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*J Immunol* 2009; 183:6489-6499; Prepublished online 19 October 2009; doi: 10.4049/jimmunol.0901037

http://www.jimmunol.org/content/183/10/6489

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Supplementary Material [http://www.jimmunol.org/content/suppl/2009/10/20/jimmunol.0901037.DC1](http://www.jimmunol.org/content/suppl/2009/10/20/jimmunol.0901037.DC1)

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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.

During an inflammatory process, damaged tissues release mediators that contribute to the mounting of immune responses by regulating the trafficking of leukocyte populations. Chemokines and other chemoattractant molecules therefore play fundamental roles in the physiology of inflammatory events, as well as in the pathological dysregulations of these processes (1, 2). APCs are multifunctional immune effector cells that act as sentinels, capturing Ags and transporting them to lymphoid tissues, where they activate naïve T cells. Dendritic cells (DCs) are the most potent APCs. They are equipped with a set of receptors for danger signals, allowing them to adapt their behavior and dictate the outcome of the resulting immune response (3–6). Given this pivotal position at the intersection of innate and adaptive immunity, DCs are attractive targets for the development of therapeutic strategies shaping the immune responses in pathological states such as cancer, inflammatory diseases, and graft rejection (7). Two main categories of DCs have been described, the conventional, or myeloid, DCs (mDCs) and the plasmacytoid DCs (pDCs), which have clearly distinct roles in the initiation of immunity against specific pathogens (8, 9). mDCs include resident immature DCs that are found in most peripheral tissues, including primary and secondary lymphoid organs. In nonlymphoid tissues, resident DCs are frequently located in close contact with the mucosal surfaces (respiratory tract, lung, intestine) where they can directly sample incoming pathogens through the epithelial barrier (10). Following maturation, mDCs migrate to the local draining lymph nodes. pDCs are recruited directly to lymphoid tissues through high endothelial venules (11–13). Whereas mDCs display a full battery of chemokine receptors, only a few receptors were demonstrated to be functional for the recruitment of immature pDCs, including CXCR4, CXCR3, and CCR9 (13–17).

Chemerin in a Lung Disease Model

Chemerin is a novel extracellular mediator that we identified as the ligand of ChemR23, a G protein-coupled receptor expressed by immature mDCs and pDCs, NK cells, and macrophages (18–21). High amounts of active chemerin were found in various inflammatory situations as in human (rheumatoid arthritis, inflammatory ascites) and ChemR23-expressing cell recruitment was described in human inflammatory diseases (21, 22). Chemerin was characterized as a strong chemoattractant factor acting at subnanomolar concentrations (18, 22). The protein is secreted as an inactive precursor, prochemerin, which is converted into a full agonist of the ChemR23 receptor by proteolytic cleavage. Human prochemerin is secreted as a large precursor and is processed by a specific protease to release the active chemerin peptide (19). 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.
Molecular Probes. Peptides at different concentrations were plated in 96-row-derived DCs (BMDCs) were loaded with 5 μM [125I]YHSFFFPGQFAFS peptide as tracer (Phoe- 

systems) and 1 mM ATP (Sigma-Aldrich). Competition binding assays were conducted using primer pairs (Eurogentec) for mouse chemerin (forward: 5'-AAGACGATGGTAAGGTAGCA-3', reverse: 5'-GACAGAGCCCGAACTCA-3', dilution 1/30; BD Pharmingen), anti I-Ab (clone AF6–120.1, mouse IgG2a, dilution 1/100; BD Pharmingen), and variable concentrations of competitors, as described previously (23). Total binding was measured in the absence of chemotactic factor and nonspecific binding was measured in the presence of a 100-fold excess of the unlabeled peptide. Data resulting from competition 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 489C (IgM) and 681 (IgG2ck) directed against the mouse ChemR23 receptor were obtained following immunization of HsdCpD Wistar Uni- 

lever rats (Harlan Netherlands) with a peptide corresponding to the second extracellular loop (acetyl-CAPESSPHIAHSQV-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 cul- 

tured for 14 days in RPMI 1640 containing 1-glutamine (Cambrex) sup- 

plemented 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 (CD1c+ 

DCs and pDCs) and NK cells were purified using the specific isolation kits (Milenyi Biotec) following the manufacturer’s instructions. The CD11c+ 

