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Mouse ChemR23 Is Expressed in Dendritic Cell Subsets and Macrophages, and Mediates an Anti-Inflammatory Activity of Chemerin in a Lung Disease Model

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Chemerin is the ligand of the ChemR23 receptor and a chemoattractant factor for human immature dendritic cells (DCs), macrophages, and NK cells. In this study, we characterized the mouse chemerin/ChemR23 system in terms of pharmacology, structure-function, distribution, and in vivo biological properties. Mouse chemerin is synthesized as an inactive precursor (prochemerin) requiring, as in human, the precise processing of its C terminus for generating an agonist of ChemR23. Mouse ChemR23 is highly expressed in immature plasmacytoid DCs and at lower levels in myeloid DCs, macrophages, and NK cells. Mouse prochemerin is expressed in most epithelial cells acting as barriers for pathogens but not in leukocytes. Chemerin promotes calcium mobilization and chemotaxis on DCs and macrophages and these functional responses were abrogated in ChemR23 knockout mice. In a mouse model of acute lung inflammation induced by LPS, chemerin displayed potent anti-inflammatory properties, reducing neutrophil infiltration and inflammatory cytokine release in a ChemR23-dependent manner. ChemR23 knockout mice were unresponsive to chemerin and displayed an increased neutrophil infiltrate following LPS challenge. Altogether, the mouse chemerin/ChemR23 system is structurally and functionally conserved between human and mouse, and mouse can therefore be considered as a good model for studying the anti-inflammatory role of this system in the regulation of immune responses and inflammatory diseases. The Journal of Immunology, 2009, 183: 6489–6499.
ChemR23 by proteolytic removal of the last six or seven amino acids (23). Extracellular proteases, such as the neutrophil-derived cathepsin G and elastase, were shown to generate chemerin from prochemerin, suggesting that the processing takes place at sites of inflammation (24, 25). Indeed, increased chemerin production was detected in psoriasis skin (26, 27) and in lupus erythematous skin lesions (22), as well as high expression of ChemR23 on pDCs, known to play a determinant role in skin inflammatory processes (26, 28). The novel chemerin/ChemR23 system therefore constitutes an attractive candidate for directing specific DC migration under inflammatory conditions. To investigate the role of ChemR23 and its ligand in physiological and pathophysiological situations, we initiated the study of animal disease models with the aim of potentially validating ChemR23 as a therapeutic target in specific diseases. As little information is presently available regarding this system in animal models, we performed a detailed pharmacological and functional characterization of chemerin and its receptor in the mouse and described the distribution of both proteins at the tissue and cellular levels. Moreover, in a mouse model of acute lung injury, we demonstrated that chemerin can act not only as a strong chemoattractant to APCs but also display potent and ChemR23-dependent anti-inflammatory properties in vivo.

Materials and Methods

Mice

Eight to 12-wk-old C57BL/6 mice (Harlan Netherlands) were used throughout these studies. ChemR23-deficient knockout (KO) mice were obtained from Deltagen and backcrossed for 10 generations into the C57BL/6 background in a specific pathogen-free environment. Wild-type (WT) littermates from F1 matings were used as controls for ChemR23 KO mice. All procedures were reviewed and approved by the local ethical committee (Commission d’Ethique du Bien Etre Animal, Université Libre de Bruxelles).

RT-PCR for mouse chemerin and ChemR23

Total RNA was isolated from mouse organs (Qiagen) and RT-PCR was conducted using primer pairs (Eurogentec) for mouse chemerin (forward: 5′-GACAGACGCGCAACCTCA-3′, reverse: 5′-GGACTATCGCCGGTAGAA-3′), ChemR23 (forward: 5′-CGACTCTCCGTICACATCT-3′, reverse: 5′-AAGACAGTGTTAAGGTAGCA-3′), and mouse GAPDH (forward: 5′-ATGCTGGAGTCTACTGGTF-3′, reverse: 5′-GTGAGAACAAGGGCCCAT-3′).

Peptides

Peptides were synthesized as previously described (23) by solid phase chemistry using 9-fluorenylmethoxycarbonyl as protecting group with an automated Symphony apparatus (Protein Technologies). The synthetic 15-mer chemerin-derived peptide C15 (143-158) was purchased from PepGraft (San Diego, CA) and validated by reversed-phase HPLC. Peptide solutions at 100 μM were stored at −80°C and thawed occasionally. Stock solutions of all peptides were adjusted to final concentrations of 10 μM before use.

Calcium mobilization and binding assays

The calcium mobilization response of mouse ChemR23 was analyzed on CHO-K1 cells stably expressing mouse ChemR23 under an aquorin-based calcium mobilization assay as previously described (23). Briefly, cells were incubated for 3 h in the dark in DMEM containing 5 μM coelenterazine H (Molecular Probes). Peptides at different concentrations were plated in 96-well plates and a cell suspension (5 × 10^4 cells) was added to the wells in a Packard luminometer. The parameters of the dose-response curves were determined with the Prism software (GraphPad Software) using nonlinear regression applied to a sigmoidal dose-response model. Mouse bone marrow cells were determined with the Prism software using nonlinear regression applied to a one-site competition model.

