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Chemerin Contributes to Inflammation by Promoting Macrophage Adhesion to VCAM-1 and Fibronectin through Clustering of VLA-4 and VLA-5

Rosie Hart and David R. Greaves

Chemerin is a potent macrophage chemoattractant protein. We used murine peritoneal exudate cells (PECs) in adhesion, flow cytometry, and confocal microscopy assays to test the hypothesis that chemerin can also contribute to inflammation by promoting macrophage adhesion. Chemerin stimulated the adhesion of PECs to the extracellular matrix protein fibronectin and to the adhesion molecule VCAM-1 within a minute, with an EC_{50} of 322 and 196 pM, respectively. Experiments using pertussis toxin and PECs from ChemR23^{-/-} mice demonstrated that chemerin stimulated the adhesion of macrophages via the Gi protein-coupled receptor ChemR23. Blocking Abs against integrin subunits revealed that 89% of chemerin-stimulated adhesion to fibronectin was dependent on increased avidity of the integrin VLA-5 (α5β1) and that 88% of adhesion to VCAM-1 was dependent on increased avidity of VLA-4 (α4β1). Although chemerin was unable to induce an increase in integrin affinity as judged by the binding of soluble ligand, experiments using confocal microscopy revealed an increase in valency resulting from integrin clustering as the mechanism responsible for chemerin-stimulated macrophage adhesion. PI3K, Akt, and p38 were identified as key signaling mediators in chemerin-stimulated adhesion. The finding that chemerin can rapidly stimulate macrophage adhesion to extracellular matrix proteins and adhesion molecules, taken together with its ability to promote chemotaxis, suggests a novel role for chemerin in the recruitment and retention of macrophages at sites of inflammation. The Journal of Immunology, 2010, 185: 3728–3739.

The coordinated recruitment of leukocytes to sites of tissue injury is central to the generation of a successful inflammatory response (1). The directed migration of leukocytes is regulated by a number of inflammatory molecules including chemokines, cytokines and complement proteins (2).

Chemerin was originally isolated from human inflammatory exudates as the protein ligand for the orphan Gi-linked G protein-coupled receptor (GPCR) ChemR23 (3). ChemR23 is structurally related to chemokine receptors, and accordingly, chemerin was discovered to possess the chemoattractant abilities of a chemokine (3, 4). Chemerin is chemotactic for ChemR23-expressing human macrophages, plasmacytoid dendritic cells (pDCs), and NK cells (3, 5–7). ChemR23 is also expressed on human monocytes (6). In the mouse, macrophages express ChemR23 and migrate toward chemerin (8, 9). Recently, murine pDCs and NK cells were also shown to be ChemR23+ (10). Unlike the majority of chemokines, chemerin is secreted in an inactive precursor form (3). It requires cleavage of the C terminus by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades to become active as a chemoattractant for cells expressing ChemR23 (11–13). Moreover, chemerin’s predicted structure, thought to be the reverse orientation of that of chemokines, distinguishes it from this family of proteins (14).

In addition to mediating chemotaxis, many chemoattractants, including chemokines, are also able to induce the adhesion of leukocytes to adhesion molecules, such as VCAM-1 and ICAM-1, and to extracellular matrix proteins, such as fibronectin, laminin, and collagen IV (15). They induce adhesion by stimulating an increase in the affinity and/or the valency of integrin receptors expressed on the cell surface (16). Leukocyte adhesion plays a key role in inflammation, contributing to leukocyte rolling and arrest on the vascular endothelium, migration through the extracellular matrix to the site of inflammation, retention of leukocytes within the inflammatory site, and leukocyte activation (17–22).

In this study, we hypothesized that chemerin may contribute to inflammation not only by mediating macrophage chemotaxis, but also by promoting the adhesion of macrophages to extracellular matrix proteins and adhesion molecules.

**Materials and Methods**

**Animals**

All animal experiments were performed with ethical approval from the Dunn School of Pathology Local Ethical Review Committee and in accordance with the U.K. Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

**Reagents**

Collagen IV, laminin and fibronectin were obtained Scientific Laboratory Supplies (Nottingham, U.K.), ICAM-1, VCAM-1, and VCAM-1-Fc were from R&D Systems (Abingdon, U.K.). The FITC-conjugated GRGDSP peptide was purchased from AnaSpec (Fremont, CA), as were all the blocking Abs and their isotype controls as follows: rat IgG1 (clone RTK2071), rat IgG2a (clone RTK2758), rat IgG2b (clone RTK4530), Armenian hamster IgG (clone HK888), anti-mouse CD18 (β2; clone M18/2), anti-mouse/rat CD29 (β1; clone FIB27). Of the inhibitors, Akt inhibitor IV, cytochalasin D, LY294002, and SB202190 were purchased from Sigma-Aldrich (Gillingham, U.K.), BAPTA-AM and pertussis toxin (PTX) were from Calbiochem (Nottingham, U.K.), piceatannol and PP2 were from Tocris Bioscience (Bristol, U.K.), and U0126 was obtained from New England Biolabs.
that had been treated with inhibitors as described previously. CellTiter-Glo was used according to the manufacturer's instructions to assays using PTX, PECs were pretreated with 200 ng/ml PTX for 1 h before their use in adhesion assays.

