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A Circadian Clock Gene, Rev-erbα, Modulates the Inflammatory Function of Macrophages through the Negative Regulation of *Ccl2* Expression

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Disruption of the circadian rhythm is a contributory factor to clinical and pathophysiological conditions, including cancer, the metabolic syndrome, and inflammation. Chronic and systemic inflammation are a potential trigger of type 2 diabetes and cardiovascular disease and are caused by the infiltration of large numbers of inflammatory macrophages into tissue. Although recent studies identified the circadian clock gene Rev-erbα, a member of the orphan nuclear receptors, as a key mediator between clockwork and inflammation, the molecular mechanism remains unknown. In this study, we demonstrate that Rev-erbα modulates the inflammatory function of macrophages through the direct regulation of *Ccl2* expression. Clinical conditions associated with chronic and systemic inflammation, such as aging or obesity, dampened *Rev-erbα* gene expression in peritoneal macrophages from C57BL/6J mice. Rev-erbα agonists or overexpression of *Rev-erbα* in the murine macrophage cell line RAW264 suppressed the induction of *Ccl2* following an LPS endotoxin challenge. We discovered that Rev-erbα represses *Ccl2* expression directly through a Rev-erbα–binding motif in the *Ccl2* promoter region. Rev-erbα also suppressed CCL2-activated signals, ERK and p38, which was recovered by the addition of exogenous CCL2. Further, Rev-erbα impaired cell adhesion and migration, which are inflammatory responses activated through the ERK- and p38-signaling pathways, respectively. Peritoneal macrophages from mice lacking *Rev-erbα* display increases in *Ccl2* expression. These data suggest that Rev-erbα regulates the inflammatory infiltration of macrophages through the suppression of *Ccl2* expression. Therefore, Rev-erbα may be a key link between aging- or obesity-associated impairment of clockwork and inflammation. *The Journal of Immunology*, **2014, 192: 407–417.**

The impact of circadian rhythm on human health has attracted increasing attention in recent years, and circadian dysfunction is now regarded as a contributory factor to the incidence and severity of a wide range of pathophysiological and clinical conditions. People exposed to constant circadian disruption, as the result of long-term shift work, frequent air travel, or chronic restriction of sleep, show an increased incidence of chronic diseases, such as diabetes, obesity, depression, sleep disorders, and cancer (1–4). Chronic and systemic inflammation is one of the important pathogenic features of these diseases, which gives rise to the hypothesis that circadian clock molecules play a crucial role in the regulation of inflammation.

Chronic and systemic inflammation is closely linked to aging and obesity and is a potent contributor to most age- or obesity-related diseases, such as metabolic disorders, cardiovascular diseases, neurodegenerative diseases, musculoskeletal disorders, and cancers (5–8). Chronically inflamed tissues are characterized by the presence of infiltrating inflammatory cells, such as macrophages (9). Increased caloric intake and physical inactivity causes white adipose tissue (WAT) hypertrophy, which initiates inflammation that is due to an influx of macrophages that secrete proinflammatory cytokines (6). Several lines of evidence indicate that CCL2 (also known as MCP-1), a member of the family of chemotactic cytokines called chemokines, and its receptor CCR2 play crucial roles in the initiation of obesity-induced inflammation and insulin resistance by the recruitment of macrophages into WAT (10).

Recent evidence demonstrated that macrophages exhibiting a rhythmic expression of several clock genes, including *Rev-erbα*, are capable of cell-autonomous gene oscillation in culture and display a robust circadian gating in the responses of the cells to LPS endotoxin challenge (11–14). Rev-erbα, an orphan nuclear receptor encoded by *Nr1d1*, is part of the clock machinery and plays an important role in maintaining proper circadian timing (15). Rev-erbα binds to a monomeric response element, ROR response element (RORE), consisting of a 6-bp core motif, (A/G)GGTCA, flanked by an A/T-rich 5′ sequence or a dimeric site (Rev-erb direct repeat 2) composed of a direct repeat of the core motif separated by two nucleotides (16, 17). Rev-erbα represses transcription of the target genes through the binding to the sites, including a clock gene *Bmal1*, which partly contributes to the formation of feedback loops that fine-tune the circadian clock and maintain its oscillations (18). Moreover, Gibbs et al. (11) dem-

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; HFD, high-fat diet; qPCR, real-time quantitative PCR; RORE, ROR response element; WAT, white adipose tissue; ZT, zeitgeber time.

onstrated that Rev-erbα has control over several genes involved in human innate immunity, including *Il6*, *Il19*, *Cxcl6*, *Cxcl11*, and *Ccl2*, suggesting that Rev-erbα acts as a critical intermediary between the core clockwork and inflammatory pathways. However, the molecular mechanism that couples inflammatory function to Rev-erbα remains unknown.

In the current study, using pharmacological and genetic targeting in murine macrophages, we discovered that Rev-erbα is a direct repressor of the *Ccl2* gene. We showed that Rev-erbα suppresses CCL2-activated intracellular signals and inflammatory functions of macrophages. Furthermore, mice lacking *Rev-erbα* display increases in *Ccl2* gene expression in macrophages. Thus, regulation of *Ccl2* by Rev-erbα represents a novel link between the impairment of clockwork and the resultant inflammation.

Materials and Methods

Animals

C57BL/6J mice, B6.Cg-Lep<ob>/J (ob/ob) mice, and B6.Cg-Nr1d1<tm1Ven>/LazJ (*Rev-erbα*$^{+/-}$) mice were obtained from Sankyo Labo Service (Tokyo, Japan), Charles River Laboratories Japan (Kanagawa, Japan), and The Jackson Laboratory (Bar Harbor, ME), respectively. The mice were housed in plastic cages and reared at 23°C with a 12-h light/dark cycle (lights on at 7 AM, zeitgeber time [ZT]0; and lights off at 7 PM, ZT12). Food and water were available ad libitum. All animals were cared for in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan, based upon the Declaration of Helsinki, 1964.

We used 12–15-mo-old male C57BL/6J mice to investigate the effects of aging on *Rev-erbα* gene expression in peritoneal macrophages. To evaluate whether obesity influences *Rev-erbα* gene expression in peritoneal macrophages, male 10-wk-old C57BL/6J mice were fed a high-fat diet (HFD) for 6 wk. The diet (HFD60; Oriental Yeast, Tokyo, Japan) consisted of 35% fat by weight and provided 62.2% of its calories from fat (19). Male 2–3-mo-old genetically obese (ob/ob) mice were also used for the experiment. As control mice for each experiment, male 2–3-mo-old C57BL/6J mice were fed a standard diet (CE-2; Japan CLEA, Tokyo, Japan) that contained 4% fat by weight and provided 10.6% of its calories from fat. In some experiments, 2-mo-old *Rev-erbα*$^{-/-}$ mice were obtained according to the supplier's genotyping information, and their wild-type ($^{+/+}$) counterparts were used as control.

Preparation and culture of peritoneal macrophages

Peritoneal macrophages were collected and cultured as described previously (19–21). Briefly, the mice were injected (i.p.) with 4 ml thioglycollate medium (Difco Laboratories, Detroit, MI) and housed under the same conditions for another 4 d to elicit macrophages to the peritoneal cavity. The peritoneal exudate cells were harvested from the peritoneal cavity of the mice by sterile lavage with 5 ml ice-cold PBS. The cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-denatured FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) and cultured for 3 h to allow macrophages to adhere to the plate. After nonadherent cells were removed, adherent cells were used as peritoneal macrophages. To investigate the effects of aging or obesity on *Rev-erbα* gene expression, the peritoneal exudate cells from each mouse were harvested at ZT10 (5 PM, light and rest phases) or ZT22 (5 AM, dark and active phase) when the expression had reached its peak and trough, respectively (12). In separate experiments, the cells were treated or not with Rev-erbα agonists—2 or 20 μM GSK4112 or 5 or 50 μM Hemin (both from Sigma-Aldrich)—for 16 h in either the absence or presence of 1 μg/ml LPS from *Escherichia coli* 055 (Sigma-Aldrich). IN a different experiment, the cells were treated with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h and then stimulated with 1 μg/ml LPS for 6 h. Each of the Rev-erbα agonists was dissolved with DMSO, and the control cells were treated using the same volume of DMSO. In some experiments, the peritoneal exudate cells from *Rev-erbα*$^{-/-}$ mice and their wild-type counterparts were harvested at ZT10 when *Rev-erbα* expression reached its peak (12). Adherent cells were used as peritoneal macrophages and were treated or not with 1 μg/ml LPS for 24 h.