Antibodies

mAbs 489C1 (IgM) and 681 (IgG2c) directed against the mouse ChemR23 receptor were obtained following immunization of HsdCdp Wistar Unilever rats (Harlan Netherlands) with a peptide corresponding to the second extracellular loop (acyl-CAPESSPHAHQSV-amide).

Mouse DC, macrophage, and NK cell preparations

Mouse BMDCs were generated as previously described (30). Briefly, BMDCs were recovered by flushing femurs and tibiae and cells were cultured for 14 days in RPMI 1640 containing l-glutamine (Cambrex) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μM 2-ME (Sigma-Aldrich), 10% heat inactivated FBS (Jacques Boy Institute), and 20 ng/ml mouse recombinant GM-CSF (BioSource International) and used as immature DCs for subsequent experiments. Spleen DCs (CD11c<sup>+</sup> DCs and pDCs) and NK cells were purified by using the specific isolation kits (Millenyi Biotec) following the manufacturer’s instructions. The CD11c<sup>+</sup> DC populations was analyzed by flow cytometry for the CD11c and I-A<sup>d</sup> DC markers (BD Pharmingen), NK cells were stained with CD49b (BD Pharmingen) and NKp46/NCR1 (R&D Systems) markers, and pDCs were fluorescently stained with anti-miPDCA-1-allophycocyanin (Millenyi Biotec) and anti-Ly-6-C-PE (BD Pharmingen). Macrophages were harvested from peritoneal exudates 5 days after intraperitoneal injection of 2 ml 4% thioglycolate (Sigma-Aldrich). Macrophages were obtained by adherence after 24 h in culture. The cell population was consistently composed of >90% macrophages, as determined by flow cytometry analysis using anti-CD11b, I-Ab (BD Pharmingen), and F4/80 (Serotec).

Chemotaxis

Migration of macrophages and DCs was measured using a 48-well microchemotaxis Boyden chamber (NeuroProbe) with polycarbonate membranes (5-μm pores; NeuroProbe) previously described for mouse BMDCs (23). All conditions were tested in triplicate. Controls were performed in the absence of chemotactic factor in the lower wells. The results were expressed as migration ratio (mean cell number per well with chemoattractant over mean cell number per well without chemoattractant).

Immunostaining

Double immunofluorescence staining on DCs was measured using CD11c<sup>+</sup> DCs (clone H1.3, Armenian hamster IgG1, dilution 1/50; BD Pharmingen), anti-I-A<sup>d</sup> (clone AF6-120.1, mouse IgG2a, dilution 1/50; BD Pharmingen), and anti-mouse ChemR23 (clone 489C, rat IgM, dilution 1/100; Euroscreen SA) mAbs. Freshly isolated mouse NK cells were stained directly on cytospin slides with anti-NKp46/NCR1 (goat IgG, dilution 1/100; R&D Systems) and anti-mouse ChemR23 (clone 489C). Appropriate Texas red-, Cy3-, and FITC-conjugated isotype-specific secondary Abs (Jackson Immunoresearch Laboratories) were used to reveal NK cells and DC markers (BD Pharmingen), and NK cells were stained with anti-Ly-6-C-PE (BD Pharmingen) and anti-CD11b, I-Ab (BD Pharmingen) and F4/80 (Serotec). Tissues were fixed in 100% methanol for 10 min and mounted onto slides with DPX mounting medium. Immunofluorescence images were captured with an Axioplan 2 imaging fluorescence microscope (Zeiss), equipped with a Diagnostic Spot digital camera and analyzed by the Spot Advance soft imaging system and Adobe Photoshop 7.0. The lenses used were 20××0.5, 40××0.75, 100××1.30, and 100××1.30, 100××0.75, 40××0.5, and 20××0.5.

LPS-induced acute lung injury model

Mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg; Pfister) and xylazine (10 mg/kg; Bayer). After visualization of the trachea’s lumen, acute lung inflammation was induced by transoral instillation into the trachea of PBS (controls) or 1 μg of Escherichia coli LPS (LPS 0111:B4; Sigma-Aldrich) in 50 μl PBS, containing or not 5 μg of mouse recombinant chemerin (R&D Systems). At selected time points, bronchoalveolar lavages (BAL) were obtained by flushing the lungs with sterile 0.9% NaCl and differential cell counts were performed on cytospin preparations after Diff-Quick staining (Dade Behring). Lungs were collected 18 h after LPS instillation for histological analysis and preparation of cell suspensions. Briefly, lungs were perfused with 10 ml PBS through the right ventricle, dissected, minced, and incubated with 2 mg/ml collagenase D and 0.02 mg/ml DNase I (Roche) for binding assays were normalized for the nonspecific binding (0%) and the specific binding in the absence of competitor (100%). Binding parameters were determined with the Prism software using nonlinear regression applied to a one-site competition model.

