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Mammalian *Clock* Gene *Cryptochrome* Regulates Arthritis via Proinflammatory Cytokine TNF-α

Akira Hashiramoto,*,†,‡ Takashi Yamane,† Ken Tsumiyama,* Kohsuke Yoshida,* Koichiro Komai,* Hiroyuki Yamada,§ Fumiyoshi Yamazaki,§ Masao Doi,§ Hitoshi Okamura,§ and Shunichi Shiozawa*,†,‡

The mammalian *clock* genes, *Period* and *Cryptochrome* (*Cry*), regulate circadian rhythm. We show that circadian rhythmicity and rhythmic expression of *Period* in the nuclei of inflammatory synovial cells and spleen cells are disturbed in mouse models of experimental arthritis. Expressions of other *clock* genes, *Bmal1* and *Dbp*, are also disturbed in spleen cells by arthritis induction. Deletion of *Cry1* and *Cry2* results in an increase in the number of activated $CD3^+$ $CD69^+$ T cells and a higher production of TNF-α from spleen cells. When arthritis is induced, $Cry1^{-/-}Cry2^{-/-}$ mice develop maximal exacerbation of joint swelling, and upregulation of essential mediators of arthritis, including TNF-α, IL-1β and IL-6, and matrix metalloproteinase-3. Wee-1 kinase is solely upregulated in $Cry1^{-/-}Cry2^{-/-}$ mice, in line with upregulation of c-Fos and Wee-1 kinase in human rheumatoid arthritis. The treatment with anti–TNF-α Ab significantly reduced the severity and halted the progression of the arthritis of $Cry1^{-/-}Cry2^{-/-}$ mice and vice versa, ectopic expression of *Cry1* in the mouse embryonic fibroblast from $Cry1^{-/-}Cry2^{-/-}$ mice significantly reduced the *trans* activation of *TNF-α* gene. Thus, the biological clock and arthritis influence each other, and this interplay can influence human health and disease. ***The Journal of Immunology*****, 2010, 184: 1560–1565.**

Rheumatoid arthritis (RA), a chronic polyarthritis of unknown etiology affecting ~1% of the population worldwide, is characterized by a "tumor-like" synovial overgrowth leading to joint destruction (1). Another important feature of RA would be a circadian manifestation of the disease such as morning stiffness, which is included in the diagnostic criteria of RA (2). Such circadian characteristic is also seen in the proinflammatory cytokines and disease-specific markers important in RA: IL-1β, IL-6, and TNF-α are all elevated in sera of rheumatoid patients reaching the peak levels in early morning (3–6), and secretion of the IgA and IgM types of the rheumatoid factor also exhibits a rhythmic pattern with a peak at morning (7).

Most organisms living on earth have an internal pacemaker, called a biological clock, and circadian rhythm represents a basic feature of life and disease (8). The circadian rhythm in mammals is organized by the transcriptional/posttranscriptional machinery regulating the *clock* genes including *Clock*, *Bmal1*, *Period* (*Per*), and *Cryptochrome* (*Cry*) (9, 10). The master circadian pacemaker resides in hypothalamic suprachiasmatic nucleus (SCN) to orchestrate several *clock* genes expressed in a tissue-specific fashion. Among that, CLOCK and BMAL1 comprise positive-acting components of the clock machinery, whereas PER and CRY function as negative regulators. This core clock machinery regulating circadian time exists in virtually all cells of the body, and controls thousands of *clock*-controlled genes. Studies have shown that, in the mice lacking both *Cry1* and *Cry2*, free-running rhythmicity is abolished (11), and a cell cycle regulator Wee-1 kinase is constitutively upregulated (12). Wee-1 is also constitutively upregulated subsequent to the overexpression of c-Fos/AP-1 in RA, and overexpressed Wee-1 affords rheumatoid synovial cells a "tumor-like" characteristic that is the fundamental attribute of RA (13–17).

In the current study, we show that arthritis significantly disturbs circadian rhythmicity and the converse, circadian clock modulates arthritis. The circadian rhythm plays an important role in the pathogenesis of arthritis: especially, a novel regulatory cross talk between circadian clock gene *Cry* and proinflammatory cytokine TNF-α is demonstrated.

Materials and Methods

Animals

The 7- to 12-wk-old female wild-type (WT) and $Cry1^{-/-}Cry2^{-/-}$ mice (C57Bl6:Ola 129 hybrid) were bred under 12 h light (6°–18°) /12 h dark (18°–6°) cycles. All animal procedures were approved by, and performed in accordance with the animal experiment guidelines of Kobe University.

Experimental arthritis

Arthritis was induced by an i.p. injection of 4 mg anti-type II collagen (anti-CII) mAb (Chondrex, Redmond, WA) on days 0 and 1, followed by injection of 50 μg LPS on day 2 (18). Arthritis was assessed daily using a clinical scoring system, 0–4 grade in each limb, with a total maximum score of 16 in each mouse. Anti–TNF-α Ab (100 μg/body; R&D Systems, Minneapolis, MN) or hamstar IgG (100 μg/body; Cappel Laboratories, West Chester, PA) were injected i.p. on days 5, 8, 11, and 14 after arthritis induction.