Cell line culture

The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured as described previously (19, 20, 22). To study the effects of Rev-erbα agonists on *Ccl2* gene expression, the cells were treated or not with 20 μM GSK4112, 50 μM Hemin, or 1 μg/ml LPS for 16 h. For further study of the effects of Rev-erbα agonists on CCL2-activated signals, the cells were treated with 1 μg/ml LPS for 6 h following pretreatment with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h. In separate experiments, cells were treated with 250 ng/ml mouse rCCL2 (R&D Systems, Minneapolis, MN) for 1 min or 10 μM CCR2 antagonist RS504393 (Tocris Bioscience, Minneapolis, MN) for 24 h.

Real-time quantitative PCR

Total cellular RNA was prepared from peritoneal macrophages using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and from RAW264 cells using RNAiso reagent (Takara Bio, Siga, Japan). Extracted RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random primers. The reaction mixture was amplified in Power SYBR Green Master Mix using a 7500 Real-Time PCR System (both from Applied Biosystems) with 200 nM oligonucleotide primers (forward and reverse). The oligonucleotide sequences used for real-time quantitative PCR (qPCR) were as follows: *Rev-erbα*, 5′-ACG ACC CTG GAC TCC AAT AA-3′ (forward), 5′-CCA TTG GAG CTG TCA CTG TAG A-3′ (reverse); *Rorα*, 5′-GGG AAG AGC TCC AGC AGA TA-3′ (forward), 5′-ACA GCT GCC ACA TCA CCT CT-3′ (reverse); *Ccl2*, 5′-GCC TGC TGT TCA CAG TTG C-3′ (forward), 5′-CAG GTG AGT GGG GCG TTA-3′ (reverse); *Integrin β1* (*Itgb1*), 5′-CAC AAC AGC TGC TTC TAA AAT TG-3′ (forward), 5′-TCC ATA AGG TAG TAG AGA TCA ATA GGG-3′ (reverse); *Integrin β2* (*Itgb2*), 5′-GTG CAG CTC ATC AAG AAT GC-3′ (forward), 5′-GCT GTG GTC CAG GAA GAC TC-3′ (reverse); and *βactin* (*Actb*, internal control), 5′-AAG GCC AAC CGT GAA AAG AT-3′ (forward), and 5′-GTG GTA CGA CCA GAG GCA TAC-3′ (reverse). The expression of the target gene was normalized to the housekeeping gene *Actb*.

Western blot analysis

Nucleic protein was extracted as described previously (20, 23). Cytoplasmic protein was extracted with radio-immunoprecipitation assay buffer containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Extracted proteins were separated by SDS-PAGE (8–10%) and then transferred to a polyvinylidene difluoride membrane (Millipore, Milford, MA). Membranes were blocked with 5% nonfat dried milk in TBST and then immunoblotted with rabbit polyclonal Abs against Rev-erbα (AB10130; Millipore), RORα (638802; BioLegend, San Diego, CA), p-ERK (Thr^{202}/Tyr^{204}; sc-16982-R; Santa Cruz Biotechnology, Santa Cruz, CA), ERK (sc-94; Santa Cruz Biotechnology), p-p38 (Thr^{180}/Tyr^{182}; #9211; Cell Signaling Technology, Danvers, MA), p38 (#9212; Cell Signaling Technology), or actin (sc-1616; Santa Cruz Biotechnology). Thereafter, HRP-conjugated donkey anti-rabbit IgG secondary Abs (GE Healthcare Japan, Tokyo, Japan) were applied. The immunoreactivity was visualized with an ECL reagent (Bio-Rad, Hercules, CA).

Rev-erbα *or* Rorα *plasmid constructs and stable transfection*

Full-length murine *Rev-erbα* and *Rorα* cDNAs were obtained by PCR using the primers 5′-CTG GAG GGC TGC AGT ATA GC-3′ (forward) and 5′-GGT GGA GAG AGC AAG AGT GG-3′ (reverse) for *Rev-erbα* and 5′-GAT CGC AGC GAT GAA AGC TC-3′ (forward) and 5′-CTC GGG CGC GAC ATT TAC-3′ (reverse) for *Rorα*. The amplified *Rev-erbα* and *Rorα* fragments were subcloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA), digested at BamHI/XhoI and KpnI/ApaI sites, respectively, and then cloned into pcDNA4/TO/myc-His B vector or pcDNA4/TO/myc-His A vector at the corresponding sites (Invitrogen). The plasmid DNA used for transfection was prepared with an EndoFree Plasmid Kit (QIAGEN). RAW264 cells were transfected with the pcDNA4 vector, pcDNA4-*Rev-erbα*, or pcDNA4-*Rorα* using a Lipofectamine reagent (Invitrogen). Selection was initiated in medium containing 500 μg/ml zeocine (Invitrogen). After selection, a stable *Rev-erbα* transfectant (RAWrev) and the control cell line (RAWvecB), as well as a stable *Rorα* transfectant (RAWror) and the control cell line (RAWvecA) were established.

ELISA

Cells were cultured at 37°C for 24 h in the absence or presence of 1 μg/ml LPS, and the supernatant was collected. CCL2 concentration was determined using an ELISA kit (eBioscience, San Diego, CA), according to the manufacturer's instructions.

Luciferase reporter assay

The murine *Ccl2* promoter (distal fragment, −6942 to +85; proximal fragment, −642 to +85) was amplified from mouse genomic DNA (Promega, Madison, WI) using an LA Taq polymerase (Takara Bio) and subcloned into pCR-XL-TOPO vector (Invitrogen). The subcloned distal fragment was digested at MluI/EcoRV sites with the blunt end and cloned into MluI- and SmaI-digested pGL3-enhancer luciferase reporter vector (pGL3 vector; Promega). The subcloned proximal fragment was also digested at KpnI/XhoI sites and cloned into pGL3 vector (Promega) at the corresponding sites. RAWrev and RAWvecB and RAWror and RAWvecA cells were transiently transfected using a Lipofectamine reagent (Invitrogen) with distal or proximal constructs containing the luciferase reporter gene, and luciferase activity was determined with a Dual Luciferase Assay System Kit (Promega). Activity was normalized relative to an internal cotransfected constitutive control (*Renilla* luciferase expression vector, pRL-TK vector; Promega), as described (20, 22).

Mutagenesis

The distal *Ccl2* promoter mutant construct was made using a KOD -Plus-Mutagenesis Kit (Toyobo, Osaka, Japan), which is simple to mutate into a long plasmid sequence. The proximal *Ccl2* promoter mutant construct was made using a QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), as described (22). The proximal RORE (−542 to −531) was mutated from ATA TCA ATG TCA to ATA TCA AGA TCT using the mutant primers 5′-AGA TCT CAA AGC TTC TTA GTG CTG CAA AAT ATC-3′ (forward) and 5′-TGA TAT GAA CTG TCT GAG TTG TAA AAG-3′ (reverse) for the distal construct mutagenesis and 5′-CAG TTC ATA TCA AGA TCT CAA AGC TTC TTA G-3′ (forward) and 5′-CTA AGA AGC TTT GAG ATC TTG ATA TGA ACT G-3′ (reverse) for the proximal construct mutagenesis (underlining indicates mutant sequences). The mutant plasmid DNAs were confirmed by sequencing.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was carried out using a EpiQuik Chromatin Immunoprecipitation Kit (Epigentek, Brooklyn, NY). Normal mouse IgG (0.5 μg) and anti–Rev-erbα (AB10130; Millipore) or anti-RORα (638802; BioLegend) Abs were used for each immunoprecipitation. Purified DNA was subjected to 40 cycles of PCR amplification. The oligonucleotide sequences of the *Ccl2* gene were designed as follows: distal ROREs, 5′-GAG GTG CAC ATC GCT TGT CTG-3′ (forward), 5′-TGA GTG TAG TCT GGG CAA TG-3′ (reverse); and proximal RORE, 5′-CAG AGT AAG CAC TAG GTG AG-3′ (forward), and 5′-CTT GGT TAT CAG GCC TTG TTG-3′ (reverse).

Adhesion assay

Adherent cells were determined using methods established by Ashida et al. (24) with a slight modification. Briefly, polystyrene 96-well flat-bottom plates were coated with 25 μl 5 μg/ml soluble mouse recombinant E-selectin, 31.25 μg/ml soluble mouse rICAM-1, or 25 μg/ml soluble mouse rVCAM-1 (all from R&D Systems) at room temperature for 1 h. After incubation, wells were blocked by incubation with 225 μl 10 mg/ml heat-denatured BSA at room temperature for 30 min. One hundred microliters of each cell, suspended at a concentration of 2×10^6/ml in 0.1% BSA–RPMI 1640, was incubated at 37°C for 20 min. After incubation, nonadherent cells were removed, and attached cells were fixed with methanol. Attached cells were washed and then stained with a Giemsa stain solution (Wako Pure Chemical, Osaka, Japan) at room temperature for 30 min. Excess dye was removed by washing, and the bound dye was solubilized with 100 μl 10% acetic acid. The absorbance of each well at 595 nm was measured. Each sample was assayed in triplicate, and each assay was performed four times.

