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# ChemR23, the Receptor for Chemerin and Resolvin E1, Is Expressed and Functional on M1 but Not on M2 Macrophages

**Magdalena Herová,*,†,‡ Mattia Schmid,*,†,‡ Claudio Gemperle,*,†,‡ and Martin Hersberger*,†,‡**

**ChemR23 is a G protein–coupled receptor that is triggered by two ligands, the peptide chemerin and the eicosapentaenoic acid–derived lipid mediator resolvin E1 (RvE1). Chemerin acts as a chemoattractant for monocytes and macrophages, whereas RvE1 promotes resolution of inflammation-inducing macrophage phagocytosis of apoptotic neutrophils. Although ChemR23-mediated signaling plays a role in mononuclear cell migration to inflamed tissue, as well as in the resolution of inflammation, its regulation in different polarization states of macrophages is largely unknown. We analyzed the expression and function of ChemR23 in monocytes and differently activated human primary macrophages. Using 5′ RACE, we identified three transcription start sites and several splice variants of ChemR23 in both monocytes and macrophages. Although the promoters P1 and P3 are used equally in unpolarized macrophages, stimulation with LPS or IFN-γ leads to increased transcription from P3 in inflammatory M1 macrophages. Such ChemR23-expressing M1 macrophages are chemotactic to chemerin, whereas M2 macrophages not expressing ChemR23 surface receptor are not. Repolarization of ChemR23-expressing M1 macrophages with 10 nM RvE1 increases IL-10 transcription and phagocytosis of microbial particles, leading to a resolution-type macrophage distinct from the M2 phenotype. These results show that ChemR23 is tightly regulated in response to inflammatory and anti-inflammatory stimuli. The restricted expression of ChemR23 in naive and M1 macrophages supports the role of ChemR23 in the attraction of macrophages to inflamed tissue by chemerin and in the initiation of resolution of inflammation through RvE1-mediated repolarization of human M1 macrophages toward resolution-type macrophages.** *The Journal of Immunology*, **2015, 194: 2330–2337.**

Macrophages are key players in innate immunity with an essential role in inflammatory processes (1). Because of their phenotype diversity and plasticity, macrophages participate in all stages of inflammation from pathogen recognition to pathogen elimination, and finally to the resolution of inflammation (2). The phenotype of macrophages changes in response to stimuli from the environment. Upon stimulation with the Th1 cytokine IFN-γ or TLR-4 ligands, macrophages undergo classical M1 activation (3). In contrast, the Th2 cytokines IL-4 and IL-13 lead to alternative M2 activation of macrophages (4). The M1 phenotype is associated with initiation and progression of inflammation, whereas M2 macrophages are implicated in tissue repair, wound healing, and parasite infections (3). In addition, it was shown that macrophages can to some extent switch from one activation state to the other (5, 6), and intermediate phenotypes have been identified in specific stages of the immune response such as during the resolution of inflammation (7).

*Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, CH-8032 Zurich, Switzerland; †Children's Research Center, University Children's Hospital Zurich, CH-8032 Zurich, Switzerland; and ‡Center for Integrative Human Physiology, University of Zurich, CH-8057 Zurich, Switzerland

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Address correspondence and reprint requests to Martin Hersberger, Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland. E-mail address: martin.hersberger@kispi.uzh.ch

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Abbreviations used in this article: poly I:C, polyinosinic-polycytidylic acid; RT, reverse transcription; RvE1, resolvin E1.



ChemR23, also known as the Chemokine-like Receptor 1, is a G protein–coupled receptor expressed on monocytes and macrophages, dendritic cells (8), and NK cells (9), as well as on adipocytes (10) and endothelial cells (11). It binds two ligands: the peptide chemerin (12) and the eicosapentaenoic acid–derived lipid mediator 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid named resolvin E1 (RvE1) (13, 14). Chemerin is present in high amounts in inflammatory fluids (15), has antimicrobial activities (16), was shown to attract ChemR23-expressing leukocytes (12, 17), and promotes adhesion of macrophages to extracellular matrix proteins (18). In addition, chemerin was recently discovered to be an adipokine (10). Secreted by mature adipocytes, it stimulates preadipocytes to differentiation. Increased serum levels of chemerin have been associated with chronic inflammatory diseases (19, 20), coronary artery disease (21), the metabolic syndrome, and obesity (22). There is indication that high chemerin production in obese adipose tissue (23) might contribute to the increased infiltration of macrophages observed in obese adipose tissue leading to low-level inflammation (24).

The second ligand of ChemR23, RvE1, promotes resolution of inflammation in animal models of acute and chronic inflammation (25). On the cellular level, RvE1 blocks TNF-α–induced NF-κB signaling (14) and enhances phagocytosis of microbial particles and apoptotic neutrophils by human macrophages in a ChemR23-dependent manner (26). RvE1 is postulated to be one of the mediators of the beneficial effects of dietary eicosapentaenoic acid, which is thought to have protective function in conditions associated with chronic inflammation (27, 28).

The earlier described findings indicate that the receptor ChemR23 plays a role during monocyte/macrophage recruitment to the inflamed tissue, as well as during the resolution of inflammation. With the aim to better understand which macrophage

phenotype responds to signals triggered through ChemR23, we analyzed the expression and function of ChemR23 in resting and differently activated primary human macrophages. We show that ChemR23 is expressed in monocytes and macrophages and is upregulated in M1 macrophages. Such M1 macrophages are chemotactic to chemerin and increase IL-10 transcription and phagocytosis of microbial particles in response to RvE1 stimulation. In contrast, M2 macrophages do not express the receptor. We further show that ChemR23 is transcribed from two promoters in monocytes and macrophages. Although promoters P1 and P3 are used equally in unpolarized macrophages, the increased transcription in inflammatory M1 macrophages is driven from promoter P3.

