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Chronotherapy targeting cytokine secretion attenuates collagen-induced arthritis in mice

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A B S T R A C T

Objective: Diurnal variation of symptoms are observed in rheumatoid arthritis, especially in productions of cytokines that show peak concentrations during mid night. In contrast, cytokines of collagen-induced arthritis (CIA) mice increase in daytimes under Mid-light condition. By using chronotherapy, differences in drug efficacies according to administration time of Baricitinib, a wide ranged cytokine blocker, were examined in CIA mice.

Methods: CIA mice were administered a dose of 3 mg/kg of Baricitinib once a day at zeitgeber time (ZT) 0 or ZT12 for 21 days. Arthritis scores, histopathology and factors related to joint destruction in sera were examined. Phosphorylation of STAT3 in liver, expressions of cytokines in spleen, and Interleukin (IL)-6 and tumor necrosis factor (TNF)-α in sera were measured.

Results: In CIA mice, diurnal variations were observed both in expressions of cytokines and phosphorylation of STAT3. Arthritis scores of ZT0/12 group decreased from day3 as compared to untreated mice, and those of ZT0 group significantly decreased as compared to ZT12 group from day12. Pathological findings, immunohistochemistry of cytokines and Receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin ratio in sera well reflected results of arthritis scores. Diurnal variation of STAT3 phosphorylation was suppressed in ZT0 group. At ZT2, expressions of *IL-6/Interferon-γ/TNF/granulocyte–macrophage colony-stimulating factor* in ZT0 group were significantly decreased as compared to untreated mice, though not in ZT12 group. In ZT0 group, IL-6 and TNF-α in sera were decreased for longer time than that in ZT12 group.

Conclusion: Chronotherapy using Baricitinib targeting cytokine secretions is effective in CIA mice. Clinical applications of chronotherapy can be expected to enhance the drug efficacy.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by polyarthritis and joint destruction. Patients with RA demonstrate a characteristic diurnal variation of disease symptoms, including joint stiffness early in the morning, peaked secretion of rheumatoid factor at midnight or sleep disorder [1–4]. Particularly, this diurnal variation is present in the production of inflammatory cytokines that interleukin (IL)-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ show their peak concentrations during mid night in sera of patients with RA [5].

Thus, in this study, we demonstrated robust 24 h variation of cytokine secretion in collagen-induced arthritis (CIA) mice and examined drug efficacies according to administration time of disease modifying anti-rheumatic drugs (DMARDs). Especially about CIA, in comparison with humans, it is reported that rodents exhibit exactly opposite patters in cytokine secretion since they are nocturnal animals; IL-1β, IFN-γ and TNF-α highly elevate at Mid-light condition as compared with Mid-dark condition [6], in which plasma TNF-α levels increase or decrease with a significant 24hr rhythm [7].

Janus kinase family of protein tyrosine kinases (JAKs) are composed of four JAK family members, JAK1, JAK2, JAK3 and Tyk2. Two of four types of JAKs are recruited to bind in various combinations to the intracellular domain of Type I / Type II cytokine receptors, and

Abbreviations: RA, Rheumatoid Arthritis; CIA, collagen-induced arthritis; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; GM-CSF, granulocyte–macrophage colony- stimulating factor; MMP, matrix metalloproteinase; NF-κB, nuclear-factor kappa-B; RANKL, receptor activator of nuclear-factor kappa-B ligand; OPG, osteoprotegerin; JAK, Janus kinase; STAT, signal transducers and activators of transcription; P-STAT3, phospho-STAT3; ZT, zeitgeber time; Bmal, Brain and muscle ARNT like; Cry, Cryptochrome; Per, Period; Rora, Retinoic-acid-receptor-related orphan receptor a; E4bp4, E4-binding protein 4; DMARDs, disease modifying anti-rheumatic drugs

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phosphorylate and activate downstream proteins to contribute the intra-cellular signal transduction [8]. Cytokines, such as IL-1, IL-6, IL-17, TNF-α, IFN-γ, and granulocyte–macrophage colony-stimulating factor (GM-CSF) are key mediators of synovial cell migration and inflammation in RA [9–11] and CIA [12–16]. Among them, IL-6, IFN-γ and GM-CSF utilize JAKs to transmit the JAK/ signal transducers and activators of transcription (STAT) pathways. Matrix metalloproteinase-3 (MMP-3) is a proteolytic enzyme that also plays a pivotal role in joint destruction of RA [17]. In addition, Receptor activator of nuclear factor kappa-B ligand (RANKL) is one of the major outputs of JAK/STAT signaling pathway [18], and the RANKL/osteoprotegerin (OPG) ratio was reported to influence on joint damage in patient of RA [19].