Flow cytometry

Flow cytometric analysis was carried out, as described previously (21), using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Prior to the immunofluorescence test, cells (1×10^6) were incubated with rat anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA) in PBS at 4°C for 10 min to avoid nonspecific binding to FcR. Thereafter, the cells were treated with PE-conjugated anti-integrin β1 mAb or FITC-conjugated anti-integrin β2 polyclonal Ab (R&D Systems).

Chemotaxis assay

The migration of each cell was determined using a QCM Chemotaxis Assay (Millipore), according to the manufacturer's instructions. Briefly, 250 μl cells, resuspended at a concentration of 1×10^6/ml in 0.5% BSA–RPMI 1640, was added to the upper chamber of a 24 Transwell apparatus (5 μm pore size). Five hundred microliters of cell-free 0.5% BSA–RPMI 1640 was added to the lower chamber. Then the plate was incubated at 37°C for 24 h in an incubator with air containing 5% CO_2. After incubation, the upper chamber was dipped into 400 μl Cell Stain Buffer at room temperature for 20 min. Excess dye was washed by dipping three times into distilled water, and the nonmigratory cells were completely removed using a cotton swab. After air drying, the upper chamber was dipped into 200 μl Extraction Buffer, and the plate was shaken, to extract the stain from the membrane, at room temperature for 15 min. One hundred microliters of reactions was transferred to a 96-well plate, and the OD was measured at 560 nm. Each sample was assayed in duplicate, and each assay was performed three times.

Statistical analysis

The results are expressed as the mean ± SE. When two means were compared, a Student *t* test for unpaired samples was used. For more than two groups, the statistical significance of the data was assessed by one-way ANOVA. When significant differences were found, individual comparisons were made between groups using the *t*-statistic and adjusting the critical value according to the Tukey–Kramer method. Differences were considered significant at $p < 0.05$.

Results

Aging or obesity impairs Rev-erbα *gene expression in murine peritoneal macrophages*

Rev-erbα mRNA expression in peritoneal macrophages from control C57BL/6J mice at 10 h after the onset of light (ZT10) was significantly higher than that at ZT22 (Fig. 1). A similar trend was observed by Hayashi et al. (12). To clarify whether this temporal difference in *Rev-erbα* gene expression in peritoneal macrophages is susceptible to chronic and systemic inflammation, we used aged mice, HFD-induced obese mice, and genetic obese ob/ob mice as the models for chronic and systemic inflammation. We observed that *Rev-erbα* gene expression was dampened at ZT10 in the peritoneal macrophages of aged, diet-induced obese, and ob/ob mice compared with control mice (Fig. 1). These results suggest a link between *Rev-erbα* gene expression and the inflammatory response of macrophages.

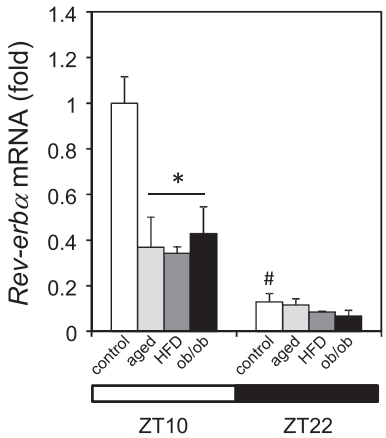

FIGURE 1. Aging and obesity dampen *Rev-erbα* gene expression in peritoneal macrophages. Peritoneal exudate cells from 2–3-mo-old C57BL/6J mice (control), 12–15-mo-old C57BL/6J mice (aged), 2–3-mo-old C57BL/6J mice fed an HFD for 6 wk (HFD), and 2–3-mo-old genetic obese mice (ob/ob) were harvested at ZT10 (5 PM) or ZT22 (5 AM). After nonadherent cells were removed, adherent cells were used as peritoneal macrophages. The gene expression of *Rev-erbα* in peritoneal macrophages was analyzed by qPCR. For normalization, *Actb* mRNA was used. Data are mean ± SE ($n = 4$–5). $^*p < 0.01$ versus control mice, $^\#p < 0.01$ versus ZT10, one-way ANOVA followed by the Tukey–Kramer test.

Rev-erbα agonists suppress Ccl2 *induction following LPS stimulation*

To determine the role of Rev-erbα in inflammatory responses, we analyzed the effects of Rev-erbα agonists GSK4112 and Hemin on the gene expression of *Ccl2* as a crucial inflammatory molecular element in macrophages. *Ccl2* mRNA induction after LPS stimulation was dose dependently repressed by the addition of GSK4112 or Hemin (Fig. 2A). Because several studies (24, 25) revealed that CCL2 activates two distinct MAPKs, ERK and p38, we next performed Western blot analysis to investigate whether Rev-erbα agonists suppress the phosphorylation of ERK and p38 in murine peritoneal macrophages. The phosphorylation of ERK and p38 following LPS stimulation was repressed by pretreatment with either GSK4112 or Hemin (Fig. 2B). Furthermore, as shown in Fig. 2C, qPCR analysis confirmed that either GSK4112 or Hemin treatment also decreased the induction of *Ccl2* mRNA after LPS stimulation in murine macrophage RAW264 cells, as well as in peritoneal macrophages. As Fig. 2D shows, pretreatment with either GSK4112 or Hemin also suppressed the phosphorylation of ERK and p38 in response to LPS challenge in RAW264 cells, as well as in peritoneal macrophages. These data suggest that activation of Rev-erbα leads to the suppression of *Ccl2* gene induction and CCL2-mediated signals in macrophages.

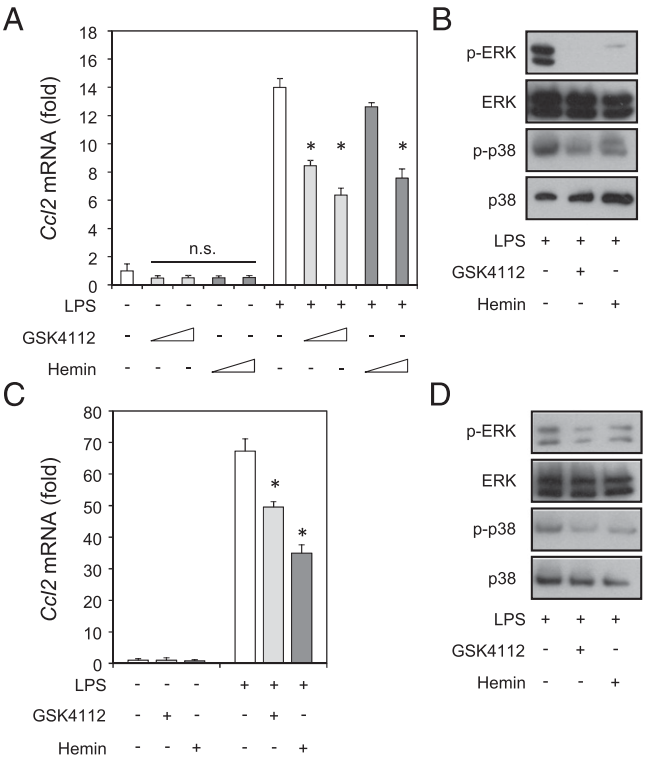

FIGURE 2. Rev-erbα agonists repress *Ccl2* gene induction and CCL2-activated signals following a LPS challenge in macrophages. (**A**) *Ccl2* mRNA induction following an LPS challenge is dose dependently repressed by Rev-erbα agonists in peritoneal macrophages. Peritoneal macrophages were harvested as adherent cells from 2–3-mo-old C57BL/6J mice and were either left untreated or treated with 1 μg/ml LPS, 2 or 20 μM GSK4112, or 5 or 50 μM Hemin for 16 h. The gene expression of *Ccl2* was analyzed by qPCR. (**B**) Phosphorylation of ERK and p38 after LPS challenge is repressed by Rev-erbα agonists in peritoneal macrophages. Peritoneal macrophages were preincubated or not with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h. After the incubation, cells were stimulated with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-ERK, ERK, p-p38, and p38. The lower band was identified as p-p38 through its m.w. determined by m.w. marker. (**C**) *Ccl2* mRNA induction following an LPS challenge is repressed by Rev-erbα agonists in RAW264 cells. Murine macrophage RAW264 cells were either left untreated or treated with 1 μg/ml LPS, 20 μM GSK4112, or 50 μM Hemin for 16 h. The gene expression of *Ccl2* was analyzed by qPCR. (**D**) Phosphorylation of ERK and p38 after LPS stimulation is repressed by Rev-erbα agonists in RAW264 cells. RAW264 cells were preincubated or not with 20 μM GSK4112 or 50 μM Hemin for 2 or 3 h. After the incubation, cells were stimulated with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-ERK, ERK, p-p38, and p38. For normalization, *Actb* mRNA was used. Data are mean ± SE ($n = 3$) and are representative of three separate experiments. *$p < 0.05$ versus LPS-stimulated cells without GSK4112 and Hemin, one-way ANOVA followed by Tukey–Kramer test.