## Materials and Methods

### *Materials*

The TLR-4 ligand LPS and the TLR3 ligand polyinosinic-polycytidylic acid (poly I:C) were purchased from Sigma Aldrich (St. Louis, MO). The recombinant human cytokines IL-4, IL-6, IL-13, IL-1β, TNF-α, TGF-β, and IFN-γ were purchased from R&D Systems (McKinley Place NE, Minneapolis, MN). The TLR9 ligand, CpG, was synthesized by Microsynth (Balgach, Switzerland). The TLR7 and TLR8 synthetic ligands 3M001 and 3M002 were purchased from 3M Pharmaceuticals (St. Paul, Minnesota, MN). Chemerin (149–157) was purchased from AnaSpec (Fremont, CA). Chemerin Receptor 23, anti-human, mAb (ChemR23 Ab) and isotype IgG3, PE-labeled anti-human CD80, monoclonal allophycocyanin-labeled anti-human CD206 Ab, and the IgG1K isotype control were purchased from R&D Systems Europe. RvE1 (5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) produced by stereospecific total synthesis (14) was purchased from Cayman Chemicals (Ann Arbor, MI) and was verified by liquid chromatography–tandem mass spectrometry analysis in the laboratory (29).

### *Primary cell purification and cell culture*

WBCs from healthy volunteers were isolated as described previously (30) from buffy coat (Blutspendezentrum, Zurich, Switzerland) using Histopaque-1077 (Sigma-Aldrich) gradient centrifugation. In brief, monocytes were purified by capture with anti-CD14 Abs coupled to MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and allowed to differentiate into macrophages for 7 d at 37˚C and 5% $CO_2$ in RPMI 1640 (Sigma-Aldrich) supplemented with 5% FCS (Bioconcept, Allschwil, Switzerland), 5% human AB serum (Sigma-Aldrich), and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA).

Human THP-1 monocytes were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (Bioconcept), 20 mM glutamine (Life Technologies), at 37˚C and 5% $CO_2$. For differentiation into macrophages, the THP-1 cells were stimulated for 48 h with PMA (Sigma-Aldrich).

### *RNA isolation and reverse transcription reaction for real-time PCR*

Total RNA from primary monocytes and macrophages was isolated using the RNeasy mini kit from Qiagen AG (Hilden, Germany). cDNA was prepared by reverse transcription (RT) reaction from 1 μg total RNA using the Superscript III reverse transcriptase (Life Technologies).

### *Relative quantification of ChemR23 expression levels*

ChemR23 expression levels were quantified by real-time PCR using the SYBR Green master mix kit (Roche Diagnostics, Rotkreuz, Switzerland) on the Light Cycler 480 (Roche Diagnostics). The reactions were performed under the following conditions: preheating 10 min at 95˚C followed by 45 cycles of denaturation 5 s at 95˚C, annealing 10 s at 60˚C, and extension 6 s at 72˚C. Relative gene expression was normalized to GAPDH. Primers are listed in Supplemental Table 1. Data were analyzed with the Light Cycler 480 software (Roche Diagnostics).

### *Rapid amplification of cDNA ends*

Total RNA was extracted from primary monocytes and macrophages as described earlier. 5′ RACE was performed using the RLM-RACE Kit (Life Technologies) according to the manufacturer's instructions. Three nested PCRs were performed using the kit's outer and inner adaptor forward primers and ChemR23-specific reverse primers. Primer sequences are listed in Supplemental Table 1. The PCR products were separated on a 2% agarose gel, and the corresponding bands were extracted from the gel and sequenced by Sanger sequencing on an ABI PRISM 3500 Genetic Analyzer (Life Technologies).

### *Analysis of splicing variants*

Novel exons detected by the 5′ RACE were verified by PCR using exon 1–, 3–, and 4–specific primers in combination with the 5′ RACE inner reverse primers. All primer sequences are listed in Supplemental Table 1. PCR products were separated on 2% agarose gel by electrophoresis, extracted from the gel, and sequenced by Sanger sequencing.

### *Generation of promoter constructs*

Promoter constructs were amplified by PCR with primers containing the XhoI and HindIII restriction sites, in forward and reverse primers, respectively (for sequence, see Supplemental Table 1). PCR products were TA subcloned into the pCRII vector (Life Technologies), and the promoter constructs were cut out using XhoI and HindIII restriction enzymes from Thermo Fisher Scientific (Waltham, MA). Constructs were further subcloned into the promoterless vector pGL4.17 (Promega, Madison, WI) from upstream of the *firefly luciferase* gene. All constructs were sequenced by Sanger sequencing.

### *Transcription assays*

Ten micrograms construct and 0.25 μg internal control phRL-SV40 (Promega) were transfected by electroporation into $6 \times 10^6$ THP-1 cells. Electroporation was done under the following conditions: 200 V, 950 μF capacitance, and ∞ resistance. After electroporation, cells were seeded in RPMI 1640 with 10% FCS and 20 mM glutamine. After 2 h, cells were stimulated with PMA for 48 h to differentiate into macrophages. THP-1 macrophages were then washed with PBS, lysed in 500 μl passive lysis buffer, and 20 μl of the lysate was used for dual-luciferase reporter assays (Promega). The luciferase and Renilla activities were measured on the luminometer Lumat LB-9507 (Berthold Technologies, Bad Wildbad, Germany). All experiments were done three times in triplicate.