*Department of Biophysics, Graduate School of Health Science, Kobe University; †Department of Medicine, Kobe University Graduate School of Medicine; ‡The Center for Rheumatic Diseases, Kobe University Hospital, Kobe; and §Department of Systems Biology, Graduate School of Pharmacological Science, Kyoto University, Kyoto, Japan

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Address correspondence and reprint requests to Prof. Shunichi Shiozawa, Department of Medicine, Kobe University Graduate School of Medicine, 7-5-2, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. E-mail address: shioz@med.kobe-u.ac.jp

The online version of this article contains supplemental material.

Abbreviations used in this paper: ADL, activity of daily living; anti-CII, anti-type II collagen; *Cry*, *Cryptochrome*; MEF, mouse embryonic fibroblast; MMP-3, matrix metalloproteinase-3; *Per*, *Period*; RA, rheumatoid arthritis; SCN, suprachiasmatic nucleus; WT, wild-type.

Preparation of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5 d postcoitus mouse embryos and maintained in DMEM (Invitrogen, San Diego, CA), supplemented with 10% heat-inactivated, FBS (Hyclone, Logan, UT), and 1% antibiotics (penicillin-streptomycin; Invitrogen) in a humidified incubator at 37°C in the presence of 5% CO_2.

Immunohistochemistry

Immunohistochemistry was performed by using Dako Envision Kit/HRP (Dako Cytomation, Carpinteria, CA). Abs to Per2 (α Diagnostic, San Antonio, TX) were reacted for 1 h, followed by reaction with HRP-conjugated Abs. Three different series of samples were stained.

Quantitative PCR

Quantitative PCR was performed with platinum SYBR green (Invitrogen) and analyzed on a StepOnePlus system (Applied Biosystems, Foster City, CA). Total RNA was extracted from frozen mouse tissues using RNase Mini kit (Qiagen, Valencia, CA). Reverse transcription was performed with SuperScriptIII (Invitrogen).

Flow cytometric measurement

Cells were incubated with Abs at 4°C for 30 min, washed, and then analyzed on a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA). All the Abs, including PE, FITC, APC, PerCP, and PECy5-conjugated mAbs, were obtained from BD Pharmingen (San Diego, CA).

Assay for cytokine production

Splenocytes from WT or *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were stimulated with 2 mg/ml anti-CD3 mAb (145-2C11; Cederlane, Ontario, Canada) and 5 mg/ml anti-CD28 mAb (37.51; BD Pharmingen) for 24 h. Cytokines in the culture supernatants were measured by ELISA (Biosource, Camarillo, CA).

Western blotting

Tissues or MEFs were lysed with RIPA buffer and subjected to SDS-PAGE. Transferred membranes were probed with anti–Wee-1 Ab (H-300; Santa Cruz Biotechnology, Santa Cruz, CA), anti–c-Fos Ab (4; Santa Cruz Biotechnology), anti–TNF-α Ab (HyCult Biotechnology, Uden, The Netherlands), or anti-actin Ab (Santa Cruz Biotechnology) and detected using an ECL detection system (Amersham, Arlington Heights, IL). Three different series of samples were examined.

ELISA for cytokines in sera

IL-1β, IL-6, and matrix metalloproteinase-3 (MMP-3) in serum samples of mice were measured by ELISA on day 15 of arthritis induction (Biosource).

Luciferase reporter gene construction

Genomic DNA obtained from a C57BL/6 mouse was amplified by PCR to generate the luciferase reporter. The primer pairs used for amplifying the 5′-flanking region of the *TNF-α* promoter were as follows: for −1322/+1 *TNF-α* (−1322 to +1), 5′-CCGCTCGAGCGGTTCTGTCTG CTTGTGTCTGTC-3′ (forward) and 5′-CCCAAGCTTGGGTGTCTT TTCTGGAGGGA-3′ (reverse). The XhoI and HindIII recognition sequences were added to the 5′ ends of the forward and reverse primers, respectively.

Luciferase reporter assay and transient transfection

For *TNF-α* promoter activity, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ MEFs and WT-MEFs were cotransfected with 400 ng reporter vector (pGL4.10–TNF-α or pGL4.13) and 400 ng expression vector (pcDNA3.1-mCRY1 or pcDNA3.1). Cell lysates (20 μl) were analyzed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI). The luciferase activities were calculated as the ratio of sample activity to pGL4.13 activity. WT MEF/pcDNA ratio was taken as background and subtracted from each sample.

Statistical analyses

We assessed statistical significance using Student *t* test and considered a probability of <5% ($p < 0.05$) to be statistically significant.

Results

We first examined whether the daily expression of *clock* genes in synovial cells of foot joints was altered by the induction of arthritis using a mixture of anti-CII mAb and LPS. In untreated WT mice, PER2 protein expression in the nuclei of the synovial cells of foot joints was essentially rhythmic under normal light-dark conditions. Few or no cells showed any positive staining of PER2 in the morning (8:00 AM: 2 h after lights on), whereas a majority of cells were positively stained at early night (8:00 PM: 2 h after lights off). However, in the mice in which arthritis had been induced, we found many PER2-positive nuclei in synovial cells regardless of light or dark condition (Fig. 1*A*).