Rev-erbα *overexpression represses* Ccl2 *expression*

To investigate the potential role of Rev-erbα in *Ccl2* expression in macrophages, a stable Rev-erbα transfectant (RAWrev) and a vector control (RAWvecB) were established. Western blot and qPCR analyses confirmed the increase in Rev-erbα protein expression and *Rev-erbα* mRNA expression, respectively (Fig. 3A). As seen in Fig. 3B, overexpression of *Rev-erbα* repressed the gene

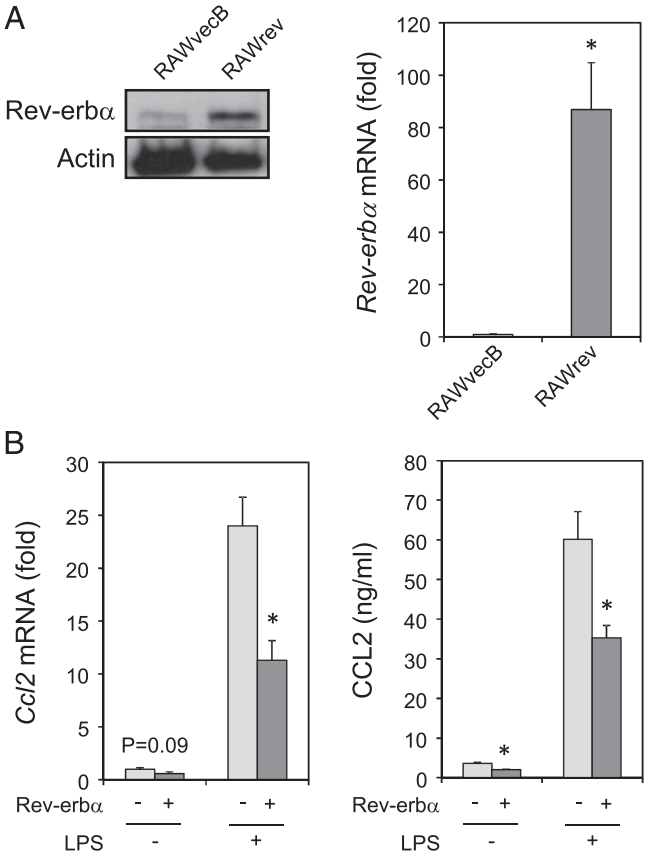

FIGURE 3. Rev-erbα represses *Ccl2* expression in RAW264 cells. (**A**) Transfected Rev-erbα expression in RAW264 cells transfected with a *Rev-erbα* construct (RAWrev) or with vector alone (RAWvecB). Nuclei lysates were analyzed by Western blot (*left panel*) for Rev-erbα and actin (loading control). The gene expression of *Rev-erbα* was analyzed by qPCR (*right panel*). (**B**) Overexpression of *Rev-erbα* in RAW264 cells represses the gene expression of *Ccl2* and the secretion of CCL2. RAW264 cells transfected or not with *Rev-erbα* were left untreated or treated with 1 μg/ml LPS for 24 h, and the gene expression of *Ccl2* or the secretion of CCL2 was analyzed by qPCR (*left panel*) and ELISA (*right panel*), respectively. For normalization, *Actb* mRNA was used. Data are mean ± SE ($n = 4$–5) and are representative of two separate experiments. *$p < 0.05$ versus vector control, Student *t* test.

expression of *Ccl2* and the secretion of CCL2 in either the absence or presence of LPS.

Rorα *overexpression enhances* Ccl2 *expression*

Rev-erbα is known to engage in cross-talk with RORα, an orphan nuclear receptor encoded by *Nr1f1*, which has a similar DNA-binding specificity to Rev-erbα, acts as a constitutive transcriptional activator, and, thus, competes with the binding of Rev-erbα (16, 17, 26–30). In a recent study (31), RORα directly *trans*-activated *Il6* gene expression in nonreactive astrocytes. From these findings, we hypothesized that RORα might positively regulate *Ccl2* expression, and we established a stable RORα transfectant (RAWror) and a vector control (RAWvecA). The increase in the protein expression of RORα and the mRNA expression of *Rorα* was confirmed by Western blot and qPCR analyses, respectively (Fig. 4A). Indeed, overexpression of *Rorα* enhanced the gene expression of *Ccl2* and the secretion of CCL2 in both the absence and presence of LPS (Fig. 4B), indicating that RORα positively regulates *Ccl2* expression in macrophages.

*Identification of putative RORE*s *in murine* Ccl2 *promoter*

Next, we analyzed the murine *Ccl2* promoter for the presence of putative ROREs using TFSEARCH. Examination of the murine *Ccl2* promoter identified three putative ROREs within 10 kb upstream from the transcription start site at −542 to −531, −6776 to −6790, and −6842 to −6830 (Fig. 5A).

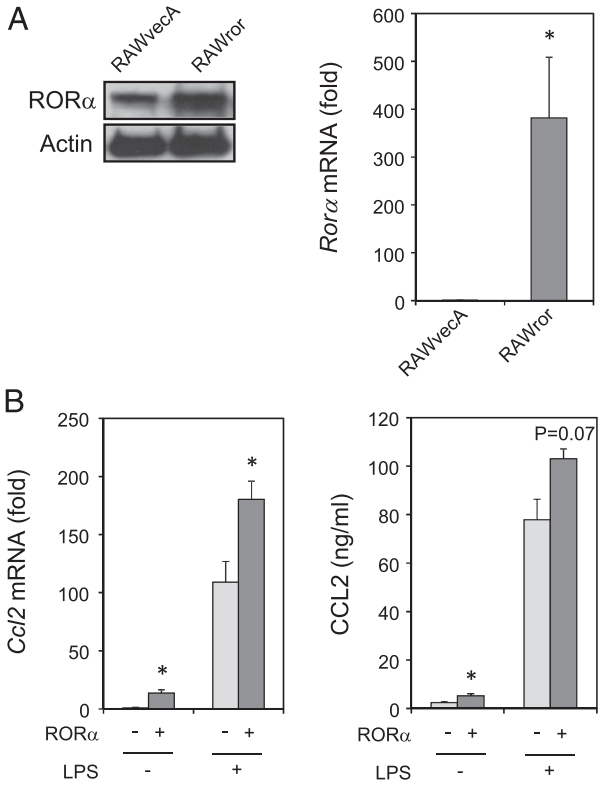

FIGURE 4. RORα enhances *Ccl2* expression in RAW264 cells. (**A**) Transfected RORα expression in RAW264 cells transfected with a *Rorα* construct (RAWror) or with vector alone (RAWvecA). Nuclei lysates were analyzed by Western blot (*left panel*) for RORα and actin (loading control). The gene expression of *Rorα* was analyzed by qPCR (*right panel*). (**B**) Overexpression of *Rorα* in RAW264 cells enhances the gene expression of *Ccl2* and the secretion of CCL2. RAW264 cells transfected or not with *Rorα* were left untreated or treated with 1 μg/ml LPS for 24 h, and the gene expression of *Ccl2* or the secretion of CCL2 was analyzed by qPCR (*left panel*) and ELISA (*right panel*), respectively. For normalization, *Actb* mRNA was used. Data are mean ± SE (n = 4–6) and are representative of two separate experiments. *$p < 0.05$ versus vector control, Student t test.

Rev-erbα represses the activity of murine Ccl2 *promoter*

To determine whether the *Ccl2* promoter is sensitive to Rev-erbα regulation, we cloned *Ccl2* promoters with different lengths—a distal promoter that included two putative ROREs located in the distal region and one putative RORE located in the proximal region, as well as a proximal promoter that included one putative RORE located in the proximal region—into a luciferase reporter vector. Then these two constructs were transiently transfected into RAWrev and RAWvecB cells. The activities of each longitudinal promoter in RAWrev cells were considerably lower than those in RAWvecB cells in both the absence and presence of LPS (Fig. 5B). We next investigated whether the proximal RORE in the *Ccl2* promoter was necessary for Rev-erbα–mediated repression. As shown in Fig. 5C, the mutation of the proximal RORE abolished the repression of the promoter activities in RAWrev transfected with the distal construct, as well as the proximal construct. These findings suggest a critical role for the proximal RORE in Rev-erbα–mediated repression of *Ccl2* expression.