### *FACS analysis*

Allophycocyanin-labeled anti-human ChemR23 Ab with the isotype control IgG3, and a monoclonal PE-labeled anti-human CD80 and a monoclonal FITC-labeled anti-human CD206 with the IgG1K isotype control were used for FACS analysis. In brief, cells were resuspended in PBS containing 2.5% FCS, and after addition of 10 μl Ab or 4 μl isotype control incubated in the dark for 30 min at 4˚C before analysis on a FACSCalibur analyzer (BD Biosciences, Franklin Lakes, NJ).

### *Chemotaxis assay*

A total of $10^5$ macrophages was placed on a 96-well membrane (5.7-mm diameter, 5-μm pore size; ChemoTX from NeuroProbe, Gaithersburg, MD) in RPMI 1640 containing 0.1% BSA (Sigma-Aldrich). The cells were allowed to migrate toward 10 nM chemerin for 60 min. Migrated cells were fixed (2% paraformaldehyde) and stained with DAPI (Sigma-Aldrich), and migration was quantified as the total pixel count of DAPI-stained nuclei under the fluorescence microscope (two photos per membrane and three replicate wells per treatment). Migration indices were calculated over control values.

### *IL-10 ELISA*

IL-10 concentrations in supernatants of human macrophages were measured using commercially available human IL-10 ELISA (Biolegend, San Diego, CA) according to the manufacturer's instructions.

### *Phagocytosis assay*

Monocytes were plated in 96-well plates ($2\times 10^5$ cells/well) and were differentiated to macrophages for 7 d in RPMI 1640 supplemented with 5% FCS, 5% human AB serum, and 1% penicillin/streptomycin. After stimulation with RvE1 or vehicle, cells were incubated for 30 min with FITC-labeled zymosan (5 particles/cell; Life Technologies) at 37˚C. After washing the cells with PBS, fluorescence was quenched using trypan blue (1:10 diluted; Sigma-Aldrich), and phagocytosis was assessed using a Tecan plate reader (Tecan, Männedorf, Switzerland).

### *Statistical analysis*

Statistical analysis was performed with GraphPad Prism 4.03. The levels of ChemR23 mRNA and the activities of the different ChemR23 promoters in the luciferase assays were compared using a two-sided *t* test. A two-sided $p$ value $< 0.05$ was considered significant.

## Results

*ChemR23 expression is upregulated during monocyte differentiation to macrophages and is regulated by cytokines and TLR ligands in macrophages*

Human primary monocytes transcribe and express ChemR23 on their surface (Fig. 1A, 1B). We quantified ChemR23 mRNA levels during 7-d differentiation of primary human monocytes to macrophages. ChemR23 mRNA levels gradually increased from days 1 to 3 and then remained on the same high level (Fig. 1A). ChemR23 protein expression also significantly increased after monocyte differentiation to macrophages (Fig. 1B, 1C).

To assess the expression of ChemR23 in primary human macrophages after activation, we stimulated macrophages with cytokines and TLR ligands. ChemR23 transcription and protein expression were downregulated by IL-13 and IL-4 (Fig. 2A–C), cytokines leading to the M2 phenotype. On the other hand, ChemR23 transcription was upregulated by the TLR-4 ligand LPS and by the proinflammatory IFN-γ, stimuli responsible for induction of the M1 phenotype. LPS stimulation also increased ChemR23 protein expression (Fig. 2D), whereas IFN-γ did not significantly change protein levels (data not shown). Stimulation with TNF-α, TGF-β, and IL-6 and the TLR ligands CpG, 3M001, and poly I:C did not significantly affect ChemR23 transcription (Fig. 2A). In a chemotaxis assay, we show that these changes on the mRNA and protein level are reflected in functionality. Naive and LPS-stimulated M1 macrophages actively migrate toward chemerin. However, IL-4– and IL-13–stimulated M2 macrophages show only unspecific migration and are not attracted by chemerin (Fig. 2E). These results indicate that alternatively activated M2 macrophages do not express functional ChemR23, and thus cannot migrate toward chemerin.

*ChemR23 is transcribed from three transcription start sites and is differentially spliced in human monocytes and macrophages*

We used 5′ RACE to detect *ChemR23* transcription start sites in primary human monocytes and macrophages. We identified three transcription starts in both monocytes and macrophages (Fig. 3). The first start site lies upstream of exon 1 in a region homologous to the promoter region and to the start site previously described in murine microglial cells (31). The other two start sites are located in previously unreported genomic regions 12,950 and 21,370 bp downstream from P1, respectively.

To analyze the functionality of the putative promoter regions upstream of the identified transcription starts, we cloned fragments of 500 and 1000 bp for each promoter upstream of the firefly luciferase gene, and luciferase activity was measured in transfected THP-1 macrophages. The promoters upstream of exon 1 (P1) and 4 (P3) increased transcription of the reporter gene, whereas the promoter upstream of exon 3 (P2) showed only basal activity similar to the promoterless control vector, suggesting it may only be essential in other cell types (Fig. 4A).

In addition, the RACE experiment indicated that several ChemR23 mRNA isoforms are transcribed from promoter P1 located upstream of exon 1. To characterize these splice forms, we performed several RT-PCRs with primers specific for each exon. We identified three splice variants in primary human macrophages and four in monocytes for mRNAs starting with exon 1 (transcribed from P1), and we confirmed the presence of ChemR23 mRNAs starting with exons 3 (transcribed from P2) and 4 (transcribed from P3; Fig. 3B) with no differential splicing.

