together. However, the combination of both NF-κB and JAK-1 inhibitors almost completely blocked CCR2L induction by combined TNF-α, LPS, and IFN-γ, suggesting that both pathways are involved and that they can independently and synergistically upregulate endothelial CCR2L (Fig. 3).

Activated bEND.3 cells bind chemerin

Excess unlabeled chemerin inhibited the binding of either 125I chemerin (IC50, 3 nM; Fig. 4A) or Fc-chemerin (IC50, 8 nM; Fig. 4B) to bEND.3 cells treated with proinflammatory stimuli. Chemerin did not bind to unstimulated bEND.3 cells (Fig. 4).

Expression and regulation of CCR2L in human EC

Primary human umbilical vein and dermal microvascular EC (HUVEC and HDMEC, respectively), as well as a human brain endothelial cell line (hCMVEC/D3), significantly upregulated CCR2L RNA following exposure to TNF-α, LPS, and IFN-γ (Fig. 5A). VCAM-1 RNA was also significantly upregulated as anticipated (data not shown). Furthermore, unstimulated HUVEC expressed CCR2L protein and bound Fc-chemerin, and stimulation with TNF-α, LPS, and IFN-γ slightly increased CCR2L protein expression and Fc-chemerin binding (Fig. 5B).

Expression and regulation of CCR2L on mouse primary EC

We next asked whether CCR2L was expressed on freshly isolated mouse vascular lung and liver EC, as these organs provided an adequate quantity of primary EC for analysis. Interestingly, CD31+
CD146+ mouse lung EC expressed high levels of CCRL2 in the absence of experimental exogenous activation, and mouse liver EC were moderately positive. Abs against CCRL2 failed to stain lung or liver EC from CCRL2-deficient mice, confirming the specificity of the Ab staining. We did not detect any genotype-dependent differences in VCAM-1, CD31, or CD146 expression.

**FIGURE 1.** Selective upregulation of CCRL2 and VCAM-1 in endothelioma cells by proinflammatory stimuli. CCRL2 (A) and VCAM-1 (B) protein expression was measured by flow cytometry on mouse brain endothelioma cells (bEND.3) treated for 24 h with the indicated soluble inflammatory mediators. The induction of CCRL2 and VCAM-1 is represented as a heat map. Histogram plots of CCRL2 (C) and VCAM-1 (D) are representative of the induction of CCRL2 and VCAM-1. Results are representative of three independent experiments.

**FIGURE 2.** Kinetic analysis of CCRL2 and VCAM-1 induction. CCRL2 (A) and VCAM-1 (B) mRNA and protein induction was measured in bEND.3 cells treated with the indicated cytokines for 24 h. The means of three different experiments ± SD are shown. TNF-α plus LPS plus IFN-γ were incubated with bEND.3 cells for the indicated times. At each time point CCRL2 (C) and VCAM-1 (D) induction was measured by either RT-qPCR (normalized to CDC42) or by mAb staining and flow cytometry. Datum points represent the mean of two to three experiments. *p < 0.05 by t test.
on lung or liver EC, suggesting that overall the endothelial cell phenotype is not altered in the CCRL2-deficient animals (Fig. 6).

**In vivo injection of LPS upregulates CCRL2 on liver EC**

LPS injection activates vascular EC in vivo (24, 25). To determine whether endothelial CCRL2 is induced by LPS in vivo, we injected mice systemically with endotoxin, isolated vascular EC from liver and lung, and assessed CCRL2 and VCAM-1 expression and chemerin binding by flow cytometry. CD31 + CD146 + liver EC from LPS-injected WT mice significantly upregulated CCRL2 and bound to Fc-chemerin, whereas similar cells from saline-injected WT mice were CCRL2 low (Fig. 7). LPS injection had no effect on CCRL2 expression or Fc-chemerin binding to WT lung EC relative to isotype control staining (Fig. 8). Furthermore, neither CCRL2 Ab nor Fc-chemerin stained liver or lung EC from LPS-injected or control CCRL2 −/− mice (Figs. 7, 8). Consistent with previous reports, LPS injection upregulated VCAM-1 on liver and lung EC in both genotypes (Figs. 7, 8) (24, 26–29).

