Research Article

Direct and Indirect Suppression of Interleukin-6 Gene Expression in Murine Macrophages by Nuclear Orphan Receptor REV-ERBα

Shogo Sato,1 Takuya Sakurai,1 Junetsu Ogawara,1 Ken Shirato,1 Yoshinaga Ishibashi,1 Shuji Oh-ishi,2 Kazuhiko Imaizumi,3 Shukoh Haga,4 Yoshiaki Hitomi,5 Tetsuya Izawa,6 Yoshinobu Ohira,6 Hideki Ohno,1 and Takako Kizaki1

1 Department of Molecular Predictive Medicine and Sport Science, Kyorin University, School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan
2 Department of Respiratory Medicine, National Hospital Organization, Ibarakihigashi National Hospital, 825 Terunuma, Tokai-mura, Naka-gun, Ibaraki 319-1113, Japan
3 Faculty of Human Sciences, Waseda University, 2-579-15 Mikajima, Tokorozawa, Saitama 359-1192, Japan
4 Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan
5 Graduate School of Medical Science, Kanazawa University, Kakumamachi, Kanazawa 920-1192, Japan
6 Faculty of Health and Sport Science, Doshisha University, Kyotanabe, Kyoto 610-0394, Japan

Correspondence should be addressed to Takako Kizaki; kizaki@ks.kyorin-u.ac.jp

Received 16 May 2014; Accepted 5 August 2014; Published 14 October 2014

Academic Editor: Austin J. Cooney

Copyright © 2014 Shogo Sato et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

It is now evident that many nuclear hormone receptors can modulate target gene expression. REV-ERBα, one of the nuclear hormone receptors with the capacity to alter clock function, is critically involved in lipid metabolism, adipogenesis, and the inflammatory response. Recent studies suggest that REV-ERBα plays a key role in the mediation between clockwork and inflammation. The purpose of the current study was to investigate the role of REV-ERBα in the regulation of interleukin-6 (il6) gene expression in murine macrophages. REV-ERBα agonists, or overexpression of rev-erbα in the murine macrophage cell line RAW264 cells, suppressed the induction of il6 mRNA following a lipopolysaccharide (LPS) endotoxin challenge. Also, rev-erbα overexpression decreased LPS-stimulated nuclear factor κB (NFκB) activation in RAW264 cells. We showed that REV-ERBα represses il6 expression not only indirectly through an NFκB binding motif but also directly through a REV-ERBα binding motif in the murine il6 promoter region. Furthermore, peritoneal macrophages from mice lacking rev-erbα increased il6 mRNA expression. These data suggest that REV-ERBα regulates the inflammatory response of macrophages through the suppression of il6 expression. REV-ERBα may therefore be identified as a potent anti-inflammatory receptor and be a therapeutic target receptor of inflammatory diseases.

1. Introduction

The human genome contains 48 nuclear hormone receptor genes, comprising a large family of ligand-dependent transcription factors. In contrast with most classic receptors, nuclear hormone receptors modulate transcription by binding directly to DNA, and ligand interactions occur primarily within the cell cytosol or nucleus. Nuclear hormone receptors are now recognized as key intermediaries between the molecular clock machinery and a wide array of physiological processes [1]. In particular, REV-ERBα, one of the nuclear hormone receptors encoded by nr1d1, is a crucial regulator of lipid, lipoprotein metabolism, and inflammation [1]. REV-ERBα, one of the key clock genes, is a part of the clock machinery and plays an important role in maintaining the proper rhythm of circadian timing [2]. REV-ERBα binds as a monomer to the retinoic acid receptor-related orphan receptor (ROR) response elements (ROREs) composed of a
6 bp A/T-rich sequence immediately preceding a site with the core motif of (A/G)GGTCA [3]. It also binds as a homodimer to the RevDR2 response element, which is composed of a 6 bp A/T-rich sequence immediately preceding a site with a tandem repeat of two (A/G)GGTCA motifs spaced by two nucleotides [4].

Our recent work has demonstrated that REV-ERBα suppresses chemokine (C-C motif) ligand 2 (ccl2) gene expression directly through a RORE in the ccl2 promoter region [5]. These results implicate REV-ERBα as a critical intermediary between the core clockwork and inflammatory pathways. Gibbs et al. [6] have demonstrated that the administration of a REV-ERBα ligand or a genetic knockdown of rev-erbα expression is effective at modulating the production and release of the proinflammatory cytokine interleukin-6 (IL6). Furthermore, Journiac and coworkers [7] have shown that 2 and 3 putative ROREs have also been found in the il6 promoter region of mice and rats, respectively [7]. However, it is unclear whether the putative ROREs in the murine il6 promoter are sensitive to REV-ERBα regulation. In some cases, there are several similarities and differences in the inflammatory response to endotoxin in mice and humans [8]. Therefore, it is important to demonstrate the impact of REV-ERBα on il6 gene in murine immune cells as well as humans.