*In M1 macrophages, ChemR23 is preferentially transcribed from promoter P3*

To assess which of the identified promoters drives the increased transcription of *ChemR23* in M1 macrophages, we quantified all mRNA variants transcribed from P1 and P3 in unpolarized, LPS-, and IFN-γ–stimulated M1 primary human macrophages. The mRNA transcribed from P3 increased 5- to 10-fold after IFN-γ and LPS stimulation, respectively (Fig. 4B), whereas the mRNA transcribed from P1 remained on the same level. To locate the regulatory elements responsible for the transcriptional activation in the promoter region P3, we performed luciferase activity assays in THP-1 macrophages transfected with a plasmid containing 1000 pb promoter P3 upstream of the *luciferase* gene. However, no increase in luciferase activity was observed after stimulation of these macrophages with LPS or IFN-γ (data not shown). Congruent with the lack of induction of luciferase activity by LPS and IFN-γ, in silico analysis of the promoter P3 using the software MatInspector from Genomatix (32) did not predict any NF-κB, AP-1, IRF-3 transcription factor binding sites, or IRF-1, IRF-9, and STAT-1 binding sites necessary for TLR-4 (33) and IFN-γ receptor signaling (34), respectively.

*RvE1 repolarizes human M1 macrophages*

Our results show that resting and M1 primary human macrophages express functional ChemR23 on their surface and are chemotactic toward chemerin, whereas M2 macrophages do not. This would indicate that RvE1 is anti-inflammatory and proresolving mainly in resting and M1 macrophages, possibly polarizing or repolarizing such macrophages toward a proresolution macrophage. To investigate whether RvE1 causes a repolarization of inflammatory M1 macrophages, we sequentially stimulated primary human macrophages with LPS followed by the stimulation with RvE1, and characterized the polarization phenotype of these macrophages. As expected, LPS-stimulated M1 macrophages exhibited an increased transcription of IL-1β, TNF-α, and cell-surface expression of CD80 and CD206 (mannose receptor) (30, 35), which returned to levels comparable with untreated macrophages upon removal of the stimulus for 4 d (data not shown). Sequential stimulation of these M1 macrophages with 10 nM RvE1 at day 1 of the 4-d incubation did

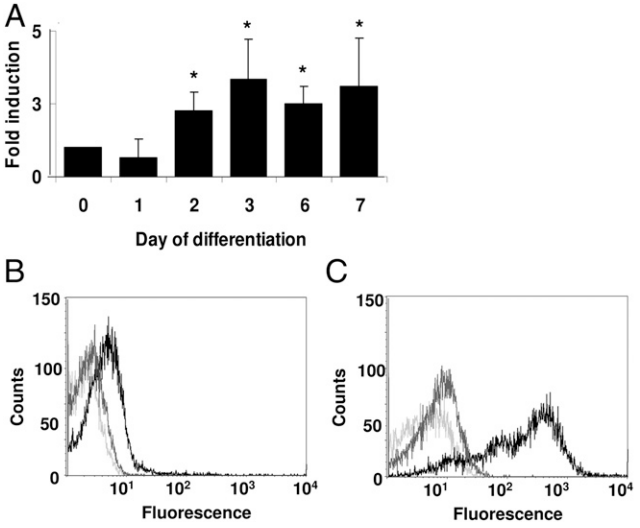


**FIGURE 1.** ChemR23 expression on primary human monocytes and macrophages. (**A**) Relative mRNA expression of ChemR23 during the 7-d differentiation of primary human monocytes to macrophages. The values were normalized for GAPDH mRNA. Bars indicate the mean of three independent experiments as fold induction of monocytes. *$p < 0.05$. (**B**) ChemR23 protein expression on monocytes measured by FACS. (**C**) ChemR23 protein expression on macrophages. Autofluorescence of the cells is shown in light gray, the isotype control in gray, and cells labeled with ChemR23 Ab in black. Representative graphs of at least three independent experiments are shown.