Antibodies

mAbs 489C1 (IgM) and 681 (IgG2c) directed against the mouse ChemR23 receptor were obtained following immunization of HsdCdp Wistar Unilever rats (Harlan Netherlands) with a peptide corresponding to the second extracellular loop (acyl-CAPESSPHAHQSV-amide).
1 h at 37°C. After lysis of erythrocytes, cells were stained for neutrophils (CD11b<sup>Hi</sup> Gr-1<sup>Hi</sup> CD11c<sup>Neg</sup>) with FITC-CD11b, PerCpCy5.5-Gr-1, and allophycocyanin-CD11c; stained for interstitial macrophages (F4/80<sup>/H11001</sup> CD11b<sup>Hi</sup> I-A/I-E<sup>Int</sup> CD11c<sup>Neg</sup>) and alveolar macrophages (F4/80<sup>/H11001</sup> CD11c<sup>Hi</sup> I-A/I-E<sup>Int</sup> CD11b<sup>Neg/Lo</sup>) with FITC-F4/80 (Serotec), PE-I-A/I-E, PerCp-Cy5.5-CD11b, and allophycocyanin-CD11c; and stained for iso-type controls (BD Pharmingen). All samples were analyzed using a dual-laser flow cytometer (FACSCalibur) using the CellQuest software (BD Pharmingen). BAL fluids (BALF) were assayed for TNF-α, IL-6, IL-1β, and KC/CXCL1 using cytometric bead array-based immunoassays (CBA flex set; BD Biosciences), a dual-laser flow cytometer (FACSCalibur; BD Biosciences) and the FCAP array software (BD Biosciences) for analysis, following the manufacturer’s instructions. Histology was obtained after lung insufflations with 1 ml of 4% paraformaldehyde and embedded in paraffin. Lung sections (5 μm) were stained with H&E and assessed by light microscopy.

Statistical analysis

Analysis of differences in BALF cytokines and differential cell counts was performed by one-way ANOVA using the Prism 4 software. The Student-Newman-Keuls test was used for pairwise comparisons. For all tests, p < 0.05 was considered as significant.

Results

The chemerin/ChemR23 system is well conserved in vertebrate species

The characterization of mouse chemerin and its receptor ChemR23 constitutes a necessary step to study the biological functions of this new leukocyte chemoattractant system. Human and mouse ChemR23 share 80% identity at the amino acid level. The mouse ChemR23 gene, also called mcmklr1 or Dez, is localized on chromosome 5. The cDNA was originally cloned from a brain library (32), and the genomic organization of the gene was previously reported (33, 34). Prochemerin (Ensembl ID ENSMUSG00000009281) and ChemR23 (Ensembl ID ENSMUSG00000042190) amino acid sequences from vertebrate species were aligned with ClustalW and a dendrogram was constructed (Fig. 1). Orthologs of chemerin and ChemR23 were unambiguously found in primate, mammalian, bird, and fish species. The six cysteines presumably involved in disulfide bridging were strictly conserved in all species. In addition, the C-terminal nonapeptide of the mature protein, previously shown to be important for the biological activity of human chemerin, was highly conserved (YFPGQFAFS) in all mammalian species. This suggests that the interaction with the cognate receptor and the mechanisms allowing generation of bioactive chemerin are well conserved across species.

Phenotype of ChemR23 KO mice

The ChemR23 KO mice, in which part of the third exon is replaced by a Neo cassette, were obtained from Deltagen. The deleted segment includes the splice acceptor site, the start codon, and the sequence encoding the first two transmembrane segments of the receptor (supplemental data 1).<sup>5</sup> The ChemR23 KO mice did not display differences with their WT littermates in terms of breeding, growth, and survival in a specific pathogen-free environment. No major changes in spleen and blood leukocyte populations were observed in physiological conditions (T and B cells, macrophages, DCs, NK, granulocytes; supplemental data 1).

Tissue and cell distribution of mouse chemerin and ChemR23

The expression of the mouse genes was determined and compared with what is known in human. By RT-PCR, preprochemerin transcripts were detected in most tissues tested (Fig. 2A). ChemR23 transcripts were found at high levels in skin, lung, colon, ovary, testis, spleen, and mesenteric lymph nodes, and were detected in other tissues as well. This broad distribution parallels what has been described in human.

<sup>5</sup>The online version of this article contains supplemental material.
To identify more precisely which cell populations express chemerin in the various tissues, we tested its distribution by immunohistochemistry, focusing on a set of tissues playing key roles in immune defense mechanisms and frequently involved in inflammatory diseases. Similar results were obtained with a rat mAb (R&D Systems) and two goat polyclonal Abs (R&D Systems and Santa Cruz Biotechnology). As shown in Fig. 2B, staining for chemerin was obtained in the epithelial cell layer of the small intestine; the ciliated epithelium of the lung bronchioles; and the granular keratinocyte layer, external root sheath, and sebaceous glands in the skin.