Results from the current study showed that REV-ERBα directly and indirectly suppresses il6 gene expression in macrophages through a RORE and a nuclear factor κB response element (NFκBRE), respectively, in the murine il6 promoter region. Furthermore, we observed increases in il6 gene expression in peritoneal macrophages from mice lacking rev-erbα. REV-ERBα may therefore be a key link between the clockwork and inflammation.

## 2. Materials and Methods

### 2.1. Animals.

C57BL/6J mice and B6.Cg-Nrl1<tm1Ven>/LazJ mice were obtained from Sankyo Labo Service (Tokyo, Japan) and Jackson Laboratories (Bar Harbor, ME), respectively. The mice were housed in plastic cages and reared at 23°C with a 12 h light/dark cycle. 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 Declarations of Helsinki, 1964.

### 2.2. Preparation and Culture of Peritoneal Macrophages.

Peritoneal macrophages were collected from 2-month-old C57BL/6J mice and rev-erbα<sup>−/−</sup> mice and cultured as described previously [5, 9–11]. The cells from C57BL/6J mice were treated with or without REV-ERBα agonist, 2 or 20 μM GSK4112 (Sigma Aldrich), for 16 h in the absence or presence of 1 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* 055 (Sigma Aldrich, St. Louis, MO). GSK4112 was dissolved with DMSO, and the control cells were treated using the same volume of DMSO. The cells from rev-erbα<sup>−/−</sup> mice were stimulated with or without LPS for 24 h.

### 2.3. Cell Line Culture.

The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Ibaraki, Japan) and cultured as described previously [5, 9, 10, 12]. To study the effects of REV-ERBα agonists on il6 gene expression, the cells were treated with or without 20 μM GSK4112 or 1 μg/mL LPS for 16 h.

### 2.4. Real-Time Quantitative PCR (qPCR).

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 a Power SYBR Green Master Mix (Applied Biosystems) using a 7500 Real-Time PCR System (Applied Biosystems) with 200 nM oligonucleotide primers (forward and reverse). The oligonucleotide sequences used for qPCR were as follows: il6: 5′-GAT GGA TGC TAC CAA ACT GGA-3′ (forward), 5′-CCA GGT AGC TAT GGT ACT CCA GAA-3′ (reverse); β-actin (actb, internal control), 5′-AAG GCC AAC CTT GAA AAG AT-3′ (forward), and 5′-GTT GCA CCA GAG GCA TAC-3′ (reverse). The expression of the target gene was normalized to the housekeeping gene actb.

### 2.5. rev-erbα or rorα Plasmid Constructs and Stable Transfection.

A stable rev-erbα transfectant (RAWrev) and the control cell line (RAWvecB) and a stable rorα transfectant (RAWror) and the control cell line (RAWvecA) were established as described previously [5].

### 2.6. Western Blot Analysis.

Nucleic and cytosolic protein was extracted as described previously [5, 10, 13]. Extracted proteins were separated by SDS-PAGE 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 NFκB P65 (sc-372, Santa Cruz Biotechnology, Santa Cruz, CA), α-Tubulin (α-Tub, ab7291, Abcam, Cambridge, UK), or TBP binding protein (TBP, ab51841, Abcam). Thereafter, HRP-conjugated donkey anti-rabbit or anti-mouse IgG secondary Abs (GE Healthcare Japan, Tokyo, Japan) was applied. The immunoreactivity was visualized with an ECL reagent (Bio-Rad, Hercules, CA).

### 2.7. EMSA.

Nuclear extracts were prepared as described [5, 10, 13]. The murine NFκB consensus oligonucleotide probe (5′-AGT GGA TGG GAC TTT CCC AGC C-3′) was labeled with biotin. The nuclear protein (2.5 μg) and labeled oligonucleotide probe (20 fmol) were incubated in 10 μL HEPESS-KOH, pH 7.8, 50 mM KCl, 0.2 mM EDTA, 10% glycerol, 1 μg poly(dI-dc), 0.05% NP-40, and 5 mM DTT at room temperature for 20 min, electrophoresed in 4.5% polyacrylamide gels, transferred onto a nylon membrane (Biodyne, Pall Corporation, Pensacola, FL), and UV cross-linked. To detect the signals, a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Rockford, IL) was used according to the manufacturer’s protocol.
2.8. Luciferase Reporter Assay. For the analysis of the promoter activity of the NFκB-responsive promoter reporter luciferase construct, the cells were transfected with pNFκB-Luc (Clontech, Palo Alto, CA) using a LipofectAMINE Reagent (Invitrogen, Carlsbad, CA), and luciferase activity was determined using a Luciferase Assay System Kit (Promega, Madison, WI).