DCs population was analyzed by flow cytometry for the CD11c and I-Aα 

DC markers (BD Pharmingen), NK cells were stained with CD49b (BD 

Pharmingen) and NK46/NCR1 (R&D Systems) markers, and pDCs were 

fluorescently stained with anti-mPDCA-1-allophycocyanin (Milenyi Biotec) 

and anti-Ly-6C-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 micro- 

chemotaxis Boyden chamber (NeuroProbe) with polycarbonate membranes (5-μm pores; Neuro Probe) previously described (23). Cells 

were tested in triplicate. Controls were performed in the absence of che- 

moattractant in the lower wells. The results were expressed as migration 

ratio (mean cell number per well with chemotactrant over mean cell number per well without chemotactrant).

Immuno-staining
Double immunofluorescence staining on mouse DCs was performed using 

anti-Cd11b (clone HI3, Armenian hamster IgG1, dilution 1/50; BD 

Pharmingen), anti I-Aα (clone AF6–120.1, mouse IgG2a, dilution 1/50; BD 

Pharmingen), and anti-mouse ChemR23 (clone 489C, rat IgM, dilution 1/100; European Screen) mAbs. Freshly isolated mouse NK cells were cultured di- 

rectly on cytopsin slides with anti-NK46/NCR1 (goat IgG, di- 

lution 1/100; R&D Systems) and anti-mouse ChemR23 (clone 489C). Ap- 

propriate Texas red-, Cy3-, and FITC-conjugated isotype-specific second-

ary Abs (Jackson Immunoresearch Laboratories) were used to reveal 

fluorescently stained with anti-mouse chemerin (goat IgG, dilution 1/200; R&D Systems) or anti-mouse ChemR23 (clone 489C and 681, rat IgM and IgG2ck re- 

spectively, dilution 1/2000 and 1/150 respectively), then with appropriate 

peroxidase-conjugated secondary Abs (Jackson Immunoresearch Laborato- 

ries), and revealed with diaminobenzidine (DakoCytomation). Images 

were obtained with an Axioskop 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, 

×0.17. 

LPS-induced acute lung injury model
Mice were anesthetized by an intraperitoneal injection of ketamine (50 mg/kg; 

Pfizer) 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 cytopsin 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
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, <i>p</i> < 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 <i>mcmklr1</i> or <i>Dez</i>, 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 by RT-PCR from a set of mouse tissues. The data are representative of three independent experiments. MLN, Mesenteric lymph nodes; PP, Peyer’s patches. B, Immunohistochemical detection of chemerin in mouse ileum, lung bronchioles, and skin. Original magnifications are ×400 on top row and ×1000 on bottom row. C, Immunohistochemical detection of ChemR23 in the ileum of WT and ChemR23 KO mice, using the rat mAb 681C. Original magnifications are ×200 (*inset*, ×1000).

<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<sup>+</sup> 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<sup>+</sup> cells, although two distinct peaks (ChemR23<sub>Lo</sub> and ChemR23<sub>Hi</sub>) were seen (Fig. 3C). A small fraction (<10%) of the CD11c<sup>+</sup> cells was also weakly positive for ChemR23 (Fig. 3C). These cells could correspond to macrophages and NK cells previously shown to be ChemR23<sup>+</sup> 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<sup>+</sup> MHCII<sup>Lo</sup>) was recovered, of which ~40% coexpressed ChemR23 at a high level (Fig. 3D). The CD11c<sup>+</sup> MHCII<sup>Hi</sup> population of mature dendritic cells was 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<sup>+</sup> cells, demonstrating coexpression of ChemR23 and the DC markers MHC class II (I-Ab<sup>+</sup>) and CD11c (Fig. 3A). FACS analysis confirmed ChemR23 expression in the majority of CD11c<sup>+</sup> cells, although two distinct peaks (ChemR23<sub>Lo</sub> and ChemR23<sub>Hi</sub>) were seen (Fig. 3C). A small fraction (<10%) of the CD11c<sup>+</sup> cells was also weakly positive for ChemR23 (Fig. 3C). These cells could correspond to macrophages and NK cells previously shown to be ChemR23<sup>+</sup> in human (18, 21, 22).