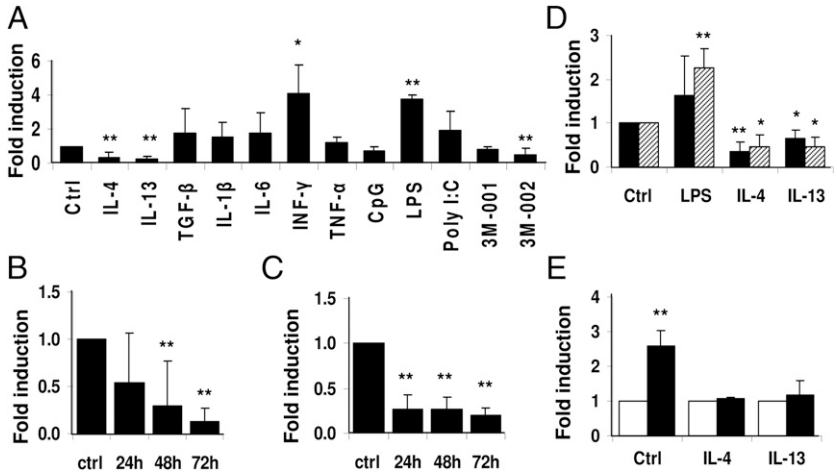


**FIGURE 2.** ChemR23 expression and function in primary human macrophages in response to inflammatory and anti-inflammatory stimuli. (**A**) Relative mRNA expression of ChemR23 after stimulation of primary human macrophages with different stimuli for 24 h (IL-4 10 ng/ml, IL-13 10 ng/ml, TGF-β 1 ng/ml, IL-1β 1 ng/ml, IL-6 10 ng/ml, IFN-γ 50 ng/ml, TNF-α 1 ng/ml, CpG 100 ng/ml, LPS 100 ng/ml, poly I:C 1 ng/ml, 3M001 3 μM, 3M002 3 μM). ChemR23 mRNA expression in macrophages stimulated for 24, 48, and 72 h with (**B**) IL-4 and (**C**) IL-13. All values were normalized for GAPDH and are presented relative to unstimulated macrophages. Bar indicates the means of three independent experiments. (**D**) ChemR23 protein expression on stimulated macrophages measured by FACS. Solid bars represent 48-h stimulation; hatched bars represent 72-h stimulation. Values are presented relative to unstimulated macrophages. Bars indicate the mean of three independent experiments. (**E**) Chemerin chemotaxis assay. Unstimulated, IL-4–, and IL-13–stimulated macrophages were allowed to migrate toward 10 nM chemerin. Bars show the mean migration index of three independent experiments. Migration of each set is shown relative to basal migration without chemerin. $*p < 0.05$, $**p < 0.01$.

not alter transcription for IL-1β and TNF-α, or cell-surface expression for ChemR23 and CD206 (Fig. 5). However, RvE1 increased IL-10 transcription, but not secretion, and led to a small increase in CD80 expression. These changes differ from the effect of IL-4 on repolarization, which reduced proinflammatory cytokines and increased the mannose receptor CD206, whereas it showed no effect on IL-10 transcription. These data indicate that RvE1 may have the potential to repolarize M1 macrophages toward some proresolution phenotype, which is, however, different from the M2 polarization triggered by IL-4.

### *RvE1 repolarized human M1 macrophages migrate less toward chemerin but phagocytose more microbial particles*

To characterize these RvE1 repolarized M1 primary human macrophages on the functional level, we analyzed chemotactic migration of macrophages toward chemerin. As expected, M1 macrophages migrated toward chemerin and toward the macro-

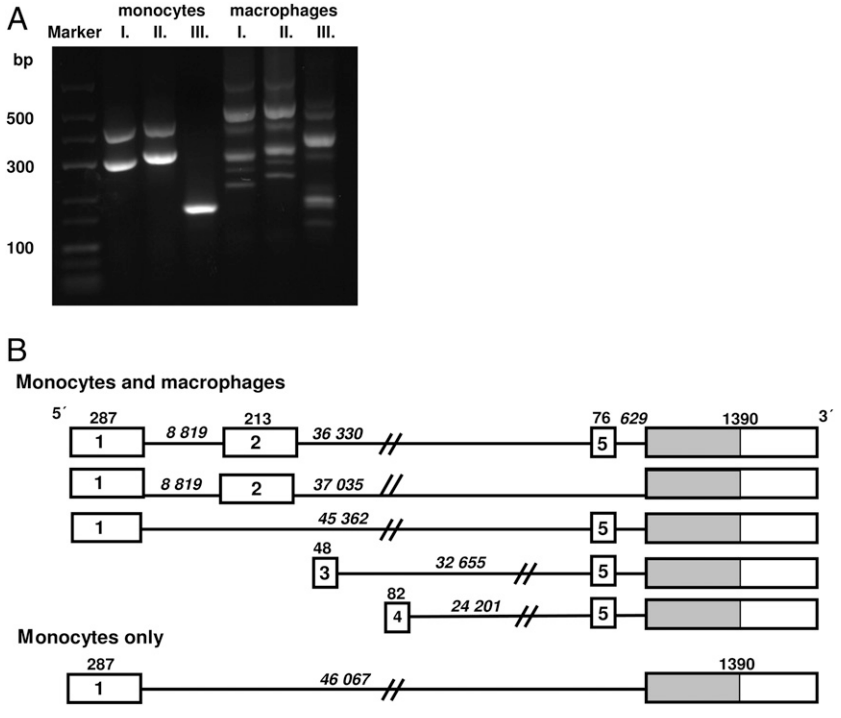


**FIGURE 3.** ChemR23 transcription start sites and mRNA isoforms in primary human monocytes and macrophages. (**A**) Nested RT-PCR products after 5′ RACE visualized on a 2% agarose gel. 5′ RACE-specific nested amplification using the following gene-specific primers in combination with the 5′ RACE adapter nested inner primer: I) coding region–specific reverse primer I, II) coding region-specific reverse primer II, and III) exon 5–specific reverse primer III. (**B**) Schematic of all differently spliced ChemR23 mRNAs identified by 5′ RACE and RT-PCR in monocytes and macrophages. Boxes represent exons and lines introns. Gray boxes represent the ChemR23 coding region. Numbers represent length of exons and introns in base pairs. ChemR23 gene is located on chromosome 12, contig 12q24.1. Intercontig position of transcription start sites: start site exon 1: 108733094; start site exon 2: 108720144; start site exon 3: 108711724.