For the analysis of il6 promoter activity, the murine il6 promoter (distal fragment, −1029 to +31; proximal fragment, −649 to +31) was amplified from mouse genomic DNA (Promega) using an LA Taq polymerase (Takara bio) and was subcloned into pCR-XL-TOPO vector (Invitrogen). The subcloned fragments were digested at KpnI/XhoI sites and cloned into pGL3 vector (Promega) at the corresponding sites. The cells were transiently transfected by using a LipofectAMINE Reagent 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 [5,10,12].

2.9. Mutagenesis. The il6 promoter mutant construct was made by using a QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as described [5,12]. The proximal RORE (−529 to −518) was mutated from AAA CTC AGG TCA to AAA CTC AGG CCT by using the mutant primers 5'-CTG AAA AAA CTC AGG CCT GAA CAT CTG TAG-3' (forward) and 5'-CTA CAG ATG TTC AGG CCT GAG TTT TTT CAG-3' (reverse) for the distal and proximal il6 promoter constructs mutagenesis (underline, mutant sequences). The NFκBRE (−91 to −82) was mutated from GGG ATT TTC C to GGG CCC TTC C by using the mutant primers 5'-GAT TTT TAT CAA ATG TGG GCC CCT CCC ATG AGT CTC-3' (forward) and 5'-GAG ACT CAT GAG AAG GGC CCA CAT TTG ATA AAA ATC-3' (reverse) for the proximal il6 promoter constructs mutagenesis.

2.10. Statistical Analysis. The results were expressed as the means ± S.E. When two means were compared, a Student's t-test for unpaired samples was conducted. For more than two groups, the statistical significance of the data was assessed by ANOVA, when significant differences were found, individual comparisons were made between groups by using the t-statistic and adjusting the critical value according to the Tukey-Kramer method. Differences were considered significant at P < 0.05.

3. Results

3.1. REV-ERBα Agonists Suppress il6 Induction following LPS Stimulation. To determine the role of REV-ERBα in inflammatory responses, we analyzed the effects of the REV-ERBα agonist, GSK4112, on the gene expression of il6 as a crucial inflammatory molecular element in macrophages. The induction of il6 mRNA after LPS stimulation was dose-dependently repressed by the addition of GSK4112 in peritoneal macrophages (Figure 1(a)). Furthermore, as shown in Figure 1(b), qPCR analysis confirmed that GSK4112 treatment also decreased the induction of il6 mRNA after LPS stimulation in murine macrophage cell line RAW264 cells as well as peritoneal macrophages. These data suggest that activation of REV-ERBα led to the suppression of il6 gene induction in macrophages.

3.2. REV-ERBα Overexpression Represses il6 Expression. To investigate the potential role of REV-ERBα in il6 expression in macrophages, a stable rev-erba transfectant (RAWrev) and the control cell line (RAWvecB) were established using RAW264 cells [5]. As seen in Figure 1(c), overexpression of rev-erba repressed the gene expression of il6 in both the absence and presence of LPS, suggesting that REV-ERBα was involved in the suppression of il6 gene expression in macrophages.

3.3. rorα Overexpression Enhances il6 Expression. REV-ERBα is known to cross-talk with RORα (orphan nuclear receptor encoded by nrlf1), another of the clock genes that has similar DNA binding specificity to REV-ERBα and competes for the binding of REV-ERBα [14–16]. Whereas REV-ERBα represses transcription from these sites of the target genes, RORα acts as a transcriptional activator [3,4,17]. From these findings, we hypothesized that RORα might positively regulate il6 expression and established a stable rorα transfectant (RAWror) and the control cell line (RAWvecA) using RAW264 cells [5]. Interestingly, overexpression of rorα enhanced the gene expression of il6 in the absence of LPS, whereas it repressed the gene expression of il6 in the presence of LPS (Figure 1(d)), indicating that regulation of il6 gene expression by RORα is different between nonactivated and activated states in macrophages.