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**FIGURE 3.** Expression of mouse ChemR23 by DCs and NK cells. Immunofluorescence detection of ChemR23 on mouse CD11c<sup>+</sup> (A) and CD49b<sup>+</sup> cells (B) purified from spleen. DCs from WT mice were labeled for ChemR23 (489C monoclonal, red) and either MHCII or CD11c (green). NK cells from WT or ChemR23 KO mice were labeled for ChemR23 (489C monoclonal, green) and NKP46 (red). Original magnifications are ×400 except inset (×1000). C, FACS analysis of ChemR23 expression on CD11c<sup>+</sup> and CD11c<sup>+</sup> cell populations prepared from mouse spleen. ChemR23 staining was seen on mPDCA<sup>+</sup> CD11c<sup>+</sup> cells (pDCs). The displayed data are representative of at least three independent experiments.

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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. 4). 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).

**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 1–155, lacking the last six amino acids of prochemerin) activated ChemR23 in the aequorin assay, whereas a variant lacking two additional amino acids (form 1–153) 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

<table>
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<tr>
<th>Peptide Nomenclature</th>
<th>Peptide Sequence</th>
<th>pEC50</th>
<th>pIC50</th>
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<tr>
<td>Rec m-chemerin 1–157</td>
<td>IAQAGEDPHGYFLPGQFAFSRALRTK</td>
<td>9.34 ± 0.06</td>
<td>9.63 ± 0.11</td>
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<tr>
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<td>IAQAGEDPHGYFLPGQFAFS</td>
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<td>≤5</td>
<td>≤6</td>
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</table>

a 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.
149–157, YFPGQFAFS, 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. 5A and Table I, the bioactive m-chemerin 138–157 peptide inhibited binding of the tracer with an IC50 of 14.8 ± 0.8 nM. However, further removal of Phe149, resulting in the octapeptide m-chemerin 150–157, decreased the potency by one order of magnitude (EC50 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.

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 (EC50 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 (EC50 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 pIC50 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 bound the receptor with an IC50 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 infiltration was not found in control animals receiving PBS and coadministration of chemerin with the LPS challenge resulted in a decrease in pathological signs and cell infiltration (Fig. 6A). Neutrophils were counted in the BAL at 6, 12, 24, and 72h. 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 (Fig. 6B), but in LPS-treated WT mice, chemerin increased the macrophage number in BALFs, whereas no effect was seen on mouse deficient for ChemR23.

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 mammalian species. It appears therefore that the chemerin-ChemR23 system has been 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 (149FLPGQFAFS157), which differs slightly from the equivalent human peptide (149YFLPGQFAFS157).

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+ cells in the stroma of the same tissues suggests a role for this system in the regulation of defense mechanisms against pathogens or other types of tissue aggression. In this context, chemerin instillation in BALFs and inhibited neutrophil recruitment and the re-
types of tissue aggression. In this context, chemerin instillation in

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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(A) The mouse ChemR23 gene is located on chromosome 5, region F and spans three exons. The first and second exons are non-coding, and the whole coding region is localized in the third exon. Homologous recombination was performed by Deltagen. In the recombinant gene, a Neo cassette is inserted in the third exon, replacing codons 55 to 104 of the ChemR23 open reading frame, which encode the first two transmembrane segments of the receptor. Screening of animals is performed by PCR, using 5’-TGATCTTTGACATGGCTCCCGAA-3’ as forward ChemR23 primer (F), 5’-GGGTGGGATTAGATAATGCCTGCTCT-3’ as forward Neo primer (N), and 5’-TACAGCTTTGGTGCTTCTCCTCGGTC-3’ as common reverse primer (R). The size of the products analyzed by agarose gel electrophoresis were 204 bp for the WT allele and 424 bp for the KO allele, both bands being present for heterozygotes (HZ).

(B) FACS analysis of cell populations isolated from spleen and blood in WT and ChemR23 KO mice. No difference was observed in terms of leukocyte populations between the two groups.
Supplemental data 2. FACS analysis of mouse ChemR23 expression on NK cells and macrophages isolated from WT and ChemR23 KO mice.