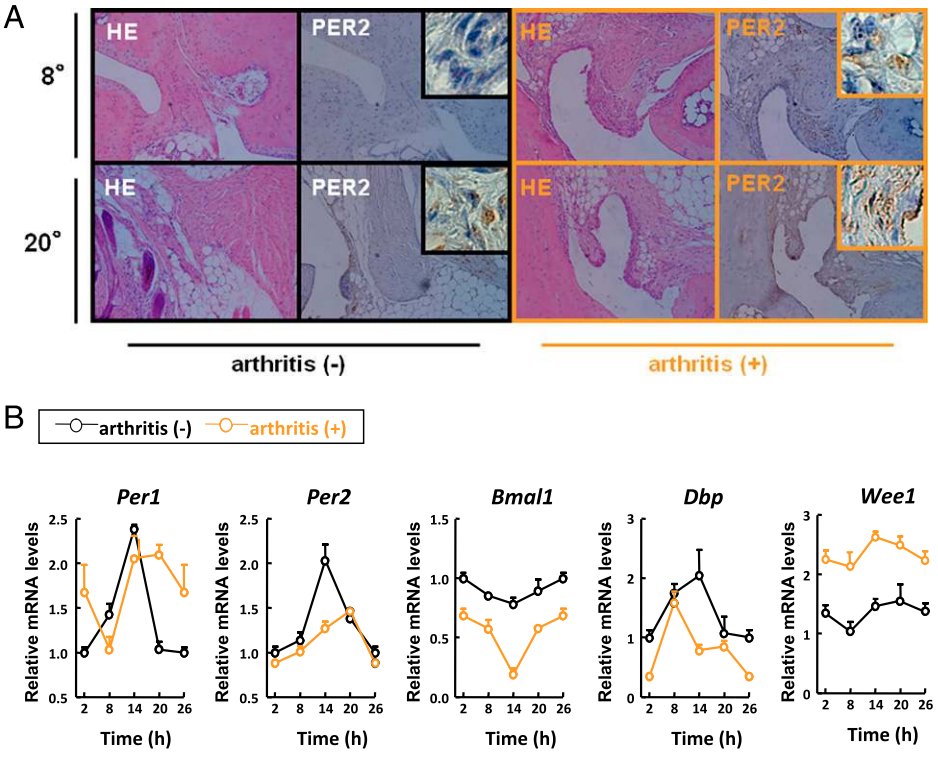

FIGURE 1. Changes of *clock* and *clock*-controlled genes expression in synovial membrane and spleen. *A*, In naive C57/BL6 mice joints, daily expression of nuclear PER2 was lower during the light period (8:00 AM/8°) and higher during the dark period (8:00 PM/20°), whereas in arthritic joints, PER2 was expressed even during the light period (8°). Three different mice were examined. *B*, Circadian expression of *clock* and *clock*-related genes in the spleen. Induction of arthritis resulted in shift *Per1/2* mRNA expression back 6 h, whereas expression of *Dbp* mRNA was shifted forward 6 h, opposite to that of *Per1/2*. Expression of *Bmal1* was lower than in the control. Expression levels were normalized to *Tbp* and are represented relative to *CT2* of nontreated animals. Values shown are means ± SEM (n = 3–4).

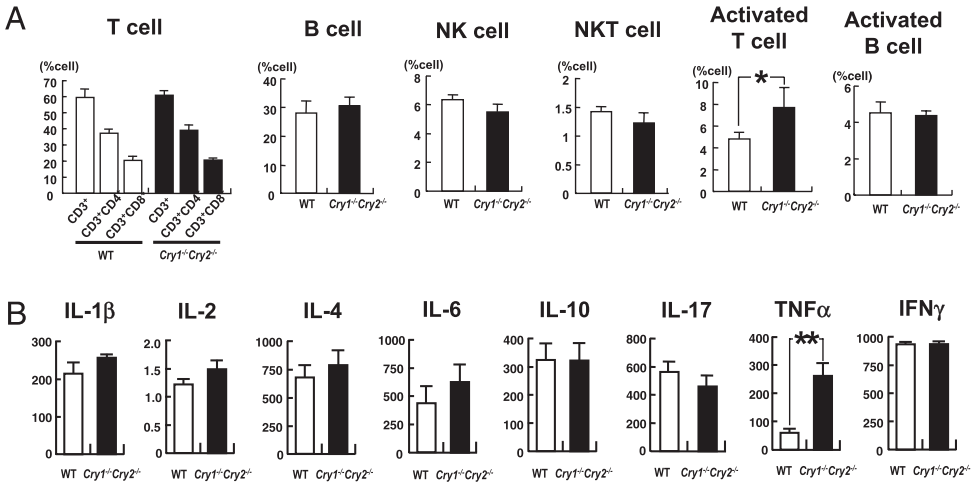

FIGURE 2. Lymphocytic profiles and cytokine production of splenocytes from WT and $Cry1^{-/-}Cry2^{-/-}$ mice. *A*, The number of activated T cells expressing $CD69^{+}CD3^{+}$ was significantly higher in lymphocytes from $Cry1^{-/-}Cry2^{-/-}$ mice. Values shown are means ± SEM ($n = 5$). *B*, Splenocytes of $Cry1^{-/-}Cry2^{-/-}$ mice produced significant amounts of TNF-α after anti-CD3/CD28 Abs stimulation as compared with WT mice. Values shown are means ± SEM ($n = 5$).