RORα enhances the activity of murine Ccl2 *promoter*

Because RORα activates target genes via ROREs in their promoters, we reasoned that RORα might be a positive regulator of the *Ccl2* promoter. Therefore, we transiently transfected the distal and the proximal *Ccl2* promoter constructs into RAWror and RAWvecA cells. The activity of each of the liner promoter in the RAWror cells was considerably higher than that in RAWvecA cells in the absence or presence of LPS (Fig. 5D). We also investigated whether the proximal RORE in the *Ccl2* promoter was essential for RORα-mediated enhancement of *Ccl2* expression. The mutation of a proximal RORE abrogated the enhancement of the promoter activities in RAWror cells transfected with either a distal or a proximal construct (Fig. 5E), suggesting that the positive regulatory effects of RORα on *Ccl2* expression are mainly dependent on the proximal RORE in the *Ccl2* promoter.

Rev-erbα directly binds proximal RORE in murine Ccl2 *promoter*

We examined whether Rev-erbα directly modulated *Ccl2* promoter activity via binding to proximal RORE in its promoter sequence by ChIP analysis in these RAW264 cells. As illustrated in Fig. 5F, proximal RORE in the murine *Ccl2* promoter was bound by Rev-erbα and RORα. However, Rev-erbα and RORα did not bind to two distal ROREs in the murine Ccl2 promoter. These results suggest that Rev-erbα transrepresses murine *Ccl2* promoter activity via direct binding to the RORE located in the proximal *Ccl2* promoter region, whereas RORα transactivates the promoter activity.

Rev-erbα suppresses the phosphorylation of ERK

CCL2 is known to activate ERK- and p38-signaling pathways, which regulate two independent signaling cascades, leading to integrin activation and chemotaxis, respectively (24, 25). To investigate whether negative or positive regulation of *Ccl2* expression by Rev-erbα or RORα influences the MAPK-signaling pathways, we first analyzed the phosphorylation of ERK in RAWrev and RAWror cells. As seen in Fig. 6A, the overexpression of *Rev-erbα* dramatically repressed the phosphorylation of ERK in both the absence and presence of LPS. In contrast, overexpression of *Rorα* considerably enhanced the phosphorylation of ERK in the presence or absence of LPS (Fig. 6A). Furthermore, the addition of mouse rCCL2 rapidly abolished the decrease in the phosphorylation of ERK in RAWrev cells, whereas treatment with

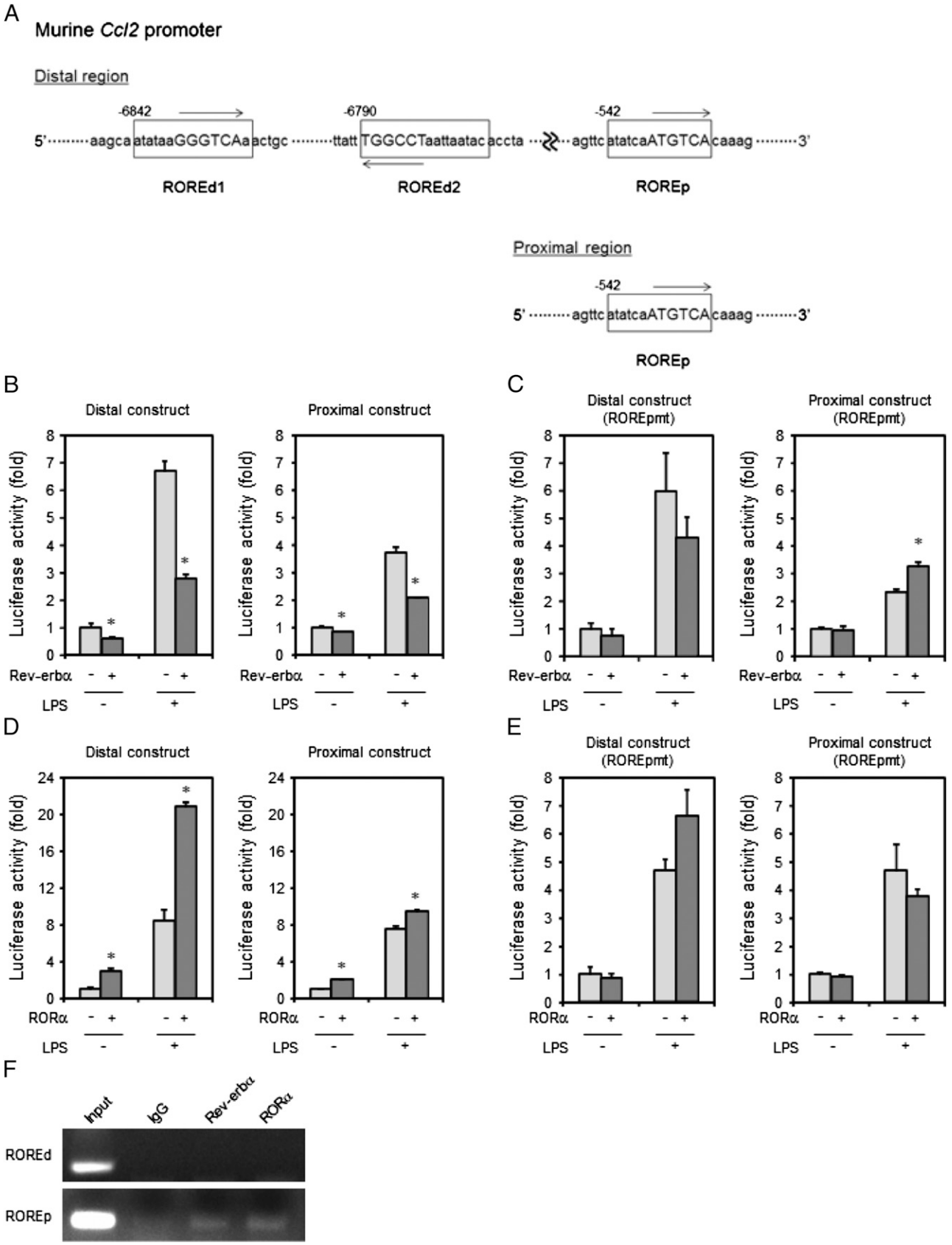

FIGURE 5. A proximal RORE in the murine *Ccl2* promoter is targeted by Rev-erbα and RORα. (**A**) Schematic representation of the murine *Ccl2* promoter sequence. The 10-kb-long *Ccl2* promoter contains two ROREs in its distal region and one RORE in its proximal region. (**B**) Rev-erbα suppresses *Ccl2*-luciferase reporter activity in RAW264 cells. Cells were transiently transfected with a luciferase reporter construct containing either a distal or a proximal construct of the *Ccl2* promoter. After no treatment or treatment with 1 μg/ml LPS for 24 h, luciferase activities were determined. (**C**) Mutation of ROREp abrogates repression. The ATGTCA half-site in the proximal RORE was changed to AGATCT by site-directed mutagenesis of nucleotides −531 (A to T), −534 (G to A), and −535 (T to G). (**D**) RORα enhances *Ccl2*-luciferase reporter activity in RAW264 cells. (**E**) (*Figure legend continues*)

the CCR2 antagonist RS504393 inhibited the increase in the phosphorylation of ERK in RAWror cells (Fig. 6B). These results suggest that Rev-erbα suppresses the phosphorylation of ERK, whereas RORα enhances the phosphorylation of ERK in macrophages, which relies on secreted CCL2 in an autocrine or a paracrine manner.

Rev-erbα impairs adhesion of RAW264 cells to VCAM1

In the development of inflammation, leukocytes roll on endothelial cells, interact with E-selectin, adhere to endothelial cells by firm adhesion to ICAM1 and VCAM1, and then migrate into the subendothelium. The process of leukocyte recruitment is tightly regulated by the expression and activation of specific adhesion molecules on the surface of leukocytes and endothelial cells. CCL2 is also known to play a key role in leukocyte recruitment by promoting both adhesion and migration to inflammatory tissues (32–35). Furthermore, the activation of ERK is reported to be involved in CCL2-dependent promotion of adhesion (24). Because of these findings, we hypothesized that adherent activity would be impaired in RAWrev cells in which *Ccl2* induction is repressed, whereas it would be improved in RAWror cells in which *Ccl2* induction is enhanced. Indeed, the adhesion of RAWrev cells to VCAM1, but not to either E-selectin or ICAM1, was significantly lower than the adhesion of RAWvecB cells (Fig. 6C). In contrast, the adhesion of RAWror cells to VCAM1, but not to either E-selectin or ICAM1, was significantly higher than was the adhesion of RAWvecA cells (Fig. 6C). These data indicate that Rev-erbα impairs the avidity of integrin molecules on macrophages, whereas RORα improves it.