We also generated rat mAbs directed against mouse ChemR23. The monoclonals 489C and 681 were validated by FACS and immunohistochemistry using mouse ChemR23-expressing CHO-K1 cells, and further used to study the distribution of the receptor on tissue sections and in leukocyte populations. The specificity was checked by using samples originating from ChemR23 KO mice as controls. In the small intestine, scattered positive cells were found in the lamina propria of the villi (Fig. 2C) using the two monoclonals. No staining was obtained for ChemR23 KO mice.

DC populations were either purified directly from spleen by magnetic bead sorting, or were generated from BMDCs (30, 35). Double immunofluorescent staining was performed on cryosections of spleen CD11c+ cells, demonstrating coexpression of ChemR23 and the DC markers MHC class II (I-Ab) and CD11c (Fig. 3A). FACS analysis confirmed ChemR23 expression in the majority of CD11c+ cells, although two distinct peaks (ChemR23Lo and ChemR23Hi) were seen (Fig. 3C). A small fraction (~10%) of the CD11c- cells was also weakly positive for ChemR23 (Fig. 3C). These cells could correspond to macrophages and NK cells previously shown to be ChemR23+ in human (18, 21, 22).

BMDCs were prepared from WT and ChemR23 KO mice, and tested by FACS for a number of DC and maturation state markers (CD11c, CD11b, I-Ab, CD40, CD86). No difference was seen between the two groups in terms of number of BMDCs recovered and maturation states (data not shown). After 14 days of culture, a majority of immature DCs (75%, CD11c+ MHCIIlo) was recovered, of which ~40% coexpressed ChemR23 at a high level (Fig. 3D). The CD11c+ MHCIIhi population of mature dendritic cells...
was essentially negative for ChemR23. As expected, no ChemR23 staining was seen on BMDCs derived from ChemR23 KO mice (Fig. 3D).

We further purified pDCs from the population of mouse spleen DCs and used CD11c, Ly-6C, and mPDCA as pDC markers. FACS costaining demonstrated that the whole population of mPDCA⁺ cells (pDCs) expressed ChemR23 at high level on their surface (Fig. 3E). It appears therefore that the ChemR23⁺ population corresponds to the pDC subset. As in human, ChemR23 is more highly expressed on pDCs than on mDCs or macrophages.

Only a fraction of macrophages (CD11b⁺ F4/80⁺) differentiated from bone marrow were positive for ChemR23 (∼10%) in FACS, while relatively weak staining was obtained for purified NK cells (CD49b⁺) from WT but not from KO mice (supplemental data 2). Immunofluorescence staining confirmed expression of ChemR23 in purified NK cells (Fig. 3B). The staining was mostly cytoplasmic, which is not unusual for chemoattractant receptors for which intracellular pools are often seen in the endosomal compartments.

**Biological activity of mouse chemerin**

The pharmacological properties of the mouse chemerin/ChemR23 system were evaluated using CHO-K1 cell lines coexpressing mouse ChemR23 and apoaequorin (23). The pEC₅₀ and pIC₅₀ values derived from binding and calcium mobilization assays are displayed in Table I. Competition binding assays were performed using the 125I-YHSFFFPGQFAFS peptide as tracer (Fig. 4F). We also tested human recombinant chemerin on mouse ChemR23 and apoaequorin (23). The pIC₅₀ and pEC₅₀ values derived from binding and calcium mobilization assays are displayed, as well as the functional (pEC₅₀) and binding (pIC₅₀) parameters of their activity on mouse ChemR23-expressing CHO-K1 cells. Data are given as mean ± SEM and were calculated from at least three independent experiments.

**Table I. Nomenclature of peptides and parameters of their binding and functional properties on mouse ChemR23**

<table>
<thead>
<tr>
<th>Peptide Nomenclature</th>
<th>Peptide Sequence</th>
<th>pEC₅₀</th>
<th>pIC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rec m-chemerin 1–157</td>
<td>IAQAGEDPHYGFLPGQFAFSRALRTK</td>
<td>9.34 ± 0.06</td>
<td>9.63 ± 0.11</td>
</tr>
<tr>
<td>m-prochemerin 138–163</td>
<td>IAQAGEDPHYGFLPGQFAFS</td>
<td>≤5</td>
<td>≤6</td>
</tr>
<tr>
<td>m-prochemerin 138–158</td>
<td>IAQAGEDPHYGFLPGQFAFS</td>
<td>7.71 ± 0.06</td>
<td>7.83 ± 0.05</td>
</tr>
<tr>
<td>m-prochemerin 150–157</td>
<td>LPGQFAFS</td>
<td>≤5</td>
<td>≤6</td>
</tr>
<tr>
<td>m-prochemerin 149–157</td>
<td>FLPGQFAFS</td>
<td>7.11 ± 0.03</td>
<td>7.18 ± 0.07</td>
</tr>
<tr>
<td>m-prochemerin 148–157</td>
<td>YFLPGQFAFS</td>
<td>8.83 ± 0.07</td>
<td>8.11 ± 0.03</td>
</tr>
<tr>
<td>m-prochemerin 147–157</td>
<td>GYFLPGQFAFS</td>
<td>8.50 ± 0.12</td>
<td>8.27 ± 0.08</td>
</tr>
<tr>
<td>m-prochemerin 146–157</td>
<td>HGYFLPGQFAFS</td>
<td>8.53 ± 0.07</td>
<td>8.42 ± 0.15</td>
</tr>
<tr>
<td>m-prochemerin 141–155 (C15)</td>
<td>AGEDPHYGFLPGQFA</td>
<td>≤5</td>
<td>≤6</td>
</tr>
</tbody>
</table>