Washes with assay buffer (RPMI 1640 supplemented with 0.1% BSA) and plates were washed twice with PBS, and nonspecific binding sites were 10% WEHI conditioned medium, 50 mM EDTA (Sigma-Aldrich). The resulting cells were used immediately, and the next day the cells were washed twice before use in further experiments. BaF3 cells stably transfected with the murine ChemR23 receptor (BaF3-ChemR23) were a gift from H. Wang and T. Schall (ChemoCentryx). ChemR23 expression was confirmed by flow cytometry using an anti-mouse ChemR23 Ab (data not shown). Wild-type (WT) BaF3 cells and BaF3-ChemR23 cells were cultured in RPMI 1640 supplemented with 10% FBS, 10% EHI conditioned medium, 50 μM 2-ME (Sigma-Aldrich), 50 μM penicillin, and 50 μg/ml streptomycin. BaF3 cells were serum starved in RPMI 1640 supplemented with 0.1% BSA and 25 μM HEPES for 24 h before their use in adhesion assays.

Adhesion assay

Ninety-six-well cell culture plates (Corning, Amsterdam, The Netherlands) were coated overnight at 4°C with 30 μg/ml collagen IV, 30 μg/ml fibronectin (Sigma-Aldrich), 2 μg/ml ICAM-1, or 2 μg/ml VCAM-1. The plates were washed twice with PBS, and nonspecific binding sites were blocked with 1% BSA in PBS for 1 h at 37°C, before an additional three washes with assay buffer (RPMI 1640 supplemented with 0.1% BSA and 25 mM HEPES). PECs or BaF3 cells were resuspended in assay buffer, and 50 μl of cell suspension in the absence or presence of chemerin (10 nM or 100 nM ChemR23, or their isotype controls (FITC-conjugated rat IgG2b or IgG2a, respectively) in combination with 1 μM piceatannol, 10 μM CCRL2, and 50 μM 1H,2[1,2]C][1,2]imidazole (Promega) or 10 nM chemerin were added to 100 μl of these cells and incubated at 37°C for up to 2 min. Tubes were immediately placed on ice to stop the reaction and the cells were fixed with 2% paraformaldehyde in PBS on ice for 1 h. Samples were washed twice with assay buffer, blocked with 10 μg/ml anti-mouse CD16/CD32 for 1 min at room temperature before the wells were washed an additional three times with assay buffer, and the slides were mounted in Dako fluorescent mounting medium. The cells were analyzed using a Carl Zeiss LSM 510 confocal laser scanning system with a 63× Plan-Apochromat objective (NA 1.4). Macrophages were identified as those cells with an average fluorescence intensity for F4/80 exceeding a predefined lower limit.

RT-PCR analysis

Total RNA was prepared from freshly obtained PECs using TRIzol (Invitrogen), isolated using an RNeasy kit (Qiagen, Crawley, U.K.), and reverse transcribed using Moloney murine leukemia virus reverse transcriptase and random primers (Promega). RT-PCR analysis for GAPDH, ChemR23, GPR1, CCR1, and BLT1 was performed using SYBRGreen (Qiagen) and the following primers: GAPDH: 5'-CTCTGACTGCTCTTTGAAAGA3' (forward) and 5'-ACCACCTTTGAGCTCATACATCT-3' (reverse); ChemR23, 5'-ACACAGTGTCTACAGAAGAAG-3' (forward) and 5'-TCTCCATGAGGACAGTCAAA-3' (reverse); GPR1, 5'-CTACGGTCCCTTGGTTACCTA-3' (forward) and 5'-CAGTGGGACTAGAAGGATTTAC3' (reverse); CCR2, 5'-ACGTCCTCTGGAGAGTGTC-3' (forward) and 5'-TCTCCCTTCAAGAGACAGAAA-3' (reverse); and BLT1, 5'-CTCTGACTGCTCTTTGAAAGA3' (forward) and 5'-TCTCCCTTCAAGAGACAGAAA-3' (reverse). The number of copies of receptor mRNA per copy of GAPDH mRNA was quantitated from a standard curve using mouse genomic DNA (Promega).
Results
Chemerin rapidly induces adhesion to fibronectin and VCAM-1
To investigate chemerin’s ability to induce macrophage adhesion to extracellular matrix proteins and recombinant adhesion molecules, we used Bio-Gel–elicited PECs, which are a good source of minimally activated macrophages, in a static adhesion assay. Bio-Gel–elicited PECs were composed of an average of 33% macrophages and 10% immature monocyte-macrophages as determined by their expression of 7/4 and F4/80 Ags (Fig. 1A).

PECs were allowed to adhere to plates coated with collagen IV, fibronectin, laminin, ICAM-1, or VCAM-1 in the presence or absence of chemerin. Treatment of PECs with 10 nM chemerin resulted in a significant increase in the number of adherent cells on fibronectin (4.6-fold; p < 0.001), ICAM-1 (5.6-fold; p < 0.001), and VCAM-1 (4.5-fold; p < 0.001) (Fig. 1B). Chemerin was not able to induce PEC adhesion to collagen IV or laminin. Chemerin stimulated the adhesion of PECs to fibronectin (Fig. 1C) and VCAM-1 (Fig. 1D) in a dose-dependent manner with maximal effects achieved at 10 nM chemerin. This corresponds well with chemerin’s chemotactic activity (8). Chemerin had an EC50 value of 322 and 196 pM for cell chemerin. This corresponds well with chemerin’s chemotactic activity (8). Chemerin had an EC50 value of 322 and 196 pM for cell

Chemerin stimulates the adhesion of macrophages through ChemR23
The effects of chemotactants on adhesion are mediated through Gi-coupled GPCRs (23–26). To determine whether this was true for chemerin’s effects, PECs from WT Sv129 mice were pretreated with PTX. Chemerin induced a significant increase in the adhesion of PECs from WT Sv129 mice to fibronectin (2.1-fold; p < 0.001) (Fig. 2A) and VCAM-1 (3.7-fold; p < 0.001) (Fig. 2B). Chemerin’s effects on cell adhesion to fibronectin and VCAM-1 were completely inhibited by PTX pretreatment, demonstrating the requirement for a Gi-coupled GPCR (Fig. 2A, 2B).