Rev-erbα decreases integrin β1 expression but not the expression of integrin β2

Integrins consist of several subtypes, and each subtype is specific to its ligand. For instance, integrin β1 adheres to VCAM1, and integrin β2 adheres to ICAM1, suggesting that the impaired adhesion of RAWrev cells to VCAM1 is closely related to the decrease in integrin β1 expression. Therefore, we analyzed integrin β1 expression on the surface of RAWrev and RAWror cells by flow cytometry. As illustrated in Fig. 6D, integrin β1 expression on the surface of RAWrev cells was obviously lower than on RAWvecB cells, without a change in integrin β2. In contrast, increased expression of integrin β1, but not integrin β2, was observed on RAWror cells compared with RAWvecA cells (Fig. 6D). In addition, qPCR analysis revealed that the gene expression of *Itgb1* was significantly downregulated in RAWrev cells with no alteration in *Itgb2*, whereas it was upregulated in RAWror cells (Fig. 6E). These results suggest that Rev-erbα impairs the cell adherent activity and integrin expression of macrophages through the CCL2-dependent repression of the ERK-signaling pathway, which is completely opposite from the positive action of RORα.

Rev-erbα *represses the phosphorylation of p38*

To gather additional evidence about whether Rev-erbα and RORα control the inflammatory function of macrophages, we next focused on the role of Rev-erbα and RORα in the p38-signaling pathway. Overexpression of *Rev-erbα* decreased the phosphorylation of p38, whereas overexpression of *Rorα* increased it (Fig. 7A). Moreover, the decrease in the phosphorylation of p38 in RAWrev cells was rapidly recovered by the addition of exogenous CCL2 (Fig. 7B). Meanwhile, the increase in the phosphorylation of p38 in RAWror cells was abolished by treatment with the CCR2 antagonist RS504393 (Fig. 7B). These results suggest that Rev-erbα represses the phosphorylation of p38, whereas RORα enhances it in macrophages, which depends on secreted CCL2 in an autocrine or a paracrine fashion.

Rev-erbα impairs the migratory activity of RAW264 cells

Several reports (24, 36) showed that p38 and its upstream Rho family of GTPases are responsible for CCL2-mediated chemotaxis. To examine whether the alteration in the phosphorylation of p38 influences the chemotaxis of RAWrev and RAWror cells, we performed a chemotaxis assay based on the Boyden chamber principle. The migratory activity of RAWrev cells was significantly lower than that of RAWvecB cells, whereas that of RAWror cells was significantly higher than that of RAWvecA cells (Fig. 7C), suggesting that Rev-erbα impairs chemotaxis through the inhibition of the CCL2-mediated p38-signaling pathway, whereas RORα activates the chemotaxis of macrophages.

Peritoneal macrophages from Rev-erbα$^{-/-}$ *mice display increases in* Ccl2 *gene expression*

To test whether the results observed in the in vitro study are physiologically relevant, we investigated the effects of *Rev-erbα* deficiency on *Ccl2* expression in peritoneal macrophages from *Rev-erbα*$^{-/-}$ mice, which mimic, at least partially, the impairment of *Rev-erbα* mRNA expression at ZT10 observed in aged and obese mice. As shown in Fig. 8A, *Ccl2* gene expression either in the absence or presence of LPS in peritoneal macrophages from *Rev-erbα*$^{-/-}$ mice was significantly higher than that in wild-type mice. These results demonstrate that *Ccl2* expression is negatively regulated by Rev-erbα in vivo, as well as in vitro. Furthermore, *Itgb1* gene expression of peritoneal macrophages cultured with or without LPS was significantly higher in *Rev-erbα*$^{-/-}$ mice than in wild-type mice, whereas *Itgb2* was not significantly different between the two genotypes (Fig. 8B), corresponding with the in vitro results. Therefore, it seems likely that Rev-erbα functions as a transcriptional repressor of *Ccl2* for physiological control of the inflammatory responses of macrophages.

Discussion

Both epidemiological and clinical data suggest circadian involvement in the predisposition, etiology, and progression of immune-related morbidities, such as cancer and autoimmune diseases (37, 38). Inflammatory diseases, in particular, exhibit strong time-of-day symptoms. For example, rheumatoid arthritis has a strong diurnal variation in disease expression, which is accompanied by fluctuations in circulating IL-6 concentration (39). LPS-induced endotoxin shock displays temporal dependency (40), and circadian disruption mimicking jet lag can greatly magnify LPS response (41). According to recent evidence, components of the circadian clock regulate the expression of innate immune molecules, such as proinflammatory cytokines (42) and pattern recognition receptors (14). Rev-erbα is a key clock gene that controls inflammatory cytokine genes, including *Il6*, in macrophages, indicating that it

Mutation of ROREp abrogates enhancement. Luciferase values were normalized using *Renilla* luciferase. Data are mean ± SE from sextuplicate cultures. (**F**) ChIP for murine *Ccl2* promoter. Proteins from RAW264 cells were immunoprecipitated using nonspecific IgG, anti–Rev-erbα, or RORα Abs. DNA extracted from the immunoprecipitated chromatin samples was amplified by PCR with *Ccl2* promoter-specific primers. Data shown are representative of two separate experiments. *$p < 0.05$ versus vector control, Student *t* test. ROREd, Distal RORE; ROREp, proximal RORE; ROREpmt, proximal RORE mutant.