**EC CCRL2 captures and concentrates chemerin on the cell surface**

Given our previous data that CCRL2 + lymphoid cells do not internalize bound chemerin, we next asked whether CCRL2 + vascular EC also concentrated chemerin on the cell surface. CCRL2 + bEND.3 cells (treated with TNF-α, LPS, and IFN-γ) bound to Fc-chemerin, whereas untreated cells were negative for chemerin binding (Fig. 9A). Upon shifting the chemerin-loaded cells to an internalization-permissive temperature (37˚C), the bEND.3 cells did not internalize bound ligand (Fig. 9A). CCRL2 + HEK293 transfectants also did not internalize bound Fc-chemerin; however, CMKL1 + HEK293 cells efficiently internalized bound Fc-chemerin when incubated at 37˚C, as evidenced by the cytoplasmic puncti and lack of membrane staining (Fig. 9B). To determine whether these results extend to primary human EC, Fc-chemerin–loaded HUVEC (stimulated with TNF-α, LPS, and IFN-γ) were incubated at 4˚C or 37˚C, washed, and then stained for surface chemerin. The staining intensity of surface Fc-chemerin on HUVEC incubated at 37˚C was similar to the staining intensity at 4˚C, indicating that HUVEC did not internalize bound chemerin (Fig. 9C).

**CCRL2 regulates circulating chemerin levels in vivo**

Given the constitutive expression of CCRL2 lung vascular EC and, to a lesser extent, liver vascular EC, we hypothesized that circulating chemerin levels may be altered in CCRL2 −/− mice owing to the lack of chemerin sequestration in the vasculature. Indeed, plasma levels of total chemerin (measured by ELISA, which detects bioactive chemerin, prochemerin, and larger fragments of...
chemotactically inert chemerin) were slightly but significantly elevated in CCRL2−/− mice compared with WT mice (Fig. 10A). There was no significant difference in the level of bioactive plasma chemerin between WT and CCRL2−/− (Fig. 10B), and there was a slight but nonsignificant increase in prochemerin (detected by ex vivo proteolytic [plasmin] activation) in CCRL2−/− plasma compared with WT (Fig. 10C), as measured by in vitro CMKLR1+ cell migration. Interestingly, in mice dosed with endotoxin to induce systemic inflammation and vascular CCRL2 expression, total chemerin plasma levels were 2-fold higher in CCRL2−/− mice versus WT mice, and 2-fold higher than untreated CCRL2−/− controls (Fig. 10A). Although there was no difference in bioactive plasma chemerin levels between LPS-treated WT and CCRL2−/− mice (Fig. 10B), prochemerin levels in CCRL2−/− plasma were significantly elevated compared with those in WT mice (Fig. 10C). Taken together, these data indicate that the increase in total circulating chemerin in LPS-treated CCRL2−/− mice is due to an increase in prochemerin and possibly inactive chemerin fragments. Interestingly, plasma levels of bioactive chemerin and prochemerin were significantly reduced in LPS-treated WT mice compared with untreated controls (Fig. 10B, 10C). Although plasma from CCRL2−/− mice showed a similar trend, the differences did not reach significance (Fig. 10B, 10C). Thus, CCRL2 regulates circulating chemerin levels and its proteolytic processing in vivo during systemic inflammation.
To isolate the role of vascular endothelium-expressed CCRL2 in regulating circulating chemerin levels, mice were injected i.v. with Fc-chemerin and the levels of plasma Fc-chemerin (as opposed to total chemerin) were measured over time. Plasma Fc-chemerin levels were significantly higher in CCRL2−/− mice compared with WT controls (area under the curve for CCRL2 mice, 40,540 ± 2,630 ng h/ml versus area under the curve for WT mice, 29,550 ± 1,240 ng h/ml; *p < 0.05 by t test; Fig. 10D).

**FIGURE 9.** CCRL2+ EC bind but do not internalize chemerin. HEK293 cells transfected with hCCRL2 or hCMKLR1 (A) as well as bEND.3 cells treated with or without TNF-α plus LPS plus IFN-γ (B) were preincubated with Fc-chemerin and goat anti-mouse IgG-Alexa 488 at 4˚C, and then shifted to 4˚C or 37˚C. Internalization of fluorescently labeled Fc-chemerin was monitored by fluorescence microscopy. The dataset shown is representative of three individual experiments with similar results. Scale bars, 25 μm. (C) HUVEC pretreated for 24 h with TNF-α plus LPS plus IFN-γ were incubated with Fc-chemerin at 4˚C for 30 min. Cells were washed to remove unbound chemerin and then shifted to either 4˚C or 37˚C for 30 min. Surface-bound Fc-chemerin was detected using rat anti-mouse IgG1-PE secondary Ab. Results are representative of three individual experiments with similar results.