Bio-Gel–elicited peritoneal macrophages from ChemR23−/− mice display no chemotaxis toward chemerin (8). To investigate whether chemerin-stimulated adhesion is mediated via ChemR23, we assessed the ability of chemerin to stimulate the adhesion of PECs from Sv129 ChemR23−/− mice (KO PECs) to both fibronectin and VCAM-1. Chemerin was unable to promote the adhesion of PECs from ChemR23−/− mice to fibronectin (Fig. 2A). Therefore, chemerin-stimulated adhesion to fibronectin is dependent on ChemR23 and its coupling to a Gi G protein. However, a significant number of PECs from ChemR23−/− mice were still capable of adhering to VCAM-1 (1.9-fold increase; p < 0.001). These results suggest that although chemerin-stimulated adhesion to VCAM-1 is dependent on activation of a Gi-coupled GPCR, just over half of this activity is independent of ChemR23.

Quantitative RT-PCR analysis of PECs from C57BL/6J mice, Sv129 mice, and Sv129 ChemR23−/− mice showed minimal levels of mRNA for GPR1 (0.02–0.03% of ChemR23 mRNA expression), another GPCR shown to bind chemerin (Fig. 2C) (27). Low levels of mRNA for CCRL2 (11.1–26.7% of ChemR23 mRNA expression), a third nonsignaling receptor for chemerin, were expressed in PECs from C57BL/6J mice, Sv129 mice, and Sv129 ChemR23−/− mice (28). PECs from these three strains of mouse also expressed leukotriene B4 receptor (BLT1) mRNA (28.4–40.3% of ChemR23 mRNA expression).

Only macrophage and monocyte-macrophage PECs expressed ChemR23 as determined by flow cytometry (Fig. 2D). Therefore we concluded that the cells that adhere to fibronectin and VCAM-1 in a ChemR23-dependent manner in response to chemerin treatment are macrophages and monocyte-macrophages.

To test the effect of macrophage polarization on chemerin-stimulated adhesion, we pretreated PECs with LPS and/or various cytokines. Macrophages that were treated overnight with IFN-γ alone expressed increased levels of CD80, a marker of classically activated M1 macrophages (Supplemental Fig. 1A) and the same level of ChemR23 expression as untreated cells (Supplemental Fig. 1B). Overnight treatment of macrophages with IFN-γ plus LPS upregulated CD80 expression to a greater extent than IFN-γ alone but completely downregulated ChemR23 expression. Consistent with the ChemR23 expression of these macrophage populations, IFN-γ–treated cells retained their ability to adhere to fibronectin and VCAM-1 in response to chemerin stimulation, whereas IFN-γ plus LPS-stimulated cells lost this activity (Supplemental Fig. 2). Macrophages treated overnight with IL-4 displayed an upregulation in expression of the mannose receptor, the classic marker of alternatively activated M2a macrophages (Supplemental Fig. 1A) and had the same level of ChemR23 expression as untreated cells (Supplemental Fig. 1B). However, in the adhesion assay, IL-4–treated cells did not adhere to fibronectin or VCAM-1 in response to chemerin stimulation (Supplemental Fig. 2).

To provide further evidence that chemerin is capable of promoting cell adhesion to fibronectin and VCAM-1 solely through ChemR23, we used BaF3 cells (a pro-B cell line) stably transfected with ChemR23 in the adhesion assay. Chemerin (10 nM) was capable of stimulating the adhesion of these cells to both fibronectin (Fig. 2E) and to VCAM-1 (Fig. 2F), but it was not able to induce the adhesion of WT BaF3 cells that did not express the ChemR23 receptor (Fig. 2E, 2F). We also tested whether two peptides derived from the C terminus of murine chemerin were able to promote adhesion of the BaF3-ChemR23 cells to fibronectin. These peptides had both been previously shown to induce ChemR23-dependent activity (8, 10, 29). C9 (FLPGQFAFS) was a partial agonist, capable of producing 58.6% of the adhesive activity of full-length chemerin (Fig. 2G). However, it was slightly more potent than chemerin with an EC50 of 0.94 nM compared with 2.48 nM for chemerin. Peptide C15 (AGEDPHGYFLPGQFA) was unable to promote the adhesion of BaF3-ChemR23 cells to fibronectin at any concentration tested.

Chemerin-stimulated adhesion to fibronectin and VCAM-1 is mediated through the VLA-5 (α5β1) and VLA-4 (α4β1) integrins, respectively
Leukocytes bind to extracellular matrix proteins and adhesion molecules, such as fibronectin and VCAM-1, through integrins expressed on their surface (30). The adhesion of macrophages to fibronectin is mediated via the integrins α4β1, α5β1, α6β1, and αβ7, whereas α5β1, α4β1, α4β2, and αβ2 are involved in macrophage adhesion to VCAM-1 (31, 32). To identify which of these integrins mediates chemerin-induced adhesion to fibronectin and VCAM-1, PECs were pretreated with blocking Abs against the integrin subunits that constitute the candidate integrins.