(A) Spleen NK cells were enriched as described in the Material and Methods section, and ChemR23 expression was tested on CD49b-positive cells. (B) Mononuclear phagocyte progenitor cells were prepared from bone marrow and differentiated into macrophages in the presence of GM-CSF for 10 days. ChemR23 expression was tested by FACS on the CD11b\(^+\)F4/80\(^+\) cells.
Supplemental data 3. Migration of dendritic cells in response to CCL5 and CXCL12. Chemotaxis assays were performed using mouse BMDCs from WT and ChemR23 KO mice in response to increasing concentrations of mouse recombinant CCL5 and CXCL12, as described in the Material and Methods section.
Supplemental data 4. Biological activity of recombinant C-terminal variants of mouse chemerin.

CHO-K1 cells were stably transfected with plasmids encoding the presumably bioactive form of mouse chemerin (1-157) and a presumably inactive C-terminally truncated variant, lacking the last eight amino acids of prochemerin (1-155). The coding region of mouse preprochemerin cDNA (amino acids 1-163) was cloned from mouse ovary cDNA (Clontech Laboratories) using 5’-CAGGAATTCAATGCGACGGCTGCTGA-3’ as forward and 5’-GCTCTAGATTTAGCTGCGGGCAGGCCTT-3’ as reverse primers. Constructs encoding a chemerin precursor without the “pro” terminal hexapeptide (amino acids 1-157) or further truncated variants of chemerin (amino acids 1-155) were amplified using respectively 5’-GTACTCTAGACTAGGAGAAGGCAAACTGTC-3’ as forward and 5’-GACTTCTAGACTGCAACACTGTCCAGGTAG-3’ as reverse primers. The resulting fragments were, cloned into pEFIN3 vector and sequenced. The plasmids were transfected in CHO-K1 cells, and G418-resistant cells were selected for over two weeks. For the characterization of chemerin variants, cells were grown to 70% confluence, and further incubated with serum-free DMEM-F12 (GIBCO BRL) which was collected after three days of culture. Conditioned media were tested for activity on CHO-K1 cells expressing mouse ChemR23 using the aequorin-based calcium mobilization assay and (pro)chemerin production was quantified by Western blotting, using mouse chemerin purchased from R&D Systems as standard. The mouse ChemR23 coding sequence was cloned into pEFIN3 and sequenced. The construct was transfected into CHO-K1 cells expressing apoaequorin, and G418-resistant clones were characterized for ChemR23 expression by RT-PCR.
Supplemental data 5. Analysis by flow cytometry of lung macrophage subpopulations.

(A) Macrophage counts in lung cell suspensions 18 hours after trans-orl instillation of PBS, chemerin (5 µg), LPS (1 µg), or LPS and chemerin in WT and ChemR23−/− mice. Alveolar macrophages (AM) are F4/80+CD11c+I-A/I-E+CD11b−/Lo cells whereas interstitial macrophages (IM) are F4/80+CD11b+I-A/I-E+CD11c− cells. (B) Percentage of alveolar macrophages in the total macrophage (alveolar and interstitial) population.
Supplemental data 6. Activity of recombinant mouse chemerin on antigen presenting cells

(A) Mouse macrophages isolated from lung and (B) the peritoneal cavity were incubated for 6 hours with medium alone, LPS (0.1 µg) or with LPS (0.1 µg) and chemerin (100 nM). Conditioned media were tested for TNF-α, IL-6 and IL-10 by ELISA. No significant differences were observed in cytokine levels according to the presence or absence of chemerin. (C) Bone marrow-derived dendritic cells (BMDCs) were incubated with increasing concentrations of recombinant chemerin (10^{-12} to 10^{2} nM) with or without LPS (0.1 µg) for 16 hours and conditioned media were assessed for TNF-α by ELISA. (D) The expression of ChemR23 and chemokine receptors (CCR5, CCR7, CXCR4 and CXCR3) were measured in mouse BMDCs by quantitative real time PCR in basal conditions and after incubation with LPS (0.1 µg) and/or chemerin (20 nM).