3.4. REV-ERBα Suppressed NFκB Activity. The il6 gene contains a functional κB element in its promoter region [18]. Thus, activation of NFκB leads to the transcription of this proinflammatory gene. Therefore, we next investigated whether REV-ERBα regulates NFκB activity in macrophages. As shown in Figure 2(a), cytosolic expression and LPS-induced nuclear translocation of NFκB subunit p65 were attenuated in RAWrev cells, compared with RAWvecB cells. No marked change in inhibitory κB (IκB) expression was observed between RAWrev and RAWvecB cells (data not shown). Furthermore, LPS-induced NFκB activation in RAWrev cells was markedly lower than that in RAWvecB cells (Figure 2(b)). In addition, REV-ERBα attenuated the promoter activity of the NFκB-responsive promoter reporter luciferase construct in both the absence and presence of LPS in macrophages (Figure 2(c)). These results strongly suggest that REV-ERBα suppresses LPS-enhanced NFκB activity in macrophages.

3.5. REV-ERBα Represses the Activity of the Murine il6 Promoter. Two putative ROREs have been found in the mouse il6 promoter sequence [7]. Therefore, to determine whether the putative ROREs in the il6 promoter are sensitive
The Scientific World Journal

Figure 1: REV-ERβα represses *il6* gene induction following a LPS challenge in macrophages. (a) Peritoneal macrophages were harvested as adherent cells from 2-month-old C57BL/6J mice and were either untreated or treated with 1 μg/mL LPS or 2 or 20 μM GSK4112 for 16 h. (b) Murine macrophage cell line RAW264 cells were either untreated or treated with 1 μg/mL LPS or 20 μM GSK4112 for 16 h. (c) RAW264 cells transfected with or without *rev-erbα* were either untreated or treated with 1 μg/mL LPS for 24 h. (d) RAW264 cells transfected with or without *rorα* were either untreated or treated with 1 μg/mL LPS for 24 h. The gene expression of *il6* was analyzed by qPCR. For normalization, *actb* mRNA was used. The data are presented as the means ± S.E. (*n* = 3-4). *P* < 0.05 versus cells treated with LPS and without GSK4112 or versus vector control.

To REV-ERβα regulation, we cloned *il6* promoters with different lengths—a distal promoter that included one RORE located in the distal region and one RORE located in the proximal region, and a proximal promoter that included one RORE located in the proximal region—into a luciferase reporter vector. Then, these two constructs were transiently transfected into cell lines, RAWrev and RAWvecB cell lines. 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 (Figure 3(a)). We...
next investigated whether two ROREs in the *il6* promoter are necessary for REV-ERBα-mediated repression. As shown in Figure 3(b), 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 in the absence of LPS. These results suggest a critical role for the proximal RORE in REV-ERBα-mediated repression of *il6* expression. However, the mutation of the proximal RORE still repressed the promoter activities in RAWrev transfected with the distal construct as well as the proximal construct in the presence of LPS (Figure 3(b)), indicating that REV-ERBα repressed *il6* promoter activity through other transcriptional regulators such as NFκB, which was independent of the direct binding of REV-ERBα to the RORE in the promoter.

### 3.6. REV-ERBα Represses *il6* Promoter Activity, Independent of NFκB.

To dissect the effect of NFκB on *il6* promoter activity, we used point-mutated variants in the response element of NFκB. The activity of an *il6* promoter containing an NFκBRE mutated construct in RAWrev cells was lower than that in RAWvecB cells in both the absence and presence of LPS (Figure 3(c)). These results show that REV-ERBα repressed *il6* promoter activity, independent of NFκB. A double mutation of RORE and NFκBRE completely abrogated the suppression of the promoter activity in RAWrev cells in both the absence and presence of LPS (Figure 3(c)). These results suggest both a direct and an indirect repression of the *il6* promoter activity by REV-ERBα.