Chemerin-induced adhesion to fibronectin was significantly inhibited by Abs that blocked the α5 integrin subunit (92.5% inhibition; p < 0.001) and the β1 integrin subunit (86.4% inhibition; p < 0.001) (Fig. 3A). This result demonstrates that chemerin stimulates the adhesion of macrophages to fibronectin through regulation of this integrin; therefore, chemerin’s effects on cell adhesion to fibronectin are mediated through the VLA-5 (α5β1).
of the VLA-5 (\(\alpha_5\beta_1\)) integrin. Blocking Abs against the \(\alpha_v\), \(\alpha_4\), and \(\beta_1\) subunits had no significant effect in this assay, and therefore, the \(\alpha_5\beta_3\), \(\alpha_5\beta_4\), and \(\alpha_5\beta_7\) integrins do not play a role in chemerin-stimulated adhesion to fibronectin.

Chemerin-stimulated adhesion of PECs to VCAM-1 was significantly reduced by blocking Abs against the \(\alpha_4\) integrin subunit (100% inhibition; \(p < 0.001\)) and the \(\beta_1\) integrin subunit (87.9% inhibition; \(p < 0.001\)) (Fig. 3B). This result indicates that chemerin-stimulated adhesion to VCAM-1 occurs through regulation of the VLA-4 (\(\alpha_5\beta_1\)) integrin. As adhesion to VCAM-1 is almost completely blocked by these Abs, it suggests that regulation of VLA-4 is critical for both the ChemR23-dependent and the ChemR23-independent adhesion to VCAM-1 in response to chemerin treatment. A blocking Ab against the \(\beta_1\) subunit also significantly reduced chemerin-induced adhesion to VCAM-1 (43.1% inhibition; \(p < 0.05\)), although to a lesser extent, suggesting a role for lymphocyte Peyer’s patch adhesion molecule (LPAM)-1 (\(\alpha_6\beta_1\)) in chemerin-stimulated adhesion to VCAM-1. A blocking Ab against the \(\beta_2\) subunit excluded the \(\alpha_5\beta_2\) and \(\alpha_5\beta_7\) integrins from playing a role in chemerin-stimulated adhesion to VCAM-1 as no significant inhibition of adhesion was observed following preincubation with this Ab.

**Chemerin does not stimulate an increase in integrin affinity**

Many chemoattractants stimulate leukocyte adhesion by promoting an increase in the affinity of an integrin for its ligand (16, 25). To investigate the hypothesis that chemerin promotes adhesion by increasing integrin affinity, we measured the binding of soluble fluorescently conjugated VLA-4 and VLA-5 ligands to chemerin-treated macrophages by flow cytometry. MnCl2 binds to the extracellular domain of integrins, inducing a conformational change that increases integrin affinity and was therefore used as a positive control (33).

GRGDSP is a peptide derived from fibronectin that contains the key RGD sequence required for binding to integrins (34). In our experiments, over a period of 5 min, MnCl2 significantly increased the amount of GRGDSP-FITC peptide bound to F4/80+ PECs compared with untreated F4/80+ PECs (Fig. 4A). An increase in the amount of peptide bound to F4/80+ PECs was seen as early as 10 s and became significant after 2 min (1.69-fold increase; \(p < 0.05\)). This indicates that MnCl2 is capable of inducing an increase in the affinity of fibronectin-binding integrins on macrophages. In contrast, chemerin (10 nM) was unable to stimulate an increase in the amount of GRGDSP-FITC peptide bound to F4/80+ PECs compared with untreated F4/80+ PECs at any time point. Therefore, chemerin does not induce macrophage adhesion to fibronectin by increasing VLA-5 affinity.

MnCl2 also stimulated a rapid and sustained increase in the amount of VCAM-1-Fc bound to F4/80+ cells compared with untreated F4/80+ PECs, demonstrating its ability to increase the affinity of VCAM-1–binding integrins on macrophages (Fig. 4B). A significant increase in the amount of VCAM-1-Fc was observed after just
30 s (2.83-fold increase; \( p < 0.001 \)). Again, chemerin had no effect on the amount of VCAM-1-Fc bound to F4/80+ PECs compared with untreated F4/80+ PECs over a 2-min period, indicating that chemerin does not induce macrophage adhesion to VCAM-1 by increasing the affinity of VLA-4 (or LPAM-1). Representative histograms are shown in Fig. 4C and 4D.

**FIGURE 2.** Chemerin’s effects on adhesion are mediated largely through ChemR23 expressed on macrophages. A and B, PECs from Sv129 mice (WT PECs) ±1 h of pretreatment with 200 ng/ml PTX at 37°C or PECs from ChemR23−/− mice (KO PECs) were added to wells that had been precoated with 30 μg/ml fibronectin (A) or 2 μg/ml VCAM-1 (B) and incubated for 1 min at 37°C in the presence of media alone or 10 nM chemerin. Plates were shaken for 20 s, and the wells washed thoroughly with media to remove nonadherent cells. CellTiter-Glo was added to each well, and the luminescence was measured to quantify the remaining adherent cells. Graphs show mean values ± SEM from three independent experiments; ***\( p < 0.001, \) two-way ANOVA with Bonferroni’s posthoc test. C, GPCR mRNA was quantified using SYBRGreen RT-PCR. Data presented as the number of copies of receptor mRNA per copy of GAPDH mRNA as quantified from a standard curve. D, PECs were stained with anti–7/4-FITC, anti–F4/80-Alexa Fluor 647, and either rat IgG2a-PE or rat anti-mouse ChemR23-PE Abs and analyzed by flow cytometry. Graphs representative of three separate experiments. E and F, WT-BaF3 cells or BaF3-ChemR23 cells were added to plates that had been precoated with 30 μg/ml fibronectin (E) or 2 μg/ml VCAM-1 (F) and incubated for 1 min at 37°C in the presence of media alone or 10 nM chemerin. Plates were washed and analyzed as described above. Graphs show mean values ± SEM from three independent experiments. G, BaF3-ChemR23 cells were added to plates precoated with 30 μg/ml fibronectin and incubated for 1 min at 37°C in the presence of media alone, 0.01–100 nM chemerin, or 0.001–1 μM C9 or C15 peptide. Plates were washed and analyzed as described above. Graphs show mean values ± SEM from three independent experiments.