**FIGURE 10.** CCRL2 regulates circulating chemerin levels and its bioactivity in vivo. (A) WT and CCRL2−/− mice were either untreated or injected with 12 mg/kg LPS for 12 h, after which chemerin levels in plasma were measured by ELISA. For untreated mice, the means of n = 100 (WT) and n = 92 (CCRL2−/−) individual mice ± SEM are shown. For LPS-treated mice, the mean of six individual mice ± SEM is shown. *p < 0.05, ***p < 0.001 by t test. (B) The chemotactic bioactivity of plasma chemerin from WT and CCRL2−/− mice with or without LPS treatment was evaluated by in vitro mCMKLR1/L1.2 cell migration. *p < 0.01 by t test. (C) Plasma prochemerin levels were evaluated by preincubating plasma samples with plasmin to proteolytically activate chemerin and then assessing mCMKLR1/L1.2 cell migration. For (B) and (C), the mean of n = 9–10 mice ± SD is shown. *p < 0.05, **p < 0.01 by t test. Results are representative of two independent experiments. (D) Fc-chemerin (3 μg) was injected i.v. in WT or CCRL2−/− mice, and Fc-chemerin plasma levels were quantified at the indicated time points. The mean of n = 3 mice ± SD is shown. Statistical significance (**p < 0.01) was determined by ANOVA followed by a Bonferroni post hoc test.

Impaired CMKLR1+ NK cell trafficking into inflamed airways in CCRL2−/− mice

Intranasal injection of LPS causes acute lung inflammation and the accumulation of leukocytes into the bronchoalveolar space (30). Given the high level of CCRL2 expression and chemerin binding by lung EC, we used this pulmonary inflammation model to determine whether CCRL2 deficiency altered the accumulation of CMKLR1+ NK cells into inflamed airways. Although there were no genotype-dependent differences in the total number of BAL infiltrating leukocytes, T cells, or neutrophils, significantly fewer NK cells (measured as absolute number and as a percentage of total BAL leukocytes) accumulated in the airways of CCRL2−/− mice (Fig. 11A–C). Blood NK cells from WT and CCRL2−/− mice expressed similar levels of CMKLR1 (Fig. 11D) and Fc-chemerin binding (data not shown), ruling out differential CMKLR1 receptor expression as a contributing factor in impaired airway NK cell trafficking in CCRL2−/− mice. NK cells themselves are CCRL2+ (data not shown). Additionally, there were no differences in total numbers (or percentages) of circulating NK cells (or other major leukocyte subsets, such as neutrophils) between CCRL2−/− and WT mice (Fig. 11E, 11F, and data not shown). Thus, CCRL2 deficiency selectively impairs the recruitment of CMKLR1+ NK cells in an in vivo model of airway inflammation.

**Chemerin bound to CCRL2+ EC triggers CMKLR1+ cell adhesion**

CCRL2 binds chemerin such that the critical cell-signaling carboxyl terminus remains exposed at the cell surface (10), and chemerin triggers CMKLR1+ macrophage adhesion by inducing α4β1 integrin clustering and binding to VCAM-1–coated plates (31). Because activated bEND.3 cells express high levels of both VCAM-1 and CCRL2 (Fig. 1), and L1.2 lymphoid cells express endogenous α4β1 integrin (32), we hypothesized that CCRL2 on bEND.3 cells could bind chemerin and trigger CMKLR1+ L1.2 cell adhesion (33). Using a static endothelial adhesion assay, we compared the ability of WT or CMKLR1+ L1.2 cells to adhere to untreated or activated CCRL2+ bEND.3 cells in the presence or absence of chemerin. Activated CCRL2+ EC loaded with chemerin triggered significant and robust adhesion of CMKLR1+ L1.2 cells compared with unstimulated CCRL2+ EC (Fig. 12A, 12B). WT L1.2 cells did not adhere to the endothelial monolayer under any condition tested, and chemerin was required for adhesion triggering (Fig. 12A). Blocking Abs against α4 integrin or VCAM-1 abolished chemerin-dependent CMKLR1+ cell adhesion to
CCRL2+ activated endothelium, confirming that the adhesion molecules that mediate cell sticking in this model are \( \alpha_4 \beta_1 \) integrin and VCAM-1 (Fig. 12C).