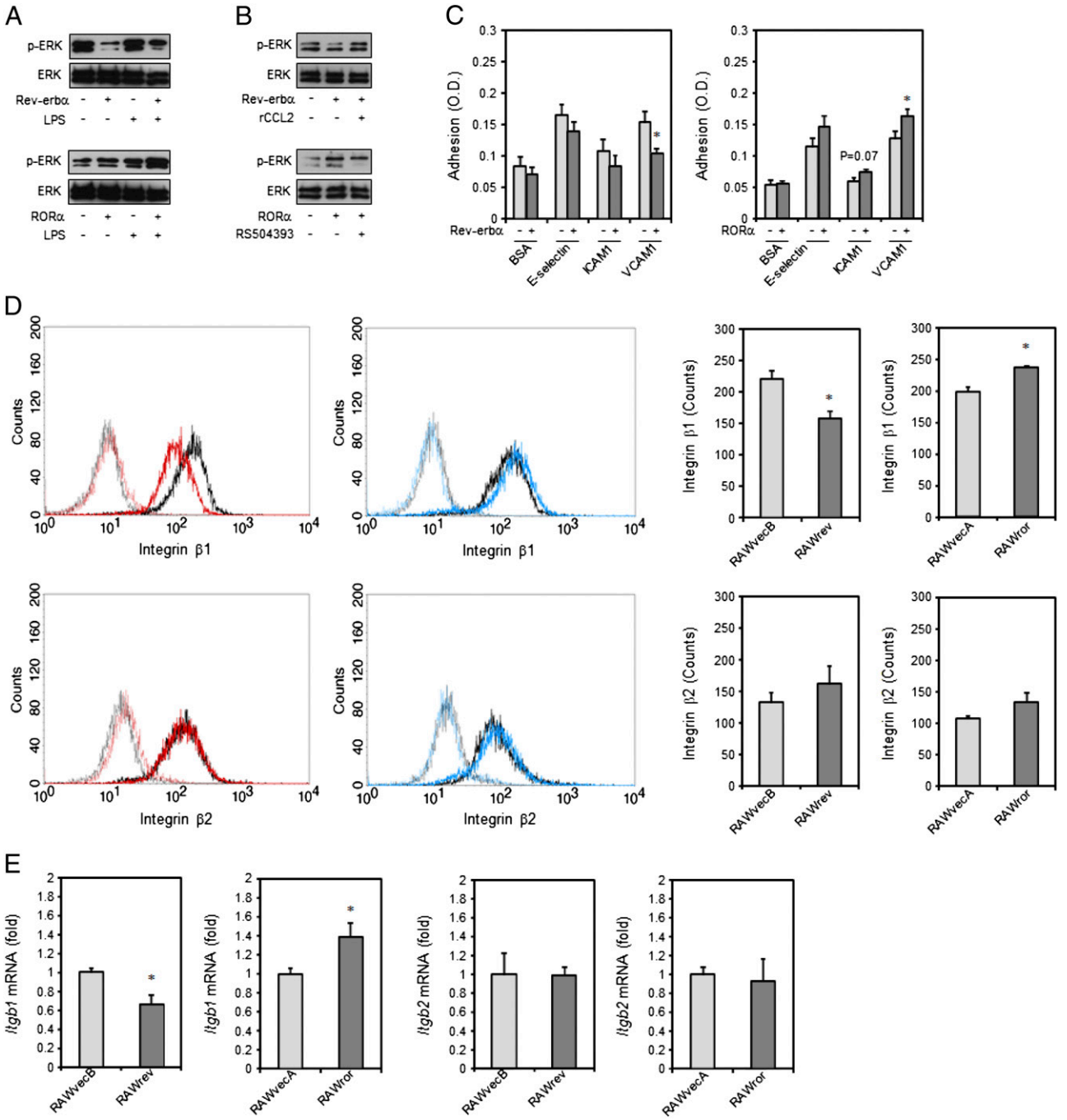

FIGURE 6. Rev-erbα and RORα regulate cell adhesion through ERK phosphorylation induced by CCL2 in RAW264 cells. (**A**) Effects of overexpression of *Rev-erbα* or *Rorα* on the phosphorylation of ERK in RAW264 cells. Cells were stimulated or not with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-ERK and ERK. Data shown are representative of three separate experiments. (**B**) Effects of overexpression of *Rev-erbα* or *RORα* on the phosphorylation of ERK are dependent on CCL2. RAWrev cells were stimulated or not with 250 ng/ml mouse rCCL2 for 1 min (*upper panel*). RAWror cells were stimulated or not with 10 μM CCR2 antagonist RS504393 for 24 h (*lower panel*). Total cell lysates were analyzed by Western blot for p-ERK and ERK. Data shown are representative of three separate experiments. (**C**) Rev-erbα and RORα regulate cell adhesion to VCAM1, but not ICAM1 or E-selectin, in RAW264 cells. Cells were subjected to adhesion assays on heat-denatured BSA (as control), E-selectin, ICAM1, or VCAM1 for 15 min. Data are mean ± SE for four separate experiments. Each experiment was assayed in triplicate cultures. (**D**) Rev-erbα and RORα change the expression of integrin β1, but not integrin β2, on the surface of RAW264 cells. Expression of integrin β1 (*upper panels*) and integrin β2 (*lower panels*) on the cell surface was analyzed by flow cytometry (*left panels*) and quantified by triplicate measurement (*right panels*). Red line, RAWrev cells; blue line, RAWror cells; black line, vector control (RAWvecB or RAWvecA cells); dotted line, isotype control. Data shown are representative of three separate experiments. (**E**) Rev-erbα and RORα change the gene expression of *Itgb1*, but not *Itgb2*, in RAW264 cells. The gene expression of *Itgb1* and *Itgb2* was analyzed by qPCR. For normalization, *Actb* mRNA was used. Data are mean ± SE ($n = 4$–7). *$p < 0.05$ versus vector control, Student *t* test.

negatively regulates the inflammatory responses in macrophages (11). In the current study, we found that chronic and systemic inflammatory conditions, aging and/or obesity, dampen *Rev-erbα* gene expression in murine peritoneal macrophages, suggesting that Rev-erbα plays a potential role in the regulation of the inflammatory functions of macrophages. However, other factors, such as free radicals and metabolic disorders associated with aging, HFD treatment, or genetic obesity, cannot be excluded. Thus, further

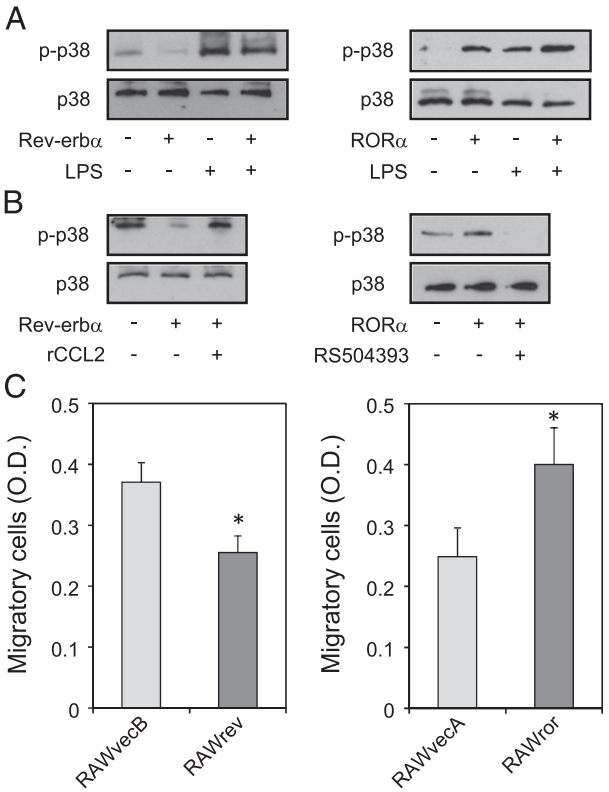

FIGURE 7. Rev-erbα and RORα mediate cell migration through p38 phosphorylation induced by CCL2 in RAW264 cells. (**A**) Effects of overexpression of *Rev-erbα* or *Rorα* on the phosphorylation of p38 in RAW264 cells. Cells were stimulated or not with 1 μg/ml LPS for 6 h, and total cell lysates were analyzed by Western blot for p-p38 and p38. Data shown are representative of four separate experiments. The lower bands were identified as p-p38 or p38 through their m.w. determined by m.w. marker. (**B**) Effects of overexpression of *Rev-erbα* or *Rorα* on the phosphorylation of p38 are dependent on CCL2. RAWrev cells were stimulated or not with 250 ng/ml mouse rCCL2 for 1 min (*left panel*). RAWror cells were stimulated or not with 10 μM CCR2 antagonist RS504393 for 24 h (*right panel*). Total cell lysates were analyzed by Western blot for p-p38 and p38. Data shown are representative of three separate experiments. (**C**) Rev-erbα and RORα regulate chemotaxis of RAW264 cells. Cells were subjected to chemotaxis assays as described in *Materials and Methods*. Data are mean ± SE for three separate experiments. Each experiment was assayed in duplicate cultures. $*p < 0.05$ versus vector control, Student *t* test.

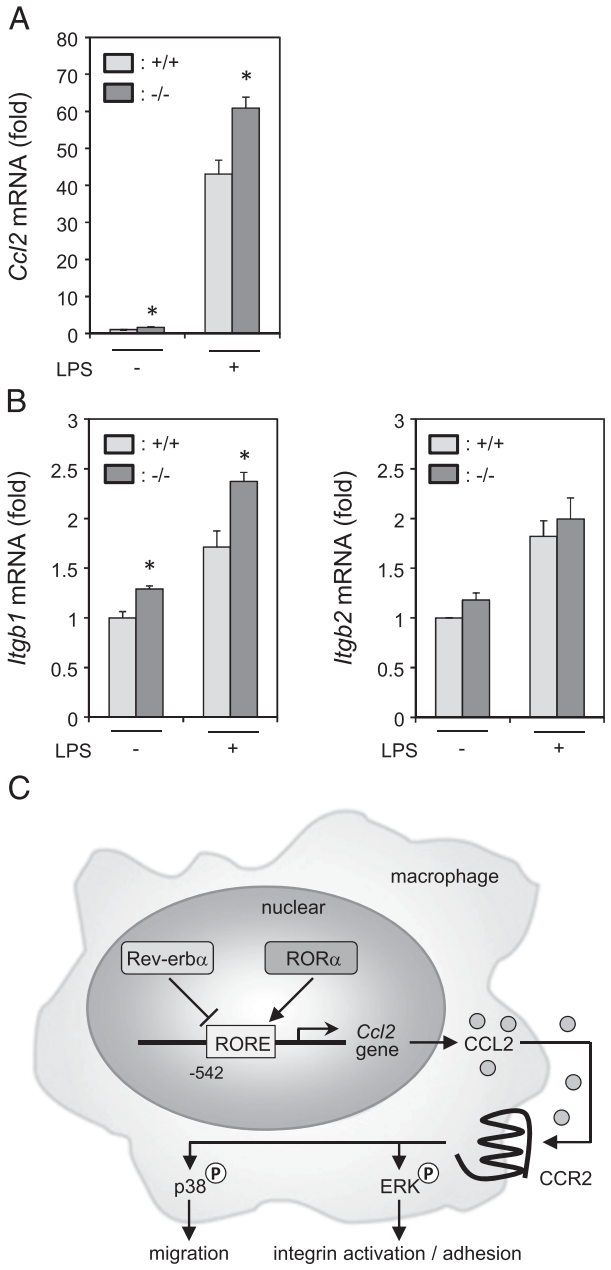

FIGURE 8. Macrophages from *Rev-erbα*$^{-/-}$ mice display increases in *Ccl2* gene expression. *Ccl2* (**A**) and *Itgb1* (**B**) mRNA expression in peritoneal macrophages cultured or not with LPS is increased in mice lacking *Rev-erbα*. Peritoneal macrophages were harvested as adherent cells from 2-mo-old *Rev-erbα*$^{-/-}$ mice and their wild-type ($^{+/+}$) counterparts. The cells were treated or not with 1 μg/ml LPS for 24 h. The gene expression was analyzed by qPCR. For normalization, *Actb* mRNA was used. Data are mean ± SE ($n = 3$). (**C**) Proposed model for the regulation of the murine *Ccl2* promoter and modulation of the inflammatory functions of macrophages by Rev-erbα and RORα. Rev-erbα represses *Ccl2* expression via a RORE in its promoter. As a result, inflammatory functions of macrophages, such as adherent and migratory activities, are suppressed by a decreased secretion of CCL2. By contrast, RORα enhances *Ccl2* expression via a RORE in its promoter. As a consequence, the inflammatory functions of macrophages are promoted by an increased secretion of CCL2. $*p < 0.05$ versus *Rev-erbα*$^{+/+}$ mice, Student *t* test.

studies on the molecular mechanisms underlying the association between inflammatory function and clockwork are needed.