This change in *clock* gene expression is not confined to arthritic joints. We quantified the expression profiles of *clock* genes in the spleen, an organ important for immune reaction, under normal light-dark conditions (Fig. 1*B*, Supplemental Fig. 1). In arthritic mice, the expression of *Per1/2* mRNA shifted ~6 h back and that of *Bmal1* mRNA remained constantly low. In contrast, expression of *Dbp* mRNA shifted 6 h forward, opposite to the direction of *Per1* and *Per2*. Interestingly, Wee-1 levels remained constantly high after the induction of arthritis. These findings suggest that normal circadian gene expression profiles are significantly disturbed in arthritic mice, at least in their joints and spleen.

We next investigated immunological states of arrhythmic $Cry1^{-/-}Cry2^{-/-}$ mice and found an increase in the number of activated $CD3^{+}$ $CD69^{+}$ T cells compared with WT mice (Fig. 2*A*,

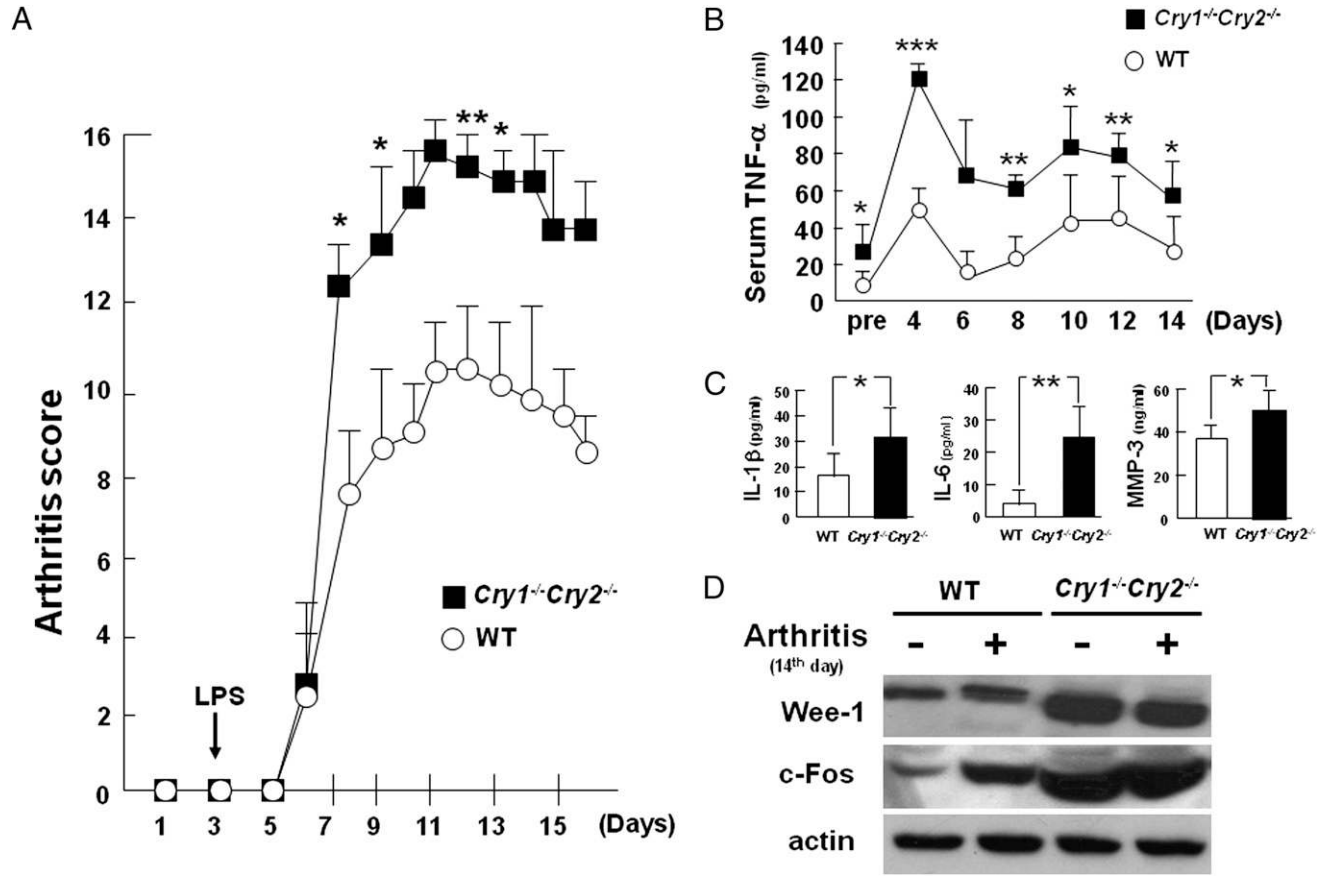

FIGURE 3. Arthritis induced by anti-CII mAb with LPS in WT and $Cry1^{-/-}Cry2^{-/-}$ mice. *A*, Severity of arthritis induced by anti-CII mAb in WT ($n = 10$) or $Cry1^{-/-}Cry2^{-/-}$ mice ($n = 12$). $*p < 0.01$; $**p < 0.05$. Values shown are means ± SEM. *B*, Measurement of serum TNF-α during anti-CII mAb-induced arthritis. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Values shown are means ± SEM. ($n = 3$–4 in each day point). *C*, Measurement of serum IL-1β, IL-6, and MMP-3 on day 14 of anti-CII mAb-induced arthritis. $*p < 0.05$; $**p < 0.01$. Values shown are means ± SEM ($n = 3$–4). *D*, Expression of Wee-1 and c-Fos protein in spleens from WT and $Cry1^{-/-}Cry2^{-/-}$ mice on day 14 after arthritis induction. Three different spleen preparations were analyzed by immunoblotting.