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**TABLE 1.** Effect of chemerin on VCAM-1-Fc binding to F4/80+ PECs.

<table>
<thead>
<tr>
<th>Condition</th>
<th>untreated</th>
<th>10 nM chemerin</th>
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<tbody>
<tr>
<td>WT PECs</td>
<td>6.25 ± 1.85</td>
<td>6.25 ± 1.85</td>
</tr>
<tr>
<td>KO PECs</td>
<td>0.11 ± 0.07</td>
<td>0.11 ± 0.07</td>
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*30 s (2.83-fold increase; \( p < 0.001 \)).*
Chemerin stimulates integrin clustering

Some chemokines have been shown to promote leukocyte adhesion by stimulating the clustering of integrins on the cell surface (16, 35). To test whether chemerin could induce the clustering of VLA-4 and/or VLA-5 integrins, we used confocal microscopy to visualize localized changes in the distribution of the $\alpha_4$ integrin subunit and the $\alpha_5$ integrin subunit, respectively, on the surface of F4/80+ macrophages. The phorbol ester PMA was used as a positive control (36, 37).

Treatment of the PECs in suspension with PMA induced the clustering of both the $\alpha_5$ (Fig. 5A) and the $\alpha_4$ (Fig. 5B) integrin subunits on the surface of F4/80+ macrophages. This was shown by an increase in fluorescence intensity on certain parts of the cell compared with the low fluorescence intensity of untreated cells in which the integrins were more evenly distributed. Treatment of the PECs for 1 min with 10 nM chemerin had a very similar effect to that of PMA. It increased both $\alpha_5$ and $\alpha_4$ fluorescent intensity on patches of the macrophage surface, suggesting that chemerin can induce rapid clustering of both VLA-5 and VLA-4 integrins, respectively. Chemerin stimulated a 2.6-fold ($p < 0.01$) increase in the average $\alpha_5$ fluorescence intensity of F4/80+ macrophages (Fig. 5C) and a 6-fold ($p < 0.01$) increase in the average $\alpha_4$ fluorescence intensity of F4/80+ macrophages (Fig. 5D) (compared with the average $\alpha_5$ fluorescence intensity of F4/80+ macrophages (Fig. 5C) and a 6-fold ($p < 0.01$) increase in the average $\alpha_4$ fluorescence intensity of F4/80+ macrophages (Fig. 5D) (compared with...
the respective 2.5-fold \( p < 0.05 \) and 5.2-fold \( p < 0.05 \) increases following PMA stimulation). The greater capacity of chemerin to stimulate VLA-4 clustering compared with VLA-5 clustering is in accordance with the adhesion data in Fig. 1C and 1D in which chemerin stimulates a greater fold change in the number of adherent cells on VCAM-1 than on fibronectin.

Low doses of cytochalasin D (0.05–5 \( \mu \)g/ml), an inhibitor of actin polymerization, have been shown to both inhibit and promote integrin clustering and leukocyte adhesion (36–38). To investigate the role of the actin cytoskeleton in chemerin-stimulated adhesion, we looked at the effect of pretreating PECs with cytochalasin D for 1 h in the adhesion assay. We used 0.015 \( \mu \)g/ml cytochalasin D, because this was the highest concentration of the inhibitor that exhibited no cytotoxic effects on the PECs (determined using Promega’s CellTiter-Glo assay) (Fig. 5E). Pretreatment with 0.015 \( \mu \)g/ml cytochalasin D had no effect on untreated PEC or chemerin-stimulated PEC adhesion to fibronectin (Fig. 5F). However, cytochalasin D pretreatment increased both the number of untreated cells (64.0% increase; \( p < 0.05 \)) and chemerin-treated cells (75.9% increase; \( p < 0.01 \)) adhering to VCAM-1 (Fig. 5G). These results suggest that actin polymerization is not required for chemerin-stimulated integrin clustering as no inhibitory effect of cytochalasin D was observed. Cytochalasin D has been shown to increase the lateral mobility of integrins by releasing them from their cytoskeletal restraints, leading to integrin clustering and adhesion. This would explain the results of Fig. 5G and implies that chemerin may act via a similar mechanism.
Akt, PI3K, and p38 play key roles in chemerin-stimulated adhesion to fibronectin and VCAM-1

To investigate which intracellular signaling pathways are important in chemerin-mediated macrophage adhesion, we pretreated PECs for 1 h with various cell signaling inhibitors at concentrations demonstrated not to have cytotoxic effects (data not shown) and used these cells in the adhesion assay. Chemerin-induced adhesion to fibronectin was significantly reduced by pretreatment of PECs with Akt inhibitor IV (54.6% reduction; \( p < 0.001 \)), LY294002 (54.3% reduction; \( p < 0.001 \)), and SB202190 (49.6% reduction; \( p < 0.001 \)), indicating a role for Akt, PI3K, and p38, respectively (Fig. 6A). Inhibitors of calcium flux (BAPTA-AM), Syk (piceatannol), Src (PP2), and ERK1/2 (U0126) had no effect on chemerin-stimulated adhesion to fibronectin.