### 3.7. RORα Enhances the Activity of Murine *il6* Promoter.

Because RORα activates target genes via ROREs in their promoters, we reasoned that RORα might positively regulate *il6* promoter activity. Therefore, we transiently transfected the distal and the proximal *il6* 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 of LPS, whereas...
REV-ERB\(\alpha\) represses \(il6\) promoter activity, independent of the inhibition of NF\(\kappa\)B signaling. (a) RAWrev and RAWvecB cells were transiently transfected with luciferase reporter construct containing either a distal or a proximal construct of \(il6\) promoter. After treatment with or without 1 \(\mu\)g/mL LPS for 24 h, luciferase activities were determined. ROREd, distal RORE; ROREp, proximal RORE. (b) The AGGTCA half-site in the proximal RORE was changed to AGGCCT by site-directed mutagenesis of nucleotides −518 (A to T) and −520 (T to C), and luciferase activities of each cell either untreated or treated with LPS for 24 h were determined. ROREpm, proximal RORE mutant. (c) The GGGATTTTCC half-site in the NF\(\kappa\)BRE was changed to GGGCCCTTCC by site-directed mutagenesis of nucleotides −86 (T to C), −87 (T to C), and −88 (A to C), and luciferase activities of each cell either untreated or treated with LPS for 24 h were determined. NF\(\kappa\)BREm, NF\(\kappa\)BRE mutant. Luciferase values were normalized using \textit{Renilla} luciferase. The data are presented as the means ± S.E. from sextuplicate cultures. * \(P < 0.05\) versus vector control.
that in RAWror cells was lower than that in RAWvecA cells in the presence of LPS (Figure 4(a)). We also investigated whether two ROREs in the il6 promoter were essential for RORα-mediated enhancement of il6 expression. The mutation of a proximal RORE abrogated the enhancement of the promoter activities in RAWror cells transfected with either distal or proximal construct in the absence of LPS (Figure 4(b)), suggesting that the positive regulatory effects of RORα on the il6 expression are mainly dependent on the proximal RORE in the il6 promoter. However, the mutation of the proximal RORE additionally repressed the promoter activities in RAWror transfected with the distal construct as well as the proximal construct in the presence of LPS (Figure 4(b)). Therefore, we hypothesized that RORα also suppressed il6 promoter activity via the inhibition of NFκB-induced transactivation after LPS stimulation as is the case with REV-ERBα. In fact, the activity of an il6 promoter containing an NFκBRE mutated construct in RAWror cells was higher than that in RAWvecA cells in both the absence and presence of LPS (Figure 4(c)). These results show that RORα activated il6 promoter activity through proximal RORE in nonactivated cells, whereas it indirectly repressed the activity through negative regulation of NFκB signaling in activated cells. A double mutation of RORα and NFκBRE showed no changes in the promoter activity between RAWror and RAWvecA cells in the absence of LPS (Figure 4(c)). However, in the presence of LPS, il6 promoter activity of RAWror cells is lower than that of RAWvecA cells, suggesting that RORα also repressed il6 promoter activity through other transcriptional regulators than NFκB.

3.8. Peritoneal Macrophages from rev-erbα Knockout Mice Display Increases in the il6 Gene Expression. To test whether results observed in the in vitro study are physiologically relevant, we investigated the effects of a rev-erbα deficiency on il6 expression in peritoneal macrophages using rev-erbα−/− mice. As shown in Figure 5, il6 gene expression in the absence of LPS in the peritoneal macrophages of rev-erbα−/− mice was significantly higher than that in wild-type mice. The induction of the il6 gene following a LPS challenge in the peritoneal macrophage of mice lacking rev-erbα was relatively higher (P = 0.08) than that found in wild-type mice. These results show that il6 expression is negatively regulated by REV-ERBα in vivo as well as in vitro.

4. Discussion

Until recently, REV-ERBα was considered to be a constitutively active nuclear orphan receptor, although heme has now been shown to bind reversibly to the receptor and to drive ligand-dependent activity [19]. This implies that REV-ERBα is responsive to the cellular redox state and perhaps to gaseous signaling molecules such as NO and CO through interactions with heme [20]. In addition, REV-ERBα acts as a transrepressor for a number of genes, including bmal1 [14], apolipoprotein A1 (apoA1) [21], apoCIII [22], fibrinogen-β [23], plasminogen activator inhibitor type 1 (pail) [24], il6 [7], and ccl2 [5], which indicates that the nuclear hormone receptor plays an important role in the regulation of metabolism, the cardiovascular system, and inflammation.

Recently, we demonstrated that REV-ERBα negatively regulates the inflammatory function of macrophages through the direct repression of ccl2 expression [5]. Furthermore, we showed that REV-ERBα suppresses not only intracellular signals such as extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK), which is known as CCL2 and the receptor chemokine (C-C motif) receptor 2- (CCR2-) activated signaling pathways, but also the inflammatory functions of macrophages such as adherent and migratory activities, the activation of which is known to be dependent on CCL2-mediated ERK and p38, respectively [5, 25]. These observations identified the nuclear hormone receptor REV-ERBα as an anti-inflammatory receptor and a therapeutic target in inflammatory disease.