The sequence of all peptides tested in the present study is displayed, as well as the functional (pEC₅₀) and binding (pIC₅₀) parameters of their activity on mouse ChemR23-expressing CHO-K1 cells. Data are given as mean ± SEM and were calculated from at least three independent experiments.

**Pharmacological characterization of synthetic peptides derived from the chemerin C terminus**

Previous studies with the human chemerin/ChemR23 system demonstrated that accurate proteolytic processing of the precursor prochemerin is required for the generation of bioactive chemerin. This processing affects the C-terminal part of the protein and only two C-terminal variants, lacking the last six or seven amino acids of the precursor, are able to exert biological activity on ChemR23 (24). To investigate whether this precise activation mechanism is conserved between the human and mouse species, we compared the activity of different recombinant C-terminal variants of mouse chemerin on ChemR23-expressing cells. The conditioned medium of CHO-K1 cells expressing mouse mature chemerin (form 1–157, lacking the last six amino acids of prochemerin) activated ChemR23 in the aequorin assay, whereas a variant lacking two additional amino acids (form 1–155) was inactive (supplemental data 4). However, Western blotting demonstrated that the two recombinant chemerin variants were present in similar amounts in the conditioned medium, confirming the weak bioactivity of chemerin 1–155. These observations strongly suggest that, as shown for human chemerin, accurate C-terminal processing of the precursor is essential for the regulation of the bioactivity of mouse chemerin.

We have previously demonstrated that human chemerin could be trimmed-down to its C-terminal nonapeptide (chemerin C₉₋₁₅) was observed in response to chemerin (Fig. 4D). We next tested the chemotactic properties of chemerin on mouse APCs. As shown in Fig. 4, E and F, mouse chemerin promoted a dose-dependent migration of immature BMDCs and macrophages, with a typical bell-shaped curve culminating for concentrations of 3 and 1 nM, respectively. The migration index was significantly higher for BMDCs than for macrophages. The efficacy of chemerin in promoting BMDC migration was higher than that of CCL5 (CCR1 ligand, peak at 10 nM) and CXCL12 (CXCR4 ligand, peak at 1 nM; supplemental data 3), as observed previously in human (22). As expected, BMDCs and macrophages prepared from ChemR23 KO mice failed to migrate in response to chemerin, while the response to CCL5 and CXCL12 was kept (supplemental data 3). Altogether, these data demonstrate that chemerin is a potent and efficient chemoattractant factor for immature DCs and macrophages, and that this effect is exclusively mediated by ChemR23. We also tested the influence of chemerin onto the maturation of DCs by analyzing maturation markers by FACS. It was found that chemerin does not promote DC maturation in vitro, nor prevents the maturation initiated by LPS (data not shown).

Ca²⁺ was observed in response to chemerin (Fig. 4D). We next tested the chemotactic properties of chemerin on mouse APCs. As shown in Fig. 4, E and F, mouse chemerin promoted a dose-dependent migration of immature BMDCs and macrophages, with a typical bell-shaped curve culminating for concentrations of 3 and 1 nM, respectively. The migration index was significantly higher for BMDCs than for macrophages. The efficacy of chemerin in promoting BMDC migration was higher than that of CCL5 (CCR1 ligand, peak at 10 nM) and CXCL12 (CXCR4 ligand, peak at 1 nM; supplemental data 3), as observed previously in human (22). As expected, BMDCs and macrophages prepared from ChemR23 KO mice failed to migrate in response to chemerin, while the response to CCL5 and CXCL12 was kept (supplemental data 3). Altogether, these data demonstrate that chemerin is a potent and efficient chemoattractant factor for immature DCs and macrophages, and that this effect is exclusively mediated by ChemR23. We also tested the influence of chemerin onto the maturation of DCs by analyzing maturation markers by FACS. It was found that chemerin does not promote DC maturation in vitro, nor prevents the maturation initiated by LPS (data not shown).