Inhibitors of Akt, PI3K and p38 also significantly reduced adhesion to VCAM-1 in response to chemerin treatment (Akt inhibitor IV, 57.3%; LY294002, 78.6%; and SB202190, 65.6% reduction; \( p < 0.001 \)), suggesting the involvement of Akt, PI3K, and p38 in chemerin-mediated adhesion to VCAM-1 as well as to fibronectin (Fig. 6B). In contrast to the adhesion to fibronectin, BAPTA-AM (34.6% reduction; \( p < 0.001 \)), piceatannol (36.8% reduction; \( p < 0.001 \)), PP2 (39.0% reduction; \( p < 0.001 \)), and U0126 (42.0% reduction; \( p < 0.001 \)) all significantly inhibited PEC adhesion to VCAM-1 resulting from chemerin treatment. This suggests that calcium flux, Syk, Src, and ERK1/2, respectively, play a role in chemerin-stimulated adhesion to VCAM-1 but not to fibronectin.

Pretreatment of PECs with a combination of Akt inhibitor IV and LY294002 almost completely blocked (96.5% reduction) chemerin-stimulated adhesion to fibronectin, suggesting that Akt and PI3K are involved in separate pathways downstream of ChemR23 (Fig. 6C). A combination of Akt inhibitor IV and SB202190 did not further inhibit adhesion to fibronectin (67.5% reduction) than either of these inhibitors alone; therefore, p38 and Akt appear to reside in the same pathway. A combination of LY294002 and SB202190 produced a significantly greater inhibition (83.6% reduction) than the two inhibitors alone but did not completely block chemerin-stimulated macrophage adhesion to fibronectin. This suggests that activation of Akt is not completely dependent upon p38 activation. A putative signaling flow diagram based on our results is presented in Fig. 7.

In contrast with the results obtained in the fibronectin adhesion assays, none of the inhibitor combinations could completely block chemerin-stimulated adhesion to VCAM-1, and they had only a slightly greater inhibitory effect than Akt inhibitor IV or SB202190 alone (Akt inhibitor IV plus LY294002, 77.4%; Akt inhibitor IV plus SB202190, 74.3%; and LY294002 plus SB202190, 84.1% reduction) (Fig. 6D).

**Discussion**

Chemerin functions as a potent macrophage chemoattractant via the GPCR ChemR23 (3, 8). In this study, we provide the first evidence that chemerin can induce the adhesion of ChemR23-expressing macrophages to recombinant adhesion molecules and extracellular matrix proteins through an increase in integrin clustering. Chemerin stimulates the adhesion of PECs to fibronectin and VCAM-1 in a dose-dependent, PTX-sensitive manner within a minute. The use of PECs from ChemR23−/− mice revealed that chemerin-stimulated adhesion to fibronectin is dependent on the expression of the GPCR ChemR23, as is nearly half of chemerin-stimulated adhesion to VCAM-1. Flow cytometry demonstrated that ChemR23 is expressed only on macrophages and immature monocyte-macrophages within this mixed cell population. Therefore, we conclude that chemerin promotes the adhesion of ChemR23-expressing macrophages to fibronectin and VCAM-1.

A peptide derived from the last nine amino acids of murine chemerin, C9, has previously been shown to bind to ChemR23 and to induce calcium flux through this receptor (10). In agreement with this,
we found that C9 is capable of stimulating the adhesion of ChemR23-transfected cells to fibronectin and is in fact slightly more potent than chemerin in this assay, although it is a partial agonist that is unable to achieve the maximal cell adhesion induced by chemerin. C15, another peptide derived from the C terminus of chemerin, has been shown to have anti-inflammatory and phagocytic effects on macrophages that are dependent on expression of the ChemR23 receptor (8, 29). We were unable to see an effect of C15 on the adhesion of ChemR23-transfected cells to fibronectin. This also agrees with the data of Luangsay et al. (10) who were unable to detect calcium release in ChemR23-transfected cells in response to C15 treatment. This does not, however, rule out the possibility that C15 activates alternative signaling pathways downstream of ChemR23 on macrophages by acting as an allosteric modulator or by binding to a heterodimer of ChemR23 and another unknown GPCR.

**Chemerin, macrophages, and inflammation**

Leukocyte adhesion and chemotaxis are important for maintaining tissue homeostasis as well as in generating a successful inflammatory response (2). Given that chemerin is activated by inflammatory proteases and that ChemR23 is predominantly expressed by macrophages in the mouse, chemerin is most likely to contribute to the development of inflammatory diseases in which macrophages play a central role (e.g., atherosclerosis or arthritis [9, 11]). Taking atherosclerosis as an example, the migration of tissue and newly differentiated macrophages from the surrounding intima and adventitia to the atherosclerotic lesion requires adhesion via integrins to extracellular matrix proteins, such as fibronectin (22, 39, 40). The adhesion of macrophages to the extracellular matrix and to VCAM-1 expressed on smooth muscle cells that have migrated into the subendothelial space, are thought to contribute to macrophage retention within the atherosclerotic plaque, thereby potentiating the pathogenic process (41, 42). Macrophage adhesion to fibronectin has been shown to increase the expression of proinflammatory molecules, such as matrix metalloproteinases, which have a deleterious effect on plaque stability (21, 43, 44). Our findings suggest that chemerin could promote any one of the above processes, thereby contributing to the progression of an inflammatory disease, such as atherosclerosis.