As in the previous report, for the current study, we analyzed the role of REV-ERBα in the gene expression of inflammatory cytokine il6 in murine macrophages. We confirmed that REV-ERBα agonist GSK4112 inhibits the induction of the il6 gene in murine peritoneal macrophages and in murine macrophage cell line RAW264 cells following LPS stimulation. Our results are consistent with the recently published results by Gibbs et al. [6] who demonstrated that GSK4112 abolishes the induction of inflammation-related genes, including il6, following a LPS challenge, using primary human monocyte-derived macrophages. In the current study, the overexpression of rev-erbα also revealed that REV-ERBα contributes to the negative regulation of il6 expression in macrophages. By contrast, peritoneal macrophages from mice lacking rev-erbα increase il6 gene expression. Reporter assay and site-directed mutagenesis identified a critical role for the proximal RORE in the murine il6 promoter in REV-ERBα-mediated repression of il6 expression. We also showed that REV-ERBα represses il6 expression not only directly through a RORE but also indirectly through an NFκBRE in the murine il6 promoter. These results strongly suggest that REV-ERBα functions as a repressor of inflammatory response in macrophages via the inhibition of the target genes, including ccl2 and il6.

REV-ERBα has been known to cross-talk with RORα, an orphan nuclear receptor encoded by nrf2, that has similar DNA binding specificity to REV-ERBα, acts as a constitutive transcriptional activator, and thus competes with the binding of REV-ERBα [3, 4, 14–17, 26]. Furthermore, Journiac and coworkers [7] have shown that REV-ERBα and/or RORα directly bind to a RORE in the human il6 promoter and acts as a transrepressor and a transactivator of il6 gene expression, respectively. We also confirmed that RORα overexpression in murine macrophage cell line enhances il6 gene expression and the promoter activity through RORE in its promoter region without any exogenous LPS stimulation, whereas it suppressed il6 gene induction and the promoter activity, at least partly, via the inhibition of NFκB-induced transactivation after LPS stimulation. These results suggest that RORα transactivates il6 expression by interacting with a RORE in the promoter of murine macrophages, whereas RORα
Figure 4: Effect of RORα on il6 promoter activity. (a) RAWror and RAWvecA cells were transiently transfected with luciferase reporter construct containing either a distal or a proximal construct of il6 promoter. After treatment with or without 1 µg/mL LPS for 24 h, luciferase activities were determined. ROReD, distal RORE; ROReP, proximal RORE. (b) The AGGTCA half-site in the proximal RORE was changed to AGGCCT by site-directed mutagenesis of nucleotides −518 (A to T) and −520 (T to C), and luciferase activities of each cell either untreated or treated with LPS for 24 h were determined. RORePm, proximal RORE mutant. (c) The GGGATTTCCTCC half-site in the NFκBRE was changed to GGGCCCTTCC by site-directed mutagenesis of nucleotides −86 (T to C), −87 (T to C), and −88 (A to C), and luciferase activities of each cell either untreated or treated with LPS for 24 h were determined. NFκBREM, NFκBRE mutant. Luciferase values were normalized using Renilla luciferase. The data are presented as the means ± S.E. from sextuplicate cultures. * P < 0.05 versus vector control.
REV-ERB of lung physiology but also increased bronchial responsiveness that can be attributed to not only daily variations in responses to exogenous inflammatory stimulation [6, 30–32]. Some asthma patients experience nighttime exacerbations accompanied by fluctuations in circulating IL6 concentration [27, 28]. Some asthma patients experience nighttime exacerbations accompanied by fluctuations in circulating IL6 concentration [27, 28].

We demonstrated that a circadian clock gene, REV-ERB\(\alpha\), represses \(i l 6\) expression not only indirectly through an NF\(\kappa\)B binding motif but also directly through a REV-ERB\(\alpha\) binding motif in the murine \(i l 6\) promoter region. Overexpression of \(rev-erb\) in murine macrophage cell line suppressed \(i l 6\) induction and NF\(\kappa\)B activity following a LPS endotoxin challenge. The present study also showed that peritoneal macrophages from mice lacking \(rev-erb\) display increases in \(i l 6\) expression. These data suggest that REV-ERB\(\alpha\) regulates the inflammatory response of macrophages through the suppression of \(i l 6\) expression. REV-ERB\(\alpha\) may therefore be a key link between clockwork and inflammation.

### Abbreviations

NF\(\kappa\)B: Nuclear factor \(\kappa\)B response element  
ROR: Retinoic acid receptor-related orphan receptor  
RORE: ROR response element

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

### Acknowledgments

This work was supported, in whole or in part, by the Japanese Ministry of Education, Culture Sports, Science and Technology (to Takako Kizaki and Hideki Ohno) and the Nakatomi foundation (to Shogo Sato).