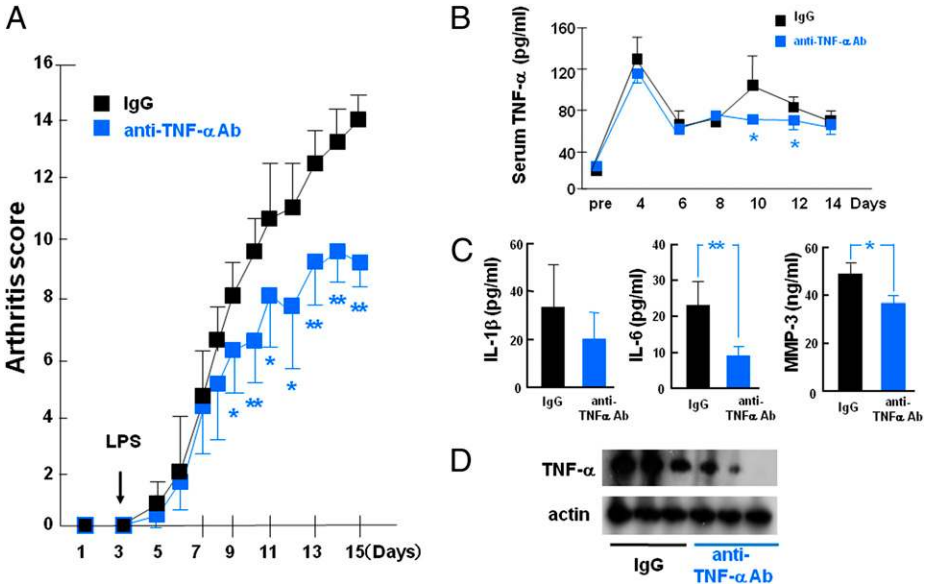

FIGURE 4. Attenuation of arthritis by the suppression of TNF-α in $Cry1^{-/-}Cry2^{-/-}$ mice. *A*, Severity of anti-CII mAb-induced arthritis in $Cry1^{-/-}Cry2^{-/-}$ mice treated with anti–TNF-α Ab (100 μg/body, $n = 5$) or control hamster IgG (100 μg/body, $n = 5$). $*p < 0.01$; $**p < 0.05$. Values shown are means ± SEM. *B*, Measurement of serum TNF-α in $Cry1^{-/-}Cry2^{-/-}$ mice having anti-CII mAb-induced arthritis and treated with anti–TNF-α Ab or control hamster IgG. $*p < 0.01$. Values shown are means ± SEM ($n = 3$ in each day point). *C*, Measurement of serum IL-1β, IL-6, and MMP-3 on day 14 of anti-CII mAb-induced arthritis in $Cry1^{-/-}Cry2^{-/-}$ mice treated with anti–TNF-α Ab (100 μg/body) or control hamster IgG (100 μg/body). $*p < 0.05$; $**p < 0.01$. Values shown are means ± SEM ($n = 5$). *D*, Expression of TNF-α protein in spleen from in $Cry1^{-/-}Cry2^{-/-}$ mice treated with anti–TNF-α Ab or control IgG treatment on day 14 after arthritis induction. Three different samples for each condition were examined.

Supplemental Fig. 2). On in vitro stimulation with anti-CD3/CD28 mAb, splenocytes taken from $Cry1^{-/-}Cry2^{-/-}$ mice showed higher production of TNF-α than those from normal mice (Fig. 2*B*), suggesting that cultured splenocytes of $Cry1^{-/-}Cry2^{-/-}$ mice may be intrinsically proinflammatory.

When experimental arthritis was induced in these proinflammatory $Cry1^{-/-}Cry2^{-/-}$ mice using a mixture of anti-CII mAb and LPS, arthritis was significantly aggravated (Fig. 3*A*): 4 of the 12 $Cry1^{-/-}Cry2^{-/-}$ mice scored the maximum value 16 for four limbs. In the arthritic joints, we observed proliferation of the synovial lining cells, infiltration of lymphocytes, and cartilage destruction. PER2 protein was undetectable in the nuclei of those cells (Supplemental Fig. 3), presumably because of rapid proteasomal degradation due to absence of accompanying CRY protein (19, 20). Serum TNF-α concentrations were significantly elevated throughout the course of the disease in $Cry1^{-/-}Cry2^{-/-}$ mice as compared with WT mice (Fig. 3*B*). The inflammatory cytokines, IL-1β and IL-6, and matrix-degrading MMP-3 in sera were also significantly increased on day 14 in these arthritis mice (Fig. 3*C*). In addition to serological markers, we found an increase in c-Fos and Wee-1 protein expression in the spleen of arthritic WT mice on day 14 (Fig. 3*D*, *left*). Further, splenic c-Fos and Wee-1 protein levels were higher in both arthritic and nonarthritic $Cry1^{-/-}Cry2^{-/-}$ mice than in arthritic WT mice (Fig. 3*D*, *right*). Thus, the lack of *Cry* gene function abrogates normal biological clock function and aggravates pathological changes in arthritis.