Discussion

Chemerin is associated with vascular endothelium in the affected tissues of multiple inflammatory disorders, such as multiple sclerosis, lupus, and psoriasis (11–14), but little is known regarding the regulation and role of its receptors on EC. In this study, we show that in a variety of EC, CCRL2, a high-affinity chemerin receptor, is either constitutively expressed (lung EC, liver EC, and HUVEC) and/or induced (bEND.3, hCMEC/D3, HDMEC, HUVEC, and liver EC) by proinflammatory stimuli. As with lymphoid cell-expressed receptor, CCRL2 on EC binds chemerin but does not internalize the ligand. Chemerin bound to CCRL2+ EC triggered robust adhesion of CMKLR1+ lymphoid cells via \( \alpha_4 \beta_1 \) integrin/VCAM-1 interactions (illustrated in Fig. 12D). In vivo, CCRL2 deficiency resulted in selective impairment of CMKLR1+ NK cell accumulation into the airways following experimental pulmonary inflammation. Thus, our data suggest that CCRL2 on EC functions to increase local concentrations of chemerin and recruit CMKLR1+ cells to sites of inflammation.

Although we tested an array of proinflammatory and immune suppressive cytokines, ILs, growth factors, and TLR ligands, only proinflammatory stimuli [TNF-\( \alpha \), IFN-\( \gamma \), IFN-\( \beta \), LPS, and poly(I:C)] induced CCRL2 on the mouse brain endothelial model cell line bEND3. Additionally, proinflammatory factors induced CCRL2 in three human endothelial model cell lines (HUVEC, HDMEC, and hCMEC/d3). We and others reported similar results for CCRL2 induction by mouse peritoneal macrophages and dendritic cells (8, 27), suggesting the involvement of shared pathways for CCRL2 regulation across cell types. Endothelial cells express TNF-\( \alpha \), IFN-\( \gamma \), IFN-\( \beta \), TLR4, and TLR3, consistent with responsiveness (as measured by CCRL2 or VCAM-1 induction) to their respective ligands (34–37). Combinations of proinflammatory mediators were significantly more robust in triggering CCRL2 induction than any individual stimuli (maximal response with TNF-\( \alpha \)/LPS/IFN-\( \gamma \)), consistent with enhanced in-
duction of CCRL2 on human neutrophils by cotreatment with TNF-α and IFN-γ (38), implying that multiple intracellular signaling pathways (NF-κB, JAK/STAT, and possibly others) work synergistically to regulate CCRL2 expression. Indeed, treating cells with pharmacoinhibitors targeting both NF-κB and JAK/STAT pathways significantly reduced CCRL2 induction by TNF-α/LPS/IFN-γ. Furthermore, the addition of immune suppressive factors such as dexamethasone, TGF-β, or IL-10 failed to inhibit the TNF-α/LPS–stimulated induction of CCRL2 or VCAM-1, indicating that the proinflammatory signals are dominant (Fig. 1).

To confirm that the endothelioma cell lines accurately reflected primary EC biology, we evaluated CCRL2 expression on freshly isolated lung and liver EC from mice dosed with endotoxin to induce systemic inflammation and vasculitis (24). Systemic administration of endotoxin has been reported to increase circulating levels of TNF-α and IFN-γ, mimicking to an extent the in vitro stimulation of CCRL2 on EC (24, 25). Indeed, liver EC upregulated CCRL2 in response to LPS challenge in vivo. Interestingly, EC isolated from the lung of normal WT mice constitutively expressed CCRL2 and bound Fc-chemerin, but LPS treatment did not alter lung CCRL2 expression. Primary human EC (HUVEC and HDMEC) treated in vitro with proinflammatory stimuli upregulated CCRL2 and bound Fc-chemerin, indicating conserved regulation in primary EC across species. Liver and lung EC from LPS-dosed CCRL2-deficient mice did not bind to Fc-chemerin, thus indicating that CCRL2 is the primary receptor for chemerin on liver and lung EC in vivo.