CCL2 is an important chemokine that binds to the CCR2 on monocytes/macrophages to stimulate their migration and initiate inflammation (32–35). The deletion of macrophage *Ccr2* or adipose tissue *Ccl2* can lead to a decrease in the numbers of macrophages infiltrating into WAT in obesity, reduce tissue markers of inflammation, and ameliorate insulin resistance (43). A decrease in atherosclerotic lesions was observed in mice deficient for *Ccr2* crossed with *ApoE*-knockout mice (44). This decrease also was observed in *Ccl2*-deficient mice crossed with *LDL receptor*–knockout mice (45). Interestingly, serum CCL2 concentration and *Ccl2* gene induction in macrophage-rich peritoneal exudate cells show significant circadian-dependent variation in the magnitude of the response to LPS in mice (11). Moreover, the expression of *Ccl2* mRNA displays robust oscillation during the day in murine peritoneal macrophages, whereas *Ccr2* is expressed constantly (12). Therefore, *Ccl2*, and not its receptor *Ccr2*, may be a direct output gene of circadian clocks. However, little is known about the regulation of *Ccl2* expression by core circadian clock proteins.

We analyzed the role of Rev-erbα in the production of the inflammatory chemokine, *Ccl2*, in murine macrophages. We con-

firmed that Rev-erbα agonists, GSK4112 and Hemin, inhibit the induction of the *Ccl2* gene in murine peritoneal macrophages and in murine macrophage RAW264 cells following LPS stimulation. Our results are consistent with the recently published results of Gibbs et al. (11), who demonstrated, using primary human monocyte–derived macrophages, that GSK4112 abolishes the induction of inflammation-related genes, including *Ccl2*, following LPS challenge. In the current study, overexpression of *Rev-erbα* also revealed that Rev-erbα contributes to the negative regulation of *Ccl2* expression in macrophages. By contrast, peritoneal macrophages from mice lacking *Rev-erbα* display increases in *Ccl2* gene expression either in the absence or presence of LPS. These results strongly suggest that Rev-erbα functions as a repressor of *Ccl2* expression in macrophages.

We next took note of three putative ROREs identified in the *Ccl2* promoter within 10 kb upstream from the transcriptional start site and demonstrated that Rev-erbα actually functions as a potent repressor at a proximal RORE in the *Ccl2* promoter region (at −542 to −531 from the transcriptional start site). In contrast, *Ccl2* promoter activity was enhanced by RORα, a clock component and orphan nuclear receptor that also recognized the RORE. Cross-talk between the two nuclear receptors has been observed for a number of genes, including *Bmal1* (30), *ApoAI* (46), *ApoCIII* (47), *fibrinogen-β* (48), *Pai1* (49), and *Il6* (31), which indicates that the two nuclear receptors play an important role in the regulation of metabolism, the cardiovascular system, and inflammation. In the current study, we discovered that this dual regulation also pertains to *Ccl2* gene expression: Rev-erbα potently represses *Ccl2* expression, whereas RORα potently enhances *Ccl2* expression.

Of note, although Rev-erbα and RORα are both implicated in circadian gene regulation, *Rorα* does not exhibit the robust diurnal oscillation of mRNA expression that *Rev-erbα* does (12, 30). These findings indicate that *Ccl2* is a direct circadian output gene and that Rev-erbα potently affects *Ccl2* rhythm through RORE in its promoter. In addition to *Ccl2* expression, signaling activities, including the phosphorylation of ERK and p38, and inflammatory activities, including adherent and migratory activities, might exhibit temporal oscillation through the negative regulation of *Ccl2* expression by Rev-erbα. However, further examination of whether Rev-erbα governs diurnal fluctuation of the *Ccl2* gene, intracellular signaling, and inflammatory functions in macrophages is obviously needed.

Scheiermann et al. (50) demonstrated that adhesion and migration of leukocytes to tissues under homeostasis are regulated through signals from the β-adrenergic nerve system and that the peak recruitment occurs at night in rodents, during a period of activity, and the minimum occurs during the day during a period of rest. Circadian-oscillated leukocyte recruitment to tissues depends on the circadian fluctuation of the expression of adhesion molecules and CCL2 in endothelial cells (50). CCL2 can play a key role in leukocyte recruitment by both integrin activation and by promoting migration (32–35). Two distinct MAPKs are known to be activated by CCL2 and regulate two independent signaling cascades, leading to integrin activation and migration: ERK is responsible for adhesion, and p38 is responsible for migration (24). The current study showed that Rev-erbα impairs adherent and migratory activities of macrophages through CCL2-dependent suppression of ERK and p38 signals. Moreover, integrin β1 expression, but not integrin β2, was decreased by Rev-erbα in macrophages, suggesting the impairment of specific adhesion to VCAM1 in RAWrev cells. Therefore, it seems likely that the circadian tissue recruitment of leukocytes is governed by the circadian oscillations of adherent molecules and chemokines in tissues mediated through signals from the autonomic nervous system (50), as well as by adherent and migratory activities of leukocytes mediated through peripheral leukocytic clockwork, including Rev-erbα and RORα in macrophages.

Leukocyte recruitment to tissues under inflammatory conditions was also shown to display circadian oscillation: higher at night (ZT13) than during the day (ZT5), as well as under homeostasis (50). In the current study, we demonstrated that *Rev-erbα* gene expression is lower at night (ZT22) than during the day (ZT10), which might influence macrophage infiltration into tissues; macrophage infiltration into tissues is suppressed during the day when *Rev-erbα* expression reaches its peak, whereas it is enhanced at night when *Rev-erbα* expression is at its minimum. Indeed, we observed that macrophage infiltration into WAT was higher at night (ZT22) than during the day (ZT10) in control, aged, and obese mice (S. Sato, T. Sakurai, J. Ogasawara, H. Ohno, T. Kizaki unpublished observations). Furthermore, we observed an increase in the gene expression of *Itgb1*, as well as *Ccl2*, in peritoneal macrophages from mice lacking *Rev-erbα*. The increase in *F4/80* mRNA expression in WAT was observed in *Rev-erbα*$^{-/-}$ mice (S. Sato, T. Sakurai, J. Ogasawara, H. Ohno, T. Kizaki unpublished observations). These observations suggest that impairment or deficiency of *Rev-erbα* heightens macrophage adherent activity, leading to the acceleration of macrophage infiltration into WAT. Meanwhile, obesity increases macrophage infiltration into WAT (6, 10, 19). The current findings that expression of the inflammatory repressor *Rev-erbα* in macrophages from HFD-induced obese mice and ob/ob mice was markedly reduced during the day (ZT10) compared with control mice suggest that it contributes, at least in part, to the increased macrophage infiltration into WAT of obese mice.

In summary, as depicted in Fig. 8C, we provide new evidence that a circadian clock gene, Rev-erbα, mediates inflammatory functions, such as cell adhesion and migration of macrophages through the regulation of *Ccl2* expression. In addition, we demonstrated that the gene expression of *Ccl2* and its downstream *Itgb1* in peritoneal macrophages from *Rev-erbα* $^{-/-}$ mice was higher than that from wild-type mice, suggesting in vivo physiological roles for Rev-erbα in the regulation of inflammatory function via the CCL2-activated signaling pathway. However, further studies are needed to clarify the precise role of Rev-erbα in the link between aging- or obesity-associated impairment of clockwork and inflammation. Thus, the current study highlights the orphan nuclear receptor Rev-erbα as an inflammatory repressor and a therapeutic target in inflammatory disease and strikes a note of warning about the impact of the disruption of clockwork induced by irregular lifestyles, such as long-term shift work, frequent air travel, and chronic restriction of sleep, on human health.

Disclosures

The authors have no financial conflicts of interest.

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