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149–157, YFPSQFAFS, also called chemerin-9) while keeping a low nanomolar affinity for its receptor (18). Such synthetic chemerin-derived peptides have been used to characterize the pharmacology of the human chemerin/ChemR23 system and to identify key residues contributing to the biological activity of chemerin. With the aim of investigating the pharmacological properties of the mouse counterpart, we have tested synthetic peptides derived from the C-terminal end of the mouse chemerin. All the peptides started after the last cysteine presumably involved in disulfide bonding. We synthesized peptides corresponding to the inactive mouse prochemerin (m-prochemerin 138–163), the active mouse chemerin (m-chemerin 138–157), a 21-amino acid peptide with one additional amino acid (m-chemerin 138–158), and m-chemerin 141–155 corresponding to the synthetic 15-mer chemerin-derived peptide C15 used by Cash et al (29) (Table I).

These peptides were tested for their ability to trigger intracellular calcium release through mouse ChemR23, using the aequorin-based assay. As shown in Fig. 5 and Table I, shortening the peptide to nine amino acids (149–157) slightly increased the potency (EC$_{50}$ of 19.7 ± 2.9 nM). However, further removal of Phe149, resulting in the octapeptide m-chemerin 150–157, decreased the potency by one order of magnitude (EC$_{50}$ of 78 ± 6 nM). In conclusion, these results identified the nonapeptide 149FLPGQFAFS157 as the shortest C-terminal peptide derived from mouse chemerin retaining high potency on ChemR23.

The binding properties of these peptides were next investigated in the competition binding assay (18). The pIC$_{50}$ values derived from the binding curves are displayed in Table I for the peptides tested in this assay. As shown in Fig. 5C and Table I, the bioactive m-chemerin 138–157 peptide inhibited binding of the tracer with an IC$_{50}$ of 14.8 ± 1.7 nM, whereas the functionally inactive peptides m-prochemerin 138–163 and m-chemerin 138–158 did not significantly compete with the tracer up to concentrations of 1 µM. The whole set of N-terminally truncated peptides (Table I) was also tested in the binding assay (Fig. 5D). The nonapeptide m-chemerin 149–157, the shortest active peptide, bound the receptor with an apparent affinity of 7.8 ± 0.6 nM. In contrast, the shorter peptide m-chemerin 150–157 had a 10-fold lower affinity, indicating that the loss of bioactivity is secondary to a decrease in affinity for ChemR23.

A second set of peptides was synthesized to explore the effect of N-terminal truncations, starting from the active m-chemerin 138–157 peptide. As shown in Fig. 5B and Table I, shortening the peptide to nine amino acids (149–157) slightly increased the potency (EC$_{50}$ of 4.8 ± 0.8 nM). However, further removal of Phe149, resulting in the octapeptide m-chemerin 150–157, decreased the potency by one order of magnitude (EC$_{50}$ of 78 ± 6 nM). In conclusion, these results identified the nonapeptide 149FLPGQFAFS157 as the shortest C-terminal peptide derived from mouse chemerin retaining high potency on ChemR23.

The binding properties of these peptides were next investigated in the competition binding assay (18). The pIC$_{50}$ values derived from the binding curves are displayed in Table I for the peptides tested in this assay. As shown in Fig. 5C, the bioactive m-chemerin 138–157 peptide inhibited binding of the tracer with an IC$_{50}$ of 14.8 ± 1.7 nM, whereas the functionally inactive peptides m-prochemerin 138–163 and m-chemerin 138–158 did not significantly compete with the tracer up to concentrations of 1 µM. The whole set of N-terminally truncated peptides (Table I) was also tested in the binding assay (Fig. 5D). The nonapeptide m-chemerin 149–157, the shortest active peptide, bound the receptor with an apparent affinity of 7.8 ± 0.6 nM. In contrast, the shorter peptide m-chemerin 150–157 had a 10-fold lower affinity, indicating that the loss of bioactivity is secondary to a decrease in affinity for ChemR23.
Anti-inflammatory role of chemerin in an LPS-induced mouse model of acute lung injury

To determine in vivo the physiological role of chemerin, we next investigated whether chemerin is able to regulate inflammation in a mouse acute lung injury model. LPS mimics the symptoms of acute lung injury, which is characterized by the accumulation in the lungs of neutrophils producing inflammatory mediators. We used whole chemerin to investigate the consequences of ChemR23 activation as synthetic chemerin-derived peptides such as the human nonapeptide (149YFPGQFAFS 157) were shown to have a short-lived and poor activity in cell cultures (i.e., chemotactic) due to their rapid degradation by proteases. Mice were injected transorally with LPS alone (1 μg) or in combination with a single dose of recombinant chemerin (5 μg). Cell counts and cytokine levels were measured in BAL and histological analyses and leukocyte typing by flow cytometry following tissue digestion were performed on lung at various time points. As shown in Fig. 6A, histological examination of lung tissues revealed a marked inflammation at 18 h in LPS-treated mice, with hyperplasia of the bronchiolar epithelium, and peribronchiolar, perivascular, and alveolar infiltration by neutrophils. Such infiltrate was not found in control animals receiving PBS or chemerin alone (Fig. 6A). Neutrophils were counted in the BAL at 6, 12, 24, and 72 h. A peak of neutrophils was reached between 12 and 24 h after LPS (Fig. 6A). Chemerin decreased neutrophil counts in BAL by 70% 12 h after the challenge, and this effect persisted at 24 (53% inhibition) and 72 h (63% inhibition) within the resolution phase, during which the neutrophil counts decreased progressively in all groups.