Macrophages that have been activated by a combination of IFN-γ plus TNF-α (LPS) are known as classically activated M1 macrophages, whereas macrophages activated by IL-4 are known as alternatively activated M2a macrophages (45). These two proinflammatory macrophage types have distinct functional properties. Our results suggest that polarized macrophages no longer adhere to fibronectin or VCAM-1 in response to chemerin stimulation, either because of a complete downregulation in ChemR23 expression in the case of M1 macrophages or, in the case of M2a macrophages that retain their ChemR23 expression, for another reason that may involve the functionality of the integrins themselves. M1 macrophages resulting from IFN-γ stimulation alone retain their ChemR23 expression and their ability to adhere to fibronectin and VCAM-1 in response to chemerin.) This suggests that chemerin-stimulated adhesion may only be relevant in the very early initiating stages of inflammation. However, M1 and M2a macrophages represent the extreme phenotypes and in inflammatory environments, where a number of stimulatory ligands are present, macrophages exhibit plasticity and may display a spectrum of functionalities for which it is hard to predict their responsiveness to chemerin (46).

Although we have focused on macrophages, ChemR23 is additionally expressed on monocytes, pDCs, and NK cells in humans and was also recently shown to be expressed by pDCs and NK cells in the mouse (5–7, 10). Because we have shown that chemerin rapidly increases murine macrophage adhesion to VCAM-1, it is possible that chemerin can induce the rapid adhesion of monocytes, NK cells, or pDCs to VCAM-1 presented on activated vascular endothelium as well as to fibronectin, thereby recruiting these cells to sites of inflammation. Chemerin has an overall positive charge that would allow it to be presented on negatively charged heparin or sulfated glycosaminoglycans, making monocyte, NK cell, and pDC recruitment from the bloodstream a feasible hypothesis (14). Indeed, chemerin immunoreactivity has been detected on endothelial cells lining blood vessels in inflamed tissues and chemerin expression has been shown to correlate with ChemR23+ pDC recruitment in psoriatic skin biopsies (6, 47–49).

**Chemerin-stimulated integrin regulation**

Integrins are the receptors expressed on leukocytes that bind to extracellular matrix proteins and adhesion molecules and are consequently responsible for leukocyte adhesion (30). The binding of many chemoattractants to their GPCRs can initiate a process termed inside-out signaling that results in an increased capacity for the integrins to bind their ligands (16, 50). Although the regulation of integrin activity has been well studied in many leukocyte subsets, such as neutrophils, monocytes and lymphocytes, this process in primary macrophages remains relatively unexplored. We used blocking Abs to identify VLA-5 and VLA-4 as the integrins responsible for chemerin-stimulated macrophage adhesion to fibronectin and VCAM-1, respectively. LPAM-1 was also partially responsible for chemerin-stimulated adhesion to VCAM-1.

Chemerin’s effects on adhesion are extremely rapid and so we considered it unlikely that an upregulation in integrin expression on the surface of macrophages was responsible for the increase in adhesion to fibronectin and VCAM-1. GPCR agonist-stimulated inside-out signaling increases integrin avidity and subsequently leukocyte adhesion (16). An increase in integrin avidity can result from either an increase in integrin affinity and/or an increase in integrin valency (51). Many chemoattractants are capable of altering the conformational state of integrins, thereby increasing their affinity. For example, CXCL12 promotes a rapid increase in the affinity of VLA-4 on monocytes for VCAM-1 (25). However, our experiments using soluble ligands revealed that chemerin is unable to increase the affinity of either VLA-4 or VLA-5 (or LPAM-1) on macrophages.

Increased integrin valency results from cytoskeletal rearrangements that result in the redistribution of integrins on the cell surface (51, 52). This increases the cooperative binding of the integrins, thereby increasing the overall bond strength and allowing leukocyte adhesion (51, 53). Using confocal microscopy, we demonstrated that chemerin stimulates the ligand-independent clustering of VLA-4 and VLA-5, thereby providing a mechanism for chemerin-stimulated adhesion to VCAM-1 and fibronectin, respectively. Some chemokines have also been shown to stimulate integrin clustering...
without stimulating an increase in integrin affinity. For example, CXCL12 promotes the rapid clustering of VLA-4 on lymphocytes to achieve adhesion to VCAM-1 while having no effect on VLA-4 affinity (35). Integrin clustering alone is sufficient to support cell adhesion under the flow conditions that model the environment found within blood vessels (54).

It is generally thought that release of integrins from cytoskeletal restraints increases integrin lateral mobility and allows integrin clustering and leukocyte adhesion to take place (52). Evidence for this comes from the stimulatory effect of cytochalasin D, an inhibitor of actin polymerization on integrin clustering and the inhibitory effect of integrin mutations on integrin clustering arising from an increase in the cytoskeletal anchoring of the integrin tail (37, 38, 55). In agreement with this, cytochalasin D pretreatment increased untreated and chemerin-stimulated adhesion to VCAM-1. It did not inhibit chemerin-stimulated adhesion to VCAM-1 or fibronectin, arguing against an active recruitment of integrins into clusters through actin polymerization (36). This implies that chemerin may function through a similar mechanism, acting to release cytoskeletal restraints holding VLA-4 and VLA-5 in place, thereby allowing them to passively diffuse into clusters. There is some evidence to suggest that movement of integrins into lipid rafts allows focused clustering to take place and is required for cell adhesion (56).