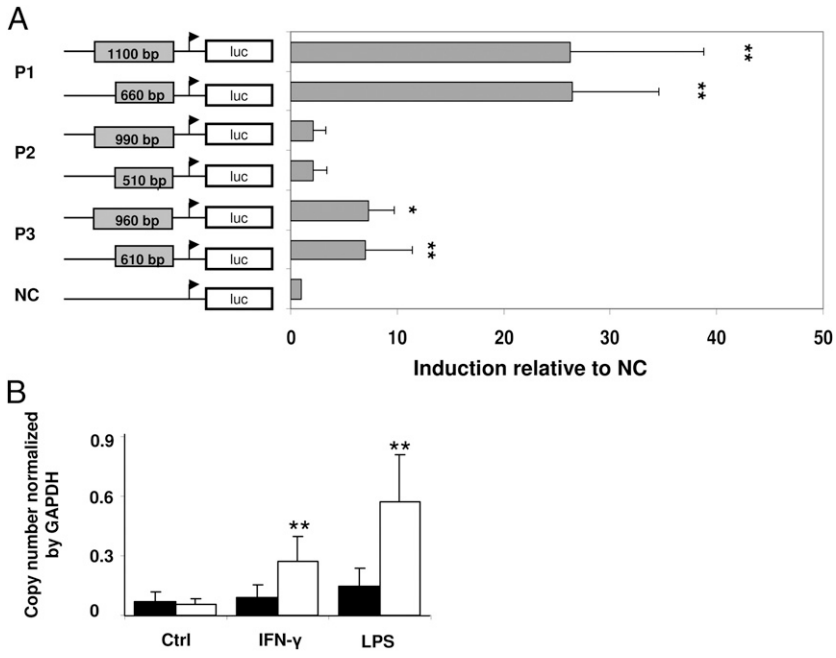


**FIGURE 4.** ChemR23 transcription from alternative promoters. (**A**) Luciferase activity was measured in THP-1 macrophages transfected with the *luciferase* gene under the control of different ChemR23 promoter fragments. Bars represent the fold induction relative to the promoterless vector pGL4.18 used as a negative control (NC). Firefly luciferase activity was normalized by the Renilla luciferase activity. All experiments were done three times in triplicate. (**B**) Absolute quantification of ChemR23 mRNA variants transcribed from P1 (black) and P3 (white) in primary human macrophages. ChemR23 mRNA was quantified in unstimulated, IFN-γ– and LPS-stimulated macrophages (24 h). Values were normalized to GAPDH mRNA copy numbers. Bars indicate the mean of three independent experiments. $*p < 0.05$, $**p < 0.01$. P1, promoter upstream of exon 1; P2, promoter upstream of exon 3; P3, promoter upstream of exon 4.

phage chemoattractant fMLF (30), whereas IL-4 repolarized macrophages lost the ability to migrate toward both chemoattractants (Fig. 6A). This is in line with the IL-4–mediated reduction in cell-surface expression for both receptors (Fig. 2D) (30). Repolarization of primary human macrophages with 10 nM RvE1 reduced but not totally ablated migration toward chemerin and did not influence migration toward fMLF. These results indicate that RvE1 repolarization specifically reduces ChemR23-mediated chemotaxis, whereas migration toward other chemoattractants is not influenced. Such a specific effect of RvE1 on ChemR23 is in line with the previously described competitive binding of chemerin and RvE1 to their common receptor ChemR23 (36).

In a second functional readout, we investigated the effect of RvE1 on phagocytosis of microbial particles by primary human M1 macrophages. Repolarization of M1 macrophages with RvE1 increased phagocytosis of zymosan particles, although the effect was smaller than the effect observed after IL-4 repolarization (Fig. 6B).

### *Short- and long-term treatment of resting primary human macrophages with RvE1 reduces migration toward chemerin and increases phagocytosis of microbial particles*

To investigate whether RvE1 also polarizes resting macrophages to resolution-type macrophages, we treated resting primary human macrophages with 1, 10, and 100 nM RvE1 and investigated the effect on polarization, chemotaxis, and phagocytosis. Short-term

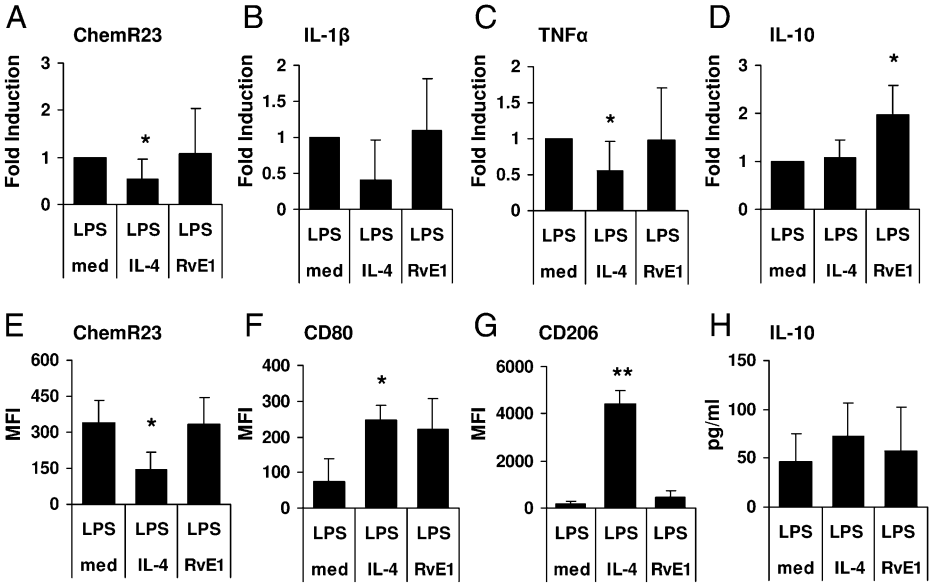


**FIGURE 5.** Repolarization of LPS-stimulated primary human M1 macrophages with RvE1 leads to a distinct macrophage phenotype with properties of proresolution macrophages. LPS-stimulated primary human macrophages were repolarized for 4 d in the presence of medium (med), IL-4, or 10 nM RvE1, and mRNA and protein expression of cytokines and cell surface markers were measured to characterize polarization of macrophages. (**A**–**D**) mRNA levels of (A) ChemR23, (B) IL-1β, (C) TNF-α, and (D) IL-10 are presented. All mRNA values were normalized for GAPDH and are presented relative to macrophages repolarized with medium alone (med). (**E**–**G**) Membrane expression of (E) ChemR23, (F) CD80, and (G) CD206 is shown, whereas in (**H**), protein secretion of IL-10 into the supernatant is shown. Bars indicate the mean of three independent experiments. $*p < 0.05$, $**p < 0.01$.