To confirm these observations and determine whether the anti-inflammatory effects of chemerin are mediated through ChemR23, the leukocyte populations recruited to lung were analyzed in both BALFs and digested lungs from WT and ChemR23 KO mice 18 h after the LPS challenge. LPS induced a stronger inflammatory response in ChemR23 KO mice as compared with WT mice, with an increase of neutrophils in BALFs and lung cell suspensions (Fig. 6B). Moreover, in WT mice receiving LPS plus chemerin, neutrophils were decreased significantly by 70% (BALF) and 50% (lung tissue) as compared with mice receiving LPS only. ChemR23 KO mice did not respond to chemerin and displayed similarly high neutrophil levels (Fig. 6B). Both WT and ChemR23-deficient mice showed increased macrophage levels in response to LPS, but macrophage recruitment was higher in BALFs and total lungs from KO mice as compared with WT mice. FACS analysis revealed that F4/80+CD11b+ interstitial macrophages and, to a lower extent, F4/80+CD11c+ alveolar macrophages (AM) increased after LPS treatment in WT and KO mice (supplemental data 5). KO mice showed a significantly lower proportion of AM as compared with WT mice (15% vs 27%, supplemental data 5), likely resulting from the relative dilution of resident macrophages by newly recruited cells. No effect of chemerin was observed on the recruitment of total lung macrophages in WT or KO mice as hallmarks of the lung inflammatory response. We also measured a set of inflammatory mediators in BALFs of WT and KO mice as hallmarks of the lung inflammatory response. The results for KC/CXCL1, IL-6, TNF-α, and IL-1β are displayed in Fig. 6C. PBS or chemerin alone did not promote any change in cytokine levels over baseline, whereas LPS treatment resulted in a strong increase of these mediators. The increase of all four cytokines was much milder in the group of WT mice receiving chemerin in addition to LPS (78% reduction for KC/CXCL1, IL-6, and IL-1β, 73% for TNF-α). In ChemR23 KO mice, chemerin treatment had no effect on inflammatory cytokines and chemokines.
in BAL. Altogether, these data clearly demonstrate that chemerin can exert an anti-inflammatory activity, which is strictly dependent on ChemR23.

Discussion

Chemerin was identified as the natural ligand of the previously orphan ChemR23 receptor (18). No other ligand has so far been described for ChemR23, with the exception of resolvin E1 (36, 37), which in our study did not act on ChemR23 either in functional or in binding assays (unpublished data). GPR1 was described recently as an additional receptor for chemerin (38). GPR1 expression was previously found in AM (39) but was not reported in other leukocyte populations so far and the signaling cascades activated by GPR1 remain elusive.

The biologically inactive precursor of chemerin, prochemerin, is expressed by most tissues in humans and is present at nanomolar concentrations in plasma. It appears therefore that a large pool of precursor is permanently available and that the activation of diverse proteolytic systems results in the local generation of active chemerin able to recruit pDCs, mDCs, macrophages, and NK cells (24, 25, 40). However, how similar the human system is to the mouse system in terms of pharmacology, precursor processing, and distribution of ChemR23 among leukocyte populations is incompletely known.

We first analyzed the conservation of chemerin and ChemR23 in mice and other species. Both the receptor and ligand are relatively well conserved between mammalian species (respectively 59% and 80% identity for chemerin and ChemR23 between human and mouse). Importantly, the C-terminal end of chemerin, which is essential for the activation of ChemR23, is particularly well conserved in evolution, suggesting important and stable functions all along the vertebrate lineage.
We have studied the pharmacology of the mouse chemerin-ChemR23 system in vitro and have tested whether the bioactivity of chemerin was dependent, as in human, on the precise proteolytic maturation of its precursor. The affinity of the mouse receptor for mouse chemerin was similar to that of the human receptor (around 1 nM). The generation of the most active chemerin form requires the proteolytic removal of the last six or seven amino acids from the mouse C terminus prochemerin. This is strictly parallel to what we observed previously for the human system (23). Moreover, the shortest peptide retaining similar bioactivity of full-size chemerin is a C terminus nonapeptide of mature chemerin (\textsuperscript{149}FLPGQFAFS\textsuperscript{157}), which differs slightly from the equivalent human peptide (\textsuperscript{149}YFPGQFAFS\textsuperscript{157}).

Using RT-PCR, immunohistochemistry, and FACS, we analyzed the expression of preprochemerin and ChemR23 in a set of mouse tissues and leukocyte populations. As described previously for human systems (18), preprochemerin transcripts can be found in all tissues. The protein was also detected at high levels by immunohistochemistry in a number of epithelial cell types, including keratinocytes, enterocytes, and bronchiolar epithelial cells. ChemR23 transcripts were found at variable levels in all tissues, but most of these signals might be attributed to the presence of resident DCs, macrophages, and NK cells.