### References

1. D. A. Bechtold, J. E. Gibbs, and A. S. I. Loudon, "Circadian dysfunction in disease," Trends in Pharmacological Sciences, vol. 31, no. 5, pp. 191–198, 2010.
2. N. Preitner, F. Damiola, L. Lopez-Molina et al., "The orphan nuclear receptor REV-ERB\(\alpha\) controls circadian transcription within the positive limb of the mammalian circadian oscillator," Cell, vol. 110, no. 2, pp. 251–260, 2002.
3. H. P. Harding and M. A. Lazar, "The orphan receptor Rev-Erb\(\alpha\) activates transcription via a novel response element," Molecular and Cellular Biology, vol. 13, no. 5, pp. 3113–3121, 1993.
4. H. P. Harding and M. A. Lazar, "The monomer-binding orphan receptor Rev-Erb represses transcription as a dimer on a novel direct repeat," Molecular and Cellular Biology, vol. 15, no. 9, pp. 4791–4802, 1995.
5. S. Sato, T. Sakurai, J. Ogasawara et al., "A circadian clock gene, Rev-erb\(\alpha\), modulates the inflammatory function of macrophages through the negative regulation of Ccl2 expression," Journal of Immunology, vol. 192, no. 1, pp. 407–417, 2014.
6. J. E. Gibbs, J. Blaikley, S. Beesley et al., "The nuclear receptor REV-ERB\(\alpha\) mediates circadian regulation of innate immunity..."
through selective regulation of inflammatory cytokines,” Proceedings
of the National Academy of Sciences of the United States of
America, vol. 109, no. 2, pp. 582–587, 2012.

[7] N. Journiac, S. Jolly, C. Jarvis et al., “The nuclear receptor ROXa
exerts a bidirectional regulation of IL-6 in resting and reactive
astrocytes,” Proceedings of the National Academy of Sciences of
the United States of America, vol. 106, no. 50, pp. 21365–21370,
2009.

[8] S. Copeland, H. S. Warren, S. F. Lowry, S. E. Calvano, and D.
Remick, “Acute inflammatory response to endotoxin in mice
and humans,” Clinical and Diagnostic Laboratory Immunology,
vol. 12, no. 1, pp. 60–67, 2005.

[9] T. Kizaki, T. Maegawa, T. Sakurai et al., “Voluntary exercise attenuates obesity-associated inflammation through ghrelin
expressed in macrophages,” Biochemical and Biophysical
Research Communications, vol. 413, no. 3, pp. 454–459, 2011.

[10] K. Shirato, T. Kizaki, T. Sakurai et al., “Hypoxia-inducible
factor-1 suppresses the expression of macrophage scavenger
receptor 1,” Pflugers Arch European Journal of Physiology, vol.
459, no. 1, pp. 93–103, 2009.

[11] T. Kizaki, S. Oh-Ishi, T. Ookawara, M. Yamamoto, T. Izawa, and
H. Ohno, “Glucocorticoid-mediated generation of suppressor
macrophages with high density FcγRII during acute cold stress,”
Endocrinology, vol. 137, no. 10, pp. 4260–4267, 1996.

[12] T. Kizaki, K. Suzuki, Y. Hitomi et al., “Uncoupling protein
2 plays an important role in nitric oxide production of
lipopolysaccharide-stimulated macrophages,” Proceedings of the
National Academy of Sciences of the United States of America,
vol. 99, no. 14, pp. 9392–9397, 2002.

[13] T. Kizaki, T. Ookawara, K. Iwabuchi et al., “Age-associated increase of basal corticosterone levels decreases ED2\textsuperscript{high}, NF-
\alphaB\textsuperscript{high} activated macrophages,” Journal of Leukocyte Biology, vol.
68, no. 1, pp. 21–30, 2000.

[14] H. R. Ueda, W. Chen, A. Adachi et al., “A transcription factor
response element for gene expression during circadian night,”
Nature, vol. 418, no. 6897, pp. 534–539, 2002.

[15] F. Guillaumond, H. Dardente, V. Giguère, and N. Cermakian,
“Differential control of Bmal1 circadian transcription by REV-
ERB and ROR nuclear receptors,” Journal of Biological Rhythms,
vol. 20, no. 5, pp. 391–403, 2005.