The lack of effect of cytochalasin D on cell adhesion to fibronectin may be explained by a low expression of VLA-5 on the macrophage surface. The number of untreated PECs adhering to fibronectin was 3.4-fold lower than the binding of these cells to VCAM-1 (data not shown). This may result from a lower expression of VLA-5 on the surface, and together with the low dose used of this inhibitor to avoid cytotoxic effects, cytochalasin D may not be able to sufficiently release enough VLA-5 integrins from the cytoskeleton to cause clustering and adhesion, unlike 10 nM chemerin.

Signaling pathways mediating chemerin-stimulated adhesion

In common with other chemoattractants, chemerin’s ability to induce adhesion to fibronectin and VCAM-1 is dependent on the activation of a Gi G protein (23–26). Many studies have looked at the signaling requirements downstream of the Gi G protein that are required for stimulation of leukocyte adhesion (16, 57). However, it has become clear that no single pathway is responsible, rather the signaling pathway depends on the specific cytoskeleton subset, the agonist, GPCR, and specific integrin involved. The inside-out pathways responsible for increased integrin avidity in macrophages, in particular, have not been studied in detail, and the same is true of the pathways responsible for increases in integrin valency in other leukocyte subsets. Agonists at Gi-coupled GPCRs are capable of activating a multitude of signaling pathways involving PI3K, phospholipase C, tyrosine kinases, MAPKs, and small GTPases (58, 59). Chemerin was previously demonstrated to induce calcium flux and activate ERK through ChemR23 (3).

In this study, we have clearly demonstrated that PI3K, Akt, and p38 are involved in chemerin-stimulated adhesion to fibronectin and therefore in the increase in VLA-5 clustering on the macrophage surface. Combinations of inhibitors revealed that Akt and PI3K are involved in separate pathways downstream of ChemR23, whereas p38 and Akt reside in the same pathway (Fig. 7). Similarly, PI3K, Akt, and p38 play a key role in chemerin-stimulated adhesion to VCAM-1 and therefore in the increase in the clustering of VLA-4. However, combinations of inhibitors against these signaling components are unable to completely block chemerin-stimulated adhesion to VCAM-1, and instead, calcium flux, Syk, Src, and ERK play a role. Taken together, our data suggest that chemerin activates at least one additional separate signaling pathway that leads to VLA-4 clustering and adhesion to VCAM-1.

Many studies have found a role for PI3K in integrin activation. For example, clustering of LFA-1 in lymphocytes was shown to be dependent on PI3K activity, but PI3K was not involved in increases in LFA-1 affinity in these cells (60). It was suggested that PI3K, through its ability to promote cytoskeletal remodeling, might release LFA-1 from its cytoskeletal restraints, resulting in integrin clustering and lymphocyte adhesion. The calcium-dependent protease calpain has also been implicated in the cleavage of a cytoskeletal component, triggering the release of LFA-1 from the cytoskeleton and integrin clustering (61). A similar mechanism may be partly responsible for chemerin-stimulated VLA-4 clustering, because inhibition of calcium flux partially inhibited cell adhesion to VCAM-1.

Chemerin- and CCRL2-dependent adhesion

Chemerin-stimulated adhesion of macrophages to fibronectin is completely dependent on the expression of ChemR23. Unexpectedly, chemerin is still capable of promoting the adhesion of PECs from ChemR23<sup>−/−</sup> mice to VCAM-1, revealing that just under half of chemerin-stimulated adhesion to VCAM-1 is dependent on ChemR23 expression. Chemerin was originally thought to bind only to ChemR3, thereby forming a highly specific receptor-ligand pair, but recent studies have identified two additional high-affinity receptors for chemerin. CCRL2 is a nonsignaling GPCR expressed on mast cells and macrophages that is proposed to bind chemerin, increasing its local concentration and presenting it for interaction with ChemR23 expressed on adjacent cells (28). Low levels of CCRL2 mRNA are expressed by the PECs used in our experiments; however, CCRL2’s reported inability to initiate intracellular signaling would seem to rule it out as the additional receptor mediating chemerin-stimulated adhesion to VCAM-1 (28). Chemerin also binds to the GPCR GPR1, initiating an increase in intracellular calcium that is also seen in cells expressing ChemR23 (27). However, negligible levels of GPR1 mRNA are expressed by PECs, suggesting the existence of a fourth unknown chemerin receptor. Pretreatment with PTX completely ablates chemerin-stimulated adhesion to VCAM-1, implying that the unknown receptor is coupled to a Gi G protein. A possible candidate for the unknown receptor is the leukotriene B<sub>4</sub> receptor (BLT1), which is expressed at the mRNA level by PECs. The lipid resolvin E1 has been reported to be a ligand for both ChemR23 and BLT1 (62, 63).

In summary, we have shown for the first time that the macrophage chemoattractant protein chemerin, which is generated at sites of inflammation, rapidly stimulates the adhesion of macrophages to fibronectin and VCAM-1 in a ChemR23-dependent manner, by promoting clustering of the integrins VLA-5 and VLA-4, respectively. We have also identified some of the key signaling components involved in the regulation of VLA-5 and VLA-4 valency on macrophages in response to chemerin treatment. Chemerin-stimulated adhesion may play an important role in recruiting and retaining macrophages at sites of inflammation and in their subsequent activation.

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References