The functionality of ChemR23 in macrophages and immature DCs was demonstrated by measuring calcium mobilization and chemotaxis. These data are consistent with the observations made previously in human models (18, 22). It was, however, reported that mouse ChemR23 was not present on mouse mDCs and pDCs (40, 41). These observations are presumably the result of the poor sensitivity of the Abs used in these studies, as in our study, ChemR23 was both present and functional in these cells. Moreover, the specificity of the labeling and the involvement of ChemR23 in the chemerin-mediated chemotaxis were clearly demonstrated for both cell populations by the lack of labeling and migration when the cells were prepared from ChemR23 knock-out mice.

The expression of chemerin in bronchiolar and other epithelial cells lining mucosal barriers and the presence of ChemR23\textsuperscript{+} cells in the stroma of the same tissues suggest a role for this system in the regulation of defense mechanisms against pathogens or other inflammatory stimuli in the stroma of the same tissues suggest a role for this system in the regulation of defense mechanisms against pathogens or other inflammatory stimuli. In this context, chemerin instillation in the regulation of defense mechanisms against pathogens or other inflammatory stimuli in the stroma of the same tissues suggest a role for this system in the regulation of defense mechanisms against pathogens or other inflammatory stimuli.

Cash et al (29) recently reported an anti-inflammatory activity of chemerin and chemerin-derived peptides. Despite the similar conclusions reached by their and our present studies, there are significant differences. Cash et al describe that mouse chemerin and a synthetic 15-mer chemerin-derived peptide (C\textsuperscript{15}, \textsuperscript{141}AGEDPH YFLPGQFA\textsuperscript{155}) inhibit macrophage activation in vitro at picomolar concentrations. The C15 peptide (but not full-sized chemerin) was also reported by Cash et al to display in vivo anti-inflammatory properties in a mouse acute peritonitis model (29). These data are difficult to reconcile with our own results because the C15 peptide lacks the amino acid F\textsuperscript{156}, which is essential for the activity of chemerin-derived peptides on ChemR23, both in human and mouse (23, 24, 31). We have tested the functional activity of the C15 peptide in an aequorin-based calcium release assay on CHO-K1 cells expressing mouse ChemR23, but could not detect any activity of this peptide, while recombinant chemerin and other peptides were fully active. Moreover, the very low concentrations (10\textsuperscript{-12}–10\textsuperscript{-10} M range) of chemerin and C15 peptide tested by Cash et al differ considerably from all previous reports showing that chemerin and its C-terminal peptides are active on ChemR23 in the 10\textsuperscript{-10}–10\textsuperscript{-8} M and 10\textsuperscript{-8}–10\textsuperscript{-7} ranges, respectively (18, 21, 22, 41).

We also tested the activity of recombinant chemerin on LPS-treated DCs and peritoneal and lung macrophages, and could not find, besides their chemotactic response, any positive or negative effect on activation and maturation of these cells in terms of cytokine production or chemokine receptor expression (supplemental data 6). Also, in contrast to our study, Cash et al did not observe a difference between WT and KO mice in terms of neutrophil and monocyte infiltration in their mouse acute peritonitis model (29), whereas an increased inflammatory response was seen in our model, suggesting a role of endogenously generated chemerin in the lung inflammatory model.

Besides the anti-inflammatory function in lung homeostasis, other functions of the chemerin-ChemR23 system must be considered. Overexpression of chemerin and recruitment of ChemR23\textsuperscript{+} cells were described in human skin diseases and chemerin was proposed to recruit immature NK cells and favor their interaction with DCs (21, 22, 26). The chemerin/ChemR23 system might therefore play a role in the bidirectional crosstalk between NK cells and DCs and potentially influence the polarity of the immune responses (52–57). The presence of chemerin on high endothelial venules in human lymph nodes and tonsils suggests a role in the recruitment of cell populations to secondary lymphoid organs (22). Also, expression of both the ligand and receptor in human adipocytes has suggested a role of chemerin as an autocrine/paracrine adipokine regulating adipogenesis and lipolysis (58–60).

In conclusion, the mouse chemerin-ChemR23 system appears very well conserved between human and mouse and, based on the available sequence data, likely in other mammalian species as well. The pharmacology of the receptor is very similar in the two species, as well as the C-terminal determinants of the ligand required for bioactivity. Mouse ChemR23 is expressed in the same leukocyte populations as in humans, namely immature pDCs and mDCs, macrophages and NK cells, and the ligand precursor displays the same broad distribution. We have in the present work demonstrated the expression of mouse chemerin in epithelial barriers and provided the demonstration of its anti-inflammatory role in vivo, through the ChemR23 receptor. Mouse appears therefore as an excellent model to help understand the physiological role of chemerin and ChemR23 in immune and inflammatory responses, as well as in models of human diseases.
References
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