[16] H. P. Harding, G. B. Atkins, A. B. Jaffe, W. J. Seo, and M. A.
Lazar, “Transcriptional activation and repression by ROXa, an
orphan nuclear receptor required for cerebellar development,”
Molecular Endocrinology, vol. 11, no. 11, pp. 1737–1746, 1997.

[17] B. M. Forman, J. Chen, B. Blumberg et al., “Cross-talk among
ROXa and the Rev-erb family of orphan nuclear receptors,”
Molecular Endocrinology, vol. 8, no. 9, pp. 1253–1261, 1994.

[18] M. J. May and S. Ghosh, “Signal transduction through NF-\alphaB,”
Immunology Today, vol. 19, no. 2, pp. 80–88, 1998.

[19] S. Raghuram, K. R. Stayrook, P. Huang et al., “Identification of
heme as the ligand for the orphan nuclear receptors REV-ERB\alpha
and REV-ERB\beta,” Nature Structural and Molecular Biology, vol.
14, no. 12, pp. 1207–1213, 2007.

[20] M. Teboul, A. Grèchez-Cassiau, F. Guillaumond, and F. Delau-
nay, “How nuclear receptors tell time,” Journal of Applied
Physiology, vol. 107, no. 6, pp. 1965–1971, 2009.

[21] N. Vu-Dac, S. Chopin-Delannoy, P. Gervois et al., “The nuclear
receptors peroxisome proliferator-activated receptor \alpha and
rev-erb\alpha mediate the species-specific regulation of apolipoprotein
A-I expression by fibrates,” Journal of Biological Chemistry, vol.
273, no. 40, pp. 25713–25720, 1998.

[22] E. Raspé, H. Duez, A. Mansén et al., “Identification of Rev-erb\alpha
as a physiological repressor of apocIII gene transcription,”
Journal of Lipid Research, vol. 43, no. 12, pp. 2172–2179, 2002.

[23] C. Chauvet, B. Bois-Joyeux, C. Fontaine et al., “The gene
encoding fibrinogen-\beta is a target for retinoic acid receptor-
related orphan receptor\alpha,” Molecular Endocrinology, vol. 19, no.
10, pp. 2517–2526, 2005.

[24] J. Wang, L. Yin, and M. A. Lazar, “The orphan nuclear receptor
Rev-erb\alpha regulates circadian expression of plasminogen activa-
tor inhibitor type I,” Journal of Biological Chemistry, vol. 281, no.
45, pp. 33842–33848, 2006.

[25] N. Ashida, H. Arai, M. Yamasaki, and T. Kita, “Distinct
signaling pathways for MCP-1-dependent integrin activation
and chemotaxis,” The Journal of Biological Chemistry, vol. 276,
no. 19, pp. 16555–16560, 2001.

[26] H. Mignita, J. Morser, and K. Kawai, “Rev-erb\alpha upregulates NF-
\beta\alpha responsive genes in vascular smooth muscle cells,” FEBS
Letters, vol. 561, no. 1–3, pp. 69–74, 2004.

[27] R. H. Straub and M. Cutole, “Circadian rhythms in rheumatoid
arthritis: implications for pathophysiology and therapeutic
management,” Arthritis & Rheumatism, vol. 56, no. 2, pp. 399–
408, 2007.

[28] M. G. Perry, J. R. Kirwan, D. S. Jessop, and L. P. Hunt,
“Overnight variations in cortisol, interleukin 6, tumor necrosis
factor \alpha and other cytokines in people with rheumatoid arthri-
tis,” Annals of the Rheumatic Diseases, vol. 68, no. 1, pp. 63–68,
2009.

[29] E. Ferraz, M. C. Borges, J. Terra-Filho, J. A. B. Martinez,
and E. O. Vianna, “Comparison of 4 AM and 4 PM bronchial
responsiveness to hypertonic saline in asthma,” Lung, vol. 184,
no. 6, pp. 341–346, 2006.

[30] M. Hayashi, S. Shimba, and M. Tezuka, “Characterization of the
molecular clock in mouse peritoneal macrophages,” Biological &
Pharmaceutical Bulletin, vol. 30, no. 4, pp. 621–626, 2007.

[31] M. Keller, J. Mazuch, U. Abraham et al., “A circadian clock
in macrophages controls inflammatory immune responses,”
Proceedings of the National Academy of Sciences of the United
States of America, vol. 106, no. 50, pp. 21407–21412, 2009.

[32] A. C. Silver, A. Arjona, W. E. Walker, and E. Fikrig, “The circadian
clock controls toll-like receptor 9-mediated innate and adaptive
immunity,” Immunity, vol. 36, no. 2, pp. 251–261, 2012.