Because TNF-α levels in sera were significantly increased in $Cry1^{-/-}Cry2^{-/-}$ mice (Fig. 3*B*), we examined the effect of anti–TNF-α Ab treatment. When experimental arthritis was induced using a mixture of anti-CII mAb and LPS, the arthritis score of

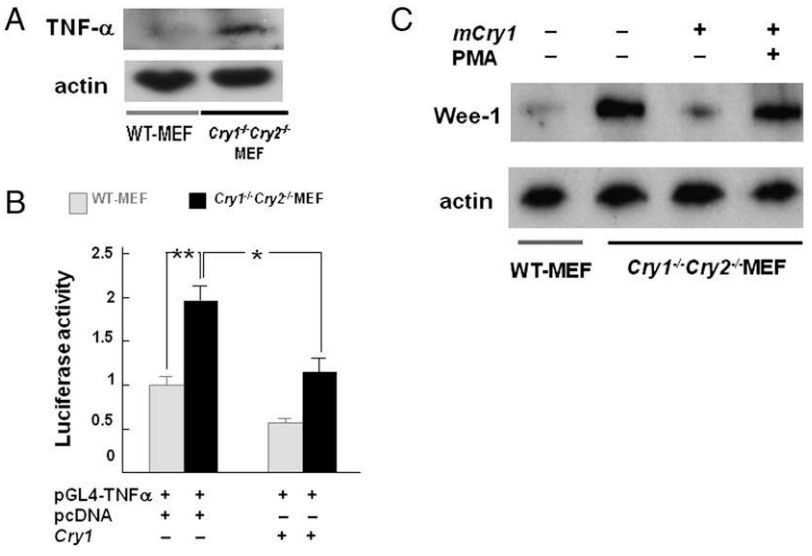

FIGURE 5. Regulation of TNF-α expression in $Cry1^{-/-}Cry2^{-/-}$ mice. *A*, TNF-α protein in MEFs detected by Western blot. Expression of TNF-α protein was higher in $Cry1^{-/-}Cry2^{-/-}$ MEFs compared with WT MEFs. Three different samples for each condition were examined. *B*, Transient reporter assay for *TNF-α trans* activation. $Cry1^{-/-}Cry2^{-/-}$ MEFs showed significantly higher TNF-α promoter activity than WT MEFs, and this activity was significantly inhibited by *mCry1* transfection. Luciferase activity was calculated by first subtracting background luminescence (WT MEF/pcDNA levels) and then calculating the ratio of activities in pGL4.10–TNF-α versus pGL4.13-transfected samples. $*p < 0.05$; $**p < 0.01$. Values shown are means ± SEM ($n = 3$). *C*, Expression of Wee-1 protein in WT or $Cry1^{-/-}Cry2^{-/-}$ MEFs with or without *mCry1* knock-in. Expressions of Wee-1 was higher in $Cry1^{-/-}Cry2^{-/-}$ MEFs than WT MEFs, and transfection of *mCry1* cDNA resulted in a decrease in Wee-1 expression. Results were confirmed by three independent experiments.

Cry1$^{-/-}$*Cry2*$^{-/-}$ mice was significantly reduced by anti–TNF-α Ab treatment (Fig. 4*A*). Serum levels of TNF-α were particularly reduced from days 10–12 (Fig. 4*B*), the exact days at which arthritis began to be ameliorated by anti–TNF-α Ab treatment. We also found that the levels of IL-1β, IL-6, and MMP-3 in sera as well as expression of TNF-α in the spleen were significantly decreased on day 14 of arthritis (Fig. 4*C*, 4*D*).

Expression of TNF-α was basically higher in the MEFs of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as compared with those of WT mice (Fig. 5*A*). This TNF-α appeared to be increased by enhanced *trans* activation of the *TNF-α* gene, because *Cry1*$^{-/-}$*Cry2*$^{-/-}$ MEFs showed a two time increase in TNF-α promoter activity, which was cancelled by ectopic expression of *Cry1* (Fig. 5*B*). These findings suggest that TNF-α can be controlled by circadian clock machinery. Finally, not only TNF-α, but also Wee-1 protein was increased in MEFs of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, and ectopic expression of *Cry1* in *Cry1*$^{-/-}$*Cry2*$^{-/-}$MEFs resulted in inhibition of Wee-1 (Fig. 5*C*).

Discussion

We show in this study, on induction of experimental arthritis in WT mice, that rhythmic expression of PER2 protein in the nuclei of synovial cells as well as mRNA expression of *clock* genes in spleen are significantly disturbed (Fig. 1). In mammals, the master circadian pacemaker SCN orchestrates several *clock* genes expressed in a tissue-specific fashion (21, 22), and delayed oscillation in the peripheral clock as compared with SCN is also reported (23). Previous study concerning the contribution of arthritis to circadian clock is not available; however, serum TNF-α has been shown to exhibit a delayed secretion rhythm in the patients with RA as compared with healthy controls, being highest at 6:00 AM and remained upregulated until 10:00 AM (7), which coincides with the sign of morning stiffness in RA (24–26). Thus, although "morning stiffness" can also be explained by hours of physical inactivity at night time because the distribution of interstitial fluid in the joints can be changed by inflammation (27), we suspect, based on our experiment, that signs and symptoms of rheumatoid patients can be modulated by the circadian clock and the converse, the circadian clock modulates arthritis.