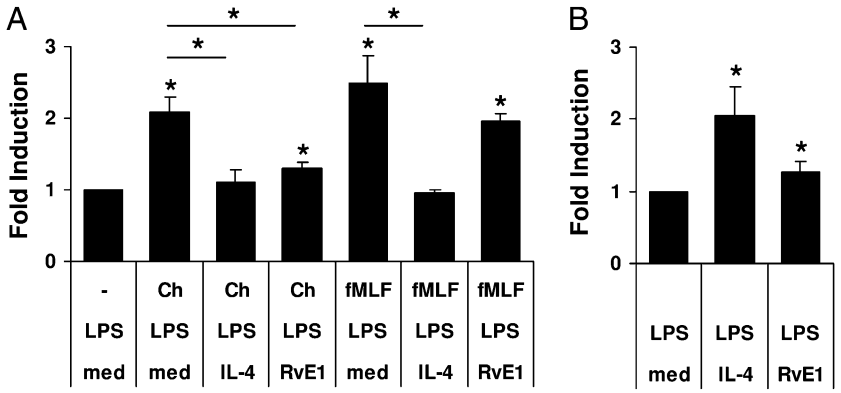


**FIGURE 6.** Chemotaxis and phagocytosis of LPS-stimulated primary human M1 macrophages after repolarization with RvE1. LPS-stimulated primary human macrophages were repolarized for 4 d in the presence of medium (med), IL-4, or 10 nM RvE1, and chemotactic migration or phagocytosis of zymosan particles was measured. (**A**) Chemotaxis of macrophages toward 10 nM chemerin (Ch) and the formylated peptide (10 nM fMLF) was measured. (**B**) Phagocytosis of zymosan particles was measured. Values are presented relative to unstimulated macrophages. Bars indicate the mean of three independent experiments. $^*p < 0.05$.

exposure of macrophages for 15 min with RvE1 blocked migration toward chemerin and increased phagocytosis of microbial particles with a maximal efficiency at 10 nM RvE1 (Fig. 7). Similarly, long-term treatment of resting macrophages for 48 h with 10 nM RvE1 reduced chemerin chemotaxis and increased phagocytosis of microbial particles, whereas there were no changes in the expression of the cytokine levels or membrane markers used for the characterization of macrophage polarization (Supplemental Fig. 1). Although there was no change in the expression of cytokines or membrane markers of macrophage polarization, these RvE1-treated resting macrophages functionally resemble the macrophages observed in the repolarization experiments.

## Discussion

Classically activated M1 macrophages secreting high levels of inflammatory cytokines are implicated in the initiation and sustaining of inflammation (37), whereas alternatively activated M2 macrophages have anti-inflammatory properties and are associated with wound healing and tissue repair (3). As a receptor for the chemoattractant chemerin and the lipid mediator RvE1, ChemR23 plays a role in migration of macrophages to the inflamed area (12), as well as in the resolution of inflammation (14, 26). However, little is known about its regulation during different stages of inflammation or in different activation states of macrophages. In this article, we show that ChemR23 transcription and protein expression increase during differentiation of monocytes to macrophages and are further amplified in classically activated human M1 macrophages in vitro. M1 macrophages migrate toward the ChemR23 ligand chemerin and increase IL-10 transcription and phagocytosis of microbial particles in response to RvE1 stimulation, suggesting that RvE1 may repolarize human M1 macrophages to an intermediate proresolution phenotype. In contrast, ChemR23 expression is not detectable in alternatively activated M2 macrophages, which are also not responsive to ChemR23 signaling.

The regulation of ChemR23 expression was previously studied in mouse macrophages, where ChemR23 was downregulated by inflammatory stimuli such as TLR ligands (LPS, CpG) and inflammatory cytokines, and upregulated by TGF-β (18, 38). The authors of these studies concluded that ChemR23 has a role in naive and anti-inflammatory macrophages only. In contrast, we and others (14) show that ChemR23 mRNA levels increase in human monocytes and macrophages after stimulation with LPS or the inflammatory cytokines TNF-α and IFN-γ, suggesting that there are differences between mouse and human in the regulation and eventual function of ChemR23. This notion would be supported by the fact that chemerin has ChemR23-dependent anti-inflammatory and protective effects in some mouse models of inflammation (39, 40), whereas in humans, chemerin was shown to induce inflammatory signaling in chondrocytes (41) and increased chemerin concentrations were associated with pathologies connected with chronic and systemic inflammation (19, 21, 42–44). The differential expression pattern of ChemR23 on stimulated macrophages and the possible opposite effect of chemerin on inflammation between mouse and human may indicate that the role of ChemR23 signaling differs between the two species.