With its lack of classical signaling responses (i.e., cell migration, intracellular calcium mobilization) and absence of a “DRY” motif in the second intracellular loop (thought to enable coupling to G proteins; see Ref. (43)), CCRL2 may be considered a member of the family of atypical chemoattractant receptors that include DARC, D6, CCX-CKR, and CXCR7 (reviewed in Ref. 44). These receptors modulate immune responses by regulating the bioavailability of chemoattractants, usually through specialized and efficient ligand internalization and degradation (44, 45). Mice deficient in D6 or DARC, for example, show increased inflammation in models of skin inflammation and endotoxemia, re-
spectively, owing to impaired chemokine clearance (46, 47). In line with their biological function to intercept excess circulating chemokines, D6, DARC, and CXCR7 are widely expressed on numerous endothelial cell types (48–50). CCR2L2 is also expressed on a variety of EC from different tissues (brain, skin, lung, and liver), suggesting a role for CCR2L2 in regulating the bioavailability of circulating chemerin. Indeed, the intravascular time-integrated chemerin levels during 2 wk following a single i.v. injection of Fe-chemerin was significantly greater (by 40%) in CCR2L2+/− mice compared with WT mice. This is also likely reflected in the small but significantly elevated plasma chemerin levels in unchallenged CCR2L2+/− mice. Furthermore, treatment of mice with endotoxin, which upregulates vascular EC CCR2L2 and enhances chemerin binding in WT mice, produced a robust 2- to 3-fold greater than WT increase in circulating chemerin levels in CCR2L2+/− mice. Other examples of increased chemoattractant levels in mice deficient for their cognate receptor include CCL2/CCR2 and chemerin/CMKLR1 in a model of pneumonia (51, 52).

Although a contribution of extravascular CCR2L2 is not formally excluded by our studies, CCR2L2 control of circulating chemerin levels most likely occurs at the level of vascular EC. Thus, in regulating the bioavailability of leukocyte attractant chemerin, CCR2L2 exhibits another trait in common with the other atypical chemoattractant “interceptors.” However, one critical difference that sets CCR2L2 apart from the other atypical receptors is that CCR2L2 does not internalize bound chemerin, demonstrated in this study with EC and previously with CCR2L2+/lymphoid cells (10).

Chemerin circulates in an inactive proform and requires proteolytic processing to increase its biological activity. It is tempting to speculate that CCR2L2 could bind and present prochemerin on the surface of EC to circulating serine proteases widely present during endotoxemia, thus removing the inhibitory peptides and presenting the active chemerin to circulating CMKLR1+ cells (reviewed in Ref. 53). Other surface-bound receptors such as endothelial protein C receptor are known to bind and concentrate their soluble ligand on the surface of EC, allowing for more efficient proteolytic activation by their cognate enzyme. Indeed, endothelial protein C receptor is widely expressed on vessels and binds and concentrates protein C, accelerating by 20-fold the activation of protein C by thrombin (54, 55). If EC CCR2L2 captures circulating prochemerin and enhances its proteolytic activation during inflammation, we would predict 1) a reduction in circulating chemerin levels in LPS-treated WT mice versus untreated WT controls, and 2) an increase in circulating prochemerin in LPS-treated CCR2L2+/− mice compared with WT mice. Indeed, there was significantly less circulating prochemerin in WT LPS-treated mice compared with untreated WT controls, likely reflecting sequestration by EC CCR2L2 during systemic inflammation (Fig. 10B). Additionally, there was significantly more plasma prochemerin in LPS-treated CCR2L2+/− mice than in WT mice (Fig. 10C), along with a significant 2- to 3-fold increase in total circulating chemerin levels (Fig. 10A). Thus, our results are consistent with the hypothesis that EC CCR2L2 binds plasma prochemerin for enhanced proteolytic activation during inflammation. Additional work is necessary to characterize protease-specific effects of CCR2L2-dependent anchoring of chemerin in its proteolytic activation.