We show that TNF-α is excessively produced from lymphocytes of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice leading to the increase of activated CD3$^+$ CD69$^+$ T cells (Fig. 2) and the converse, *Cry* inhibits the *trans* activation of *TNF-α* gene (Fig. 5). Interestingly, experimental arthritis as induced in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice was exaggerated to maximal levels in one-third of the mice (Fig. 3).

We found that c-Fos and Wee-1 proteins were significantly upregulated in the spleens of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Fig. 3), which suggests that expression of Wee-1 can be regulated by Cry. Studies show that *Wee-1* transcription can indeed be modulated by Cry proteins in cultured fibroblasts (12). Wee-1 is an important cell cycle regulator (28). Wee-1 phosphorylates Tyr15 of cdc2, a component of mitosis-promoting factor, and inhibits the cells going into aberrant mitosis during the G1/S phase of cell cycle (16). Because Wee-1 is under direct control of c-Fos/AP-1 (16), Wee-1 can naturally be upregulated when c-Fos/AP-1 was abnormally expressed in RA (17). Consequently, the cells cannot divide because of heightened Wee-1 despite increased proliferation because of upregulated c-Fos/AP-1 in RA, which is indeed "tumor-like." Because Wee-1 is upregulated in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice as shown in this study, it is likely that Cry can also contribute to the tumor-like cellular overgrowth of not only arthritis but also other tumor-like conditions. In relation to this, the finding that translocation of *clock* gene product BMAL1 into nuclei parallels with rhythmic expression of Wee-1 in tumor cell seems interesting (29).

The sleep of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice seems to be significantly disturbed because their free-running rhythmicity is abolished (11). Further, a cell cycle regulator Wee-1 kinase is constitutively upregulated in the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (12). Such findings would indicate a close association between oscillation of circadian rhythm and cell cycle regulation that is one of the fundamental systems of life, potentially affecting the physiological daily activity, including sleep disturbance.

Together, the findings appear to show that normalizing the sleep disturbance of rheumatoid patients (30) would be beneficial not only to the activity of daily living (ADL), but also to the arthritis of patients and the converse, treatment of arthritis can also improve the ADL of patients. In this sense, it would be reasonable that TNF-α is regarded as a good target for RA therapy (1, 31). Importantly, we now show that an inflammatory cytokine TNF-α is under the control of *Cry* (Fig. 5) and anti–TNF-α Ab treatment did reduce the arthritis of *Cry1*$^{-/-}$*Cry2*$^{-/-}$mice: not only TNF-α in sera and the spleen, but also inflammatory cytokines such as IL-1β or IL-6 and matrix-degrading MMP-3 in sera were significantly reduced (Fig. 4).

In conclusion, we show that arthritis significantly disturbs the biological clock of host. The *trans* activation of *TNF-α* gene is under the control of *Cry,* and the arthritis of *Cry1*$^{-/-}$*Cry2*$^{-/-}$mice can be ameliorated by anti–TNF-α Ab treatment. The association between arthritis and the biological clock shown in this study may enlighten future studies aimed at improving the ADL of patients who have chronic inflammatory diseases, including RA.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Olsen, N. J., and C. M. Stein. 2004. New drugs for rheumatoid arthritis. *N. Engl. J. Med.* 350: 2167–2179.
2. Arnett, F. C., S. M. Edworthy, and D. A. Bloch, D. J. McShane, J. F. Fries, N S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31: 315–324.
3. Cutolo, M., R. H. Straub, and F. Buttgereit. 2008. Circadian rhythms of nocturnal hormones in rheumatoid arthritis: translation from bench to bedside. *Ann. Rheum. Dis.* 67: 905–908.
4. Danis, V. A., G. M. Franic, D. A. Rathjen, R. M. Laurent, and P. M. Brooks. 1992. Circulating cytokine levels in patients with rheumatoid arthritis: results of a double blind trial with sulphasalazine. *Ann. Rheum. Dis.* 51: 946–950.
5. Dasgupta, B., M. Corkill, B. Kirkham, T. Gibson, and G. Panayi. 1992. Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. *J. Rheumatol.* 19: 22–25.
6. Choy, E. H. S., and G. S. Panayi. 2001. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N. Engl. J. Med.* 344: 907–916.
7. Straub, R. H., and M. Cutolo. 2007. Circadian rhythms in rheumatoid arthritis: implications for pathophysiology and therapeutic management. *Arthritis Rheum.* 56: 399–408.
8. Cutolo, M., B. Villaggio, K. Otsa, O. Aakre, A. Sulli, and B. Seriolo. 2005. Altered circadian rhythms in rheumatoid arthritis patients play a role in the disease's symptoms. *Autoimmun. Rev.* 4: 497–502.
9. Cuninkova, L., and S. A. Brown. 2008. Peripheral circadian oscillators: interesting mechanisms and powerful tools. *Ann. N. Y. Acad. Sci.* 1129: 358–370.
10. Sato, T. K., R. G. Yamada, H. Ukai, J. E. Baggs, L. J. Miraglia, T. J. Kobayashi, D. K. Welsh, S. A. Kay, H. R. Ueda, and J. B. Hogenesch. 2006. Feedback repression is required for mammalian circadian clock function. *Nat. Genet.* 38: 312–319.
11. Okamura, H., S. Miyake, Y. Sumi, S. Yamaguchi, A. Yasui, M. Muijtjens, J. H. Hoeijmakers, and G. T. van der Horst. 1999. Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. *Science* 286: 2531–2534.
12. Matsuo, T., S. Yamaguchi, S. Mitsui, A. Emi, F. Shimoda, and H. Okamura. 2003. Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 302: 255–259.
13. Shiozawa, S., K. Shimizu, K. Tanaka, and K. Hino. 1997. Studies on the contribution of c-fos/AP-1 to arthritic joint destruction. *J. Clin. Invest.* 99: 1210–1216.