We report the identification of three ChemR23 transcription start sites in human primary monocytes and macrophages, and show that ChemR23 is differentially spliced. Basal transcription in human monocytes and naive macrophages is driven equally from promoters P1 and P3, and transcripts from promoter P1 are differentially spliced leading to four splicing variants in monocytes and three in macrophages. All differentially spliced exons are noncoding and have no effect on the final size and sequence of the

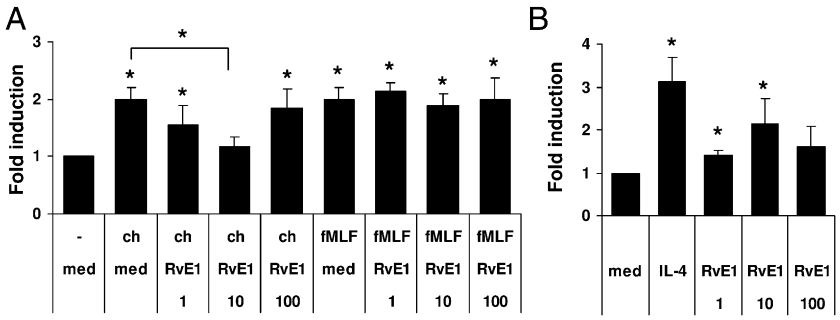


**FIGURE 7.** Chemotaxis and phagocytosis of resting primary human macrophages after short-term stimulation with RvE1. Resting macrophages were stimulated for 15 min in the presence of medium (med), 1, 10, or 100 nM RvE1, and chemotactic migration or phagocytosis of zymosan particles was measured. (**A**) Chemotaxis of macrophages toward 10 nM chemerin (Ch) and the formylated peptide (10 nM fMLF) was measured. (**B**) Phagocytosis of zymosan particles was measured. Values are presented relative to unstimulated macrophages. Bars indicate the mean of three independent experiments. $^*p < 0.05$.

protein. However, such differential splicing and the presence of various mRNA isoforms may represent an additional way of ChemR23 regulation, because sequence characteristics within the 5′ UTR have an essential impact on the differential regulation of translation efficiency and mRNA stability (45).

From the three identified promoters only P1 and P3 are used in monocytes and macrophages, whereas transcription from promoter P2 is basically absent in these cells. Similarly, two distinct start sites and several differentially spliced isoforms were reported for the mouse homolog of *ChemR23* (*DEZ*) in mouse neuroblastoma and microglia cells (31, 46). Such usage of alternative promoters has been previously described to enable differential transcriptional regulation in different cell types, developmental stages, or upon stimulation (47). Recent evidence suggests that >50% of human protein coding genes have multiple alternative promoters (48). Promoter P2 is therefore likely to be used in cell types other than monocytes and macrophages. In this line, endothelial cells express ChemR23 and secrete chemerin upon retinoic acid stimulation, which was shown to facilitate dendritic cell transmigration (49).

Stimulation of primary human macrophages with LPS or IFN-γ increased *ChemR23* transcription downstream of promoter P3, indicating a preferential use of this promoter in M1 macrophages. However, we did not detect transcription factor binding sites necessary for LPS (33) or IFN-γ signaling (34) within 1000 bp upstream of the transcriptional start site, neither by transcriptional activity experiments using reporter constructs nor by in silico analysis. These results indicate that the regulatory elements necessary for TLR-4 and IFN-γ signaling are located either further upstream or downstream of the transcription start site, in agreement with the findings that transcriptional enhancer elements can be located in the first intron (50, 51) or even several kb from the transcription start site (52).

Our results show that only resting and inflammatory M1 macrophages are responsive to ChemR23-mediated triggering, which suggests that RvE1 exerts its proresolving actions mainly on these macrophage phenotypes. In an effort to assess whether RvE1 initiates repolarization of M1 macrophages, we measured the expression of cytokines and surface markers characteristic for M1 and M2 macrophages after restimulation of M1 macrophages with RvE1. Restimulation resulted in a mild increase in CD80 expression, similar to the typical M2 stimulus IL-4, whereas in contrast with IL-4, RvE1 did not induce CD206 expression but increased IL-10 transcription. Hence restimulation of M1 macrophages with RvE1 may have a proresolving effect on M1 macrophages, which does not result in M2 polarization but rather leads to an intermediate macrophage phenotype.

Intermediate macrophage phenotypes have recently been identified in mouse models during the resolution phase of inflammation in vivo. Bystrom et al. (7) isolated resolution phase macrophages that secreted high levels of anti-inflammatory IL-10 but little inflammatory cytokines and that expressed the mannose receptor (properties of M2 macrophages), as well as high levels of COX-2 (a property of M1). A transcriptomic analysis showed that resolution phase macrophages transcribe high levels of IL-10, COX-2, and of the mannose receptor, and despite the low secretion, also high levels of inflammatory cytokines (53). In the same mouse model, peritoneal injection of RvE1 induced the formation of $CD11b^{low}$ macrophages with high phagocytic capacity, reduced TNF-α, and increased IL-10 secretion reflecting the proresolution effect of RvE1 in mouse macrophages (54). Although some characteristics of the resolution phase mouse macrophages described in the earlier mentioned studies differ from the characteristics of the RvE1 restimulated M1 human macrophages in our in vitro experiments, we also observed an increased IL-10 expression and an increased phagocytic capacity after RvE1 treatment, suggesting that RvE1 does repolarize human primary M1 macrophages toward a resolution phase macrophage. Differences in the experimental setup may well account for the not fully congruent results between the in vivo mouse studies and our in vitro human studies. Although multiple cell types are affected after systemic application of RvE1 in mice, including endothelial cells expressing ChemR23 (11, 14) and neutrophils expressing the BLT1 receptor (36), these additional stimuli are lacking in our in vitro study on primary human macrophages.

In summary, we show that ChemR23 is tightly regulated in response to inflammatory and anti-inflammatory stimuli. The restricted expression of ChemR23 in naive and M1 macrophages supports the role of ChemR23 in the attraction of macrophages to inflamed tissue by chemerin and in the initiation of resolution of inflammation through RvE1 signaling and possibly some chemerin cleavage products (40) in human M1 macrophages leading to the repolarization toward resolution phase macrophages.

## Disclosures

The authors have no financial conflicts of interest.

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