Depending on the model, chemerin and its receptors CCR2L2 and CMKLR1 can play a pathogenic or protective role in pulmonary inflammation: CMKLR1 plays a pathogenic role in cigarette smoke-induced lung inflammation (56) and CCR2L2 plays a pathogenic role in an OVA model of lung inflammation (57), whereas CMKLR1 plays a protective role in viral pneumonia and an LPS airway challenge model (30, 51). Given the robust expression of CCR2L2 on lung EC and the recent reported contributions of the chemerin receptors to leukocyte recruitment during pulmonary inflammation, we investigated the role of CCR2L2 in CMKLR1+ NK cell recruitment to the airways in response to intranasal LPS challenge. We hypothesized that EC CCR2L2-dependent anchoring/accumulation of bioactive chemerin contributes to the recruitment of CMKLR1+ NK cells to inflamed airways, an effect that should be attenuated in CCR2L2-deficient mice. Indeed, significantly fewer CMKLR1+ NK cells accumulated in the airways of CCR2L2+/− mice compared with WT mice (Fig. 11). There were no differences in the recruitment of CMKLR1+ neutrophils or CD3+ cells. Additionally, there were similar numbers of circulating NK cells and other major WBC subsets in CCR2L2+/− and WT mice, a similar expression of CMKLR1 on NK cells from both genotypes, and a lack of expression of CCR2L2 on NK cells. Taken together, these results indicate that CCR2L2 selectively coordinates the recruitment of CMKLR1+ NK cells in a manner consistent with our model of EC CCR2L2-dependent chemerin anchoring.

When bound to CCR2L2, the carboxyl terminus of chemerin important for CMKLR1 signaling remains exposed at the cell surface (10). Recently, Hart and Greaves (31) demonstrated that chemerin is a potent inducer of CMKLR1+ peritoneal macrophage adhesion to VCAM-1 by inducing αβ1 integrin clustering. Thus, we hypothesized that CCR2L2+ EC could bind and effectively present chemerin to CMKLR1+ lymphoid cells to trigger cell adhesion. Adhesion of L1.2 lymphoid cells to EC required the following components: 1) CCR2L2+ activated EC, 2) CMKLR1+ L1.2 cells, and 3) chemerin. Furthermore, adhesion of CMKLR1+ cells was completely dependent on αβ1 integrin and VCAM-1.

Thus, we propose the following possible mechanisms to describe the concerted actions of CCR2L2, CMKLR1, and chemerin: 1) Direct mechanism: CCR2L2 directly presents chemerin to CMKLR1+ cells and thus creates a trio with CCR2L2 binding the N terminus, whereas CMKLR1 interacts with the critical signaling C terminus of chemerin. 2) Indirect mechanism: Chemerin binds to CCR2L2 with an affinity typical for a chemoattractant/receptor pair (low nanomolar Kd) (10); coupled with the lack of ligand internalization, it is likely that chemerin is released from the cell surface at a certain rate dependent on, for example, receptor density, temperature, pH, and salt concentration, and at some rate is reacquired by CCR2L2. Elevated local concentrations of soluble chemerin in the media near the CCR2L2 cells, however, may trigger CMKLR1 activation and, subsequently, αβ1 integrin avidity upregulation, without requiring a trio complex.

In conclusion, our results provide a novel mechanism by which the chemoattractant chemerin is presented by CCR2L2+ EC to trigger CMKLR1+ cell adhesion. Extracellular matrix glycosaminoglycans (GAGs) on the luminal side of the endothelium and are thought to immobilize and present chemokines to rolling leukocytes, which triggers integrin activation and leukocyte extravasation (58). In several human inflammatory disorders (multiple sclerosis, lupus, and psoriasis) in which chemerin is associated with inflamed endothelium, CMKLR1+ leukocytes (NK cells, plasmacytoid dendritic cells) are found to infiltrate into the affected tissues (11–14). Furthermore, in two separate in vivo inflammatory models, CCR2L2+/− mice displayed less severe allergic inflammation (10) and less severe OVA-induced airway inflammation than in WT counterparts (57); however, it is not clear whether this protective effect is linked with a decrease in CMKLR1+ cell recruitment. Although GAGs likely play a role in chemerin binding (chemerin binds to heparin, a type of GAG), we hypothesize that CCR2L2 expressed on inflamed endothelium provides a novel specific and selective mechanism to bind and concentrate chemerin (3). A recent report indicates that CCL19
may be an alternate chemoattractant ligand for CCR2, thus widening the biological spectrum of action for CCR2 (59).

Nevertheless, selective inhibition of CCR2 binding to chemerin, rather than inhibition of GAGs, which bind all chemokines, could be a novel targeted therapeutic strategy to block chemerin-mediated recruitment of CMKL1

leukocytes in chemerin-associated inflammatory diseases, such as extracellular autoimmune encephalomyelitis/multiple sclerosis (13, 16).

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