14. Shiozawa, S., Y. Tanaka, T. Fujita, and T. Tokuhisa. 1992. Destructive arthritis without lymphocyte infiltration in H2-c-fos transgenic mice. *J. Immunol.* 148: 3100–3104.
15. Aikawa, Y., K. Morimoto, T. Yamamoto, H. Chaki, A. Hashiramoto, H. Narita, S. Hirono, and S. Shiozawa. 2008. Treatment of arthritis with a selective inhibitor of c-Fos/activator protein-1. *Nat. Biotechnol.* 26: 817–823.
16. Kawasaki, H., K. Komai, Z. Ouyang, M. Murata, M. Hikasa, M. Ohgiri, and S. Shiozawa. 2001. c-Fos/activator protein-1 transactivates wee1 kinase at G(1)/S to inhibit premature mitosis in antigen-specific Th1 cells. *EMBO J.* 20: 4618–4627.
17. Kawasaki, H., K. Komai, M. Nakamura, E. Yamamoto, Z. Ouyang, T. Nakashima, T. Morisawa, A. Hashiramoto, K. Shiozawa, H. Ishikawa, et al. 2003. Human wee1 kinase is directly transactivated by and increased in association with c-Fos/AP-1: rheumatoid synovial cells overexpressing these genes go into aberrant mitosis. *Oncogene* 22: 6839–6844.
18. Itoh, T., H. Matsuda, M. Tanioka, K. Kuwabara, S. Itohara, and R. Suzuki. 2002. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. *J. Immunol.* 169: 2643–2647.
19. Shigeyoshi, Y., K. Taguchi, S. Yamamoto, S. Takekida, L. Yan, H. Tei, T. Moriya, S. Shibata, J. J. Loros, J. C. Dunlap, and H. Okamura. 1997. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* 91: 1043–1053.
20. Yagita, K., S. Yamaguchi, F. Tamanini, G. T. van Der Horst, J. H. Hoeijmakers, A. Yasui, J. J. Loros, J. C. Dunlap, and H. Okamura. 2000. Dimerization and nuclear entry of mPER proteins in mammalian cells. *Genes Dev.* 14: 1353–1363.
21. Okamura, H. 2003. Integration of mammalian circadian clock signals: from molecule to behavior. *J. Endocrinol.* 177: 3–6.
22. Esquifino, A. I., P. Cano, V. Jiménez-Ortega, P. Fernández-Mateos, and D. P. Cardinali. 2007. Neuroendocrine-immune correlates of circadian physiology: studies in experimental models of arthritis, ethanol feeding, aging, social isolation, and calorie restriction. *Endocrine* 32: 1–19.
23. Kobayashi, H., K. Oishi, S. Hanai, and N. Ishida. 2004. Effect of feeding on peripheral circadian rhythms and behaviour in mammals. *Genes Cells* 9: 857–864.
24. Harkness, J. A., M. B. Richter, G. S. Panayi, K. Van de Pette, A. Unger, R. Pownall, and M. Geddawi. 1982. Circadian variation in disease activity in rheumatoid arthritis. *Br. Med. J. (Clin. Res. Ed.)* 284: 551–554.
25. Drewes, A. M., L. Svendsen, S. J. Taagholt, K. Bjerregård, K. D. Nielsen, and B. Hansen. 1998. Sleep in rheumatoid arthritis: a comparison with healthy subjects and studies of sleep/wake interactions. *Br. J. Rheumatol.* 37: 71–81.
26. Latman, N. S. 1983. Relation of menstrual cycle phase to symptoms of rheumatoid arthritis. *Am. J. Med.* 74: 957–960.
27. Cutolo, M., and R. H. Straub. 2008. Circadian rhythms in arthritis: hormonal effects on the immune/inflammatory reaction. *Autoimmun. Rev.* 7: 223–228.
28. Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature* 344: 503–508.
29. Wood, P. A., J. Du-Quiton, S. You, and W. J. Hrushesky. 2006. Circadian clock coordinates cancer cell cycle progression, thymidylate synthase, and 5-fluorouracil therapeutic index. *Mol. Cancer Ther.* 5: 2023–2033.
30. Abad, V. C., P. S. Sarinas, and C. Guilleminault. 2008. Sleep and rheumatologic disorders. *Sleep Med. Rev.* 12: 211–228.
31. Ranganathan, P. 2008. An update on pharmacogenomics in rheumatoid arthritis with a focus on TNF-blocking agents. *Curr. Opin. Mol. Ther.* 10: 562–567.