Morning stiffness of joints and sleep disorder, as described above, symbolize the modulation of circadian rhythm of RA patients in which inflammatory cytokines are closely related [20], and we have conducted research on chronobiology of RA. So far, model arthritis experiments using clock gene *Cryptochrome* (*Cry*) knock-out mice [21], functional analysis of clock genes *Period* (*Per*) / *Brain and muscle ARNT like-1* (*Bmal-1*) using RA synovial cells [22], and novel pharmacological actions of methotrexate (MTX) *via* clock gene transcription factors [23].

Recently, a chronotherapy trial, drug administration ingenuities targeting the period of symptoms' exacerbation in accordance with the circadian rhythm, is reported for patients with RA. By the night time administration of modified-release prednisone, cytokine production during midnight was effectively suppressed to recover "morning stiffness" of patients [24,25]. However, results of chronotherapy with DMARDs have not been reported to date.

In this study, a JAK inhibitor Baricitinib is orally administrated to CIA mice once a day in the morning or evening because this small molecular-weight compounds is metabolized in a very short time (t_{max} 0.88hrs in human) and exerts superior effects comparable to other biologic DMARDs [26,27]. Results of chronotherapy targeting cytokine secretion using Baricitinib were demonstrated.

2. Materials and Methods

2.1. Animals

The 7 to 12-week-old female wild-type mice (DBA/1JJmsSlc) were bred under 12 h light (Mid-light) /12 h dark (Mid-dark) cycles. DBA/1j mice (7-week-old females) were purchased from Japan SLC (Shizuoka, Japan). Mice were divided into one group of healthy controls and three groups of CIA with or without Baricitinib treatments. All animal procedures were approved by and performed in accordance with the animal experiment guidelines of Kobe University.

2.2. Collagen-induced arthritis

To induce CIA, mice were immunized intradermally with 100 μl chick type II collagen solution (Chondrex, Redmond, WA) in Complete Freund's Adjuvant (Chondrex). 3 weeks later, second immunizations were performed with 100 μl chick type II collagen solution in Incomplete Freund's Adjuvant (Chondrex). Arthritis was assessed using a clinical scoring system from three days after second immunization: 0–4 grade in each limb, with a total maximum score of 16 in each mouse, every third day.

CIA mice were divided into 3 groups containing similar mean arthritis scores, and two of those groups were orally given 3 mg/kg Baricitinib, suspended in 0.5% methylcellulose (Chemscene LLC, Monmouth Junction, NJ); one group was administrated Baricitinib at zeitgeber time (ZT) 0 when light condition starts (i.e., Baricitinib ZT0 treated group), and another group was given at ZT12 when dark condition starts (i.e., Baricitinib ZT12 treated group) for 21 days. ZT means time of the body clock during the 12/12 h light / dark cycle that the start of the light cycle is ZT0 and the start of the dark cycle is ZT12, depending on conditions of animal breeding facilities where lights are turn on and off every 12 h.

2.3. Enzyme linked immunosorbent assay (ELISA) for cytokines in serum

IL-6 and TNF-α in sera were measured by ELISA at ZT2, 4, 6, 10, 12, 14, 18 and 22 on day 21 (R&D systems, Minneapolis, MN). Total MMP-3, RANKL and OPG in sera at ZT2 on day 21 were measured by ELISA (R &D systems).

2.4. Immunohistochemistry

Hind limbs of mice were examined by hematoxylin eosin or toluidine blue staining, and immunohistochemistry. Formalin-fixed and paraffin-embedded tissue were visualized using Peroxidase Stain DAB Kit (NACALAI TESQUE, Kyoto, Japan) with Mayer's hematoxylin (FUJIFILM Wako Pure Chemical, Osaka, Japan), and 0.05% toluidine blue (pH2.5) (FUJIFILM).

For immunohistochemistry, samples were incubated with anti-TNF-α antibody (Novus biologicals, Centennial, CO), anti-IL-6 antibody (proteintech, Rosemont, IL) followed by reaction with Signal Strain® Boost IHC Detection Reagent HRP Rabbit (Cell signaling technology, Danvers, MA).

2.5. RNA extraction, reverse transcription and Real-time polymerase chain reaction

2.5.1. Expressions of cytokines

Splenic lymphocytes were isolated at ZT2, 6, 10, 14, 18 and 22 on day21. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Then, reverse transcription was performed with the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) and analyzed on the Step One Plus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). The TaqMan probes used were: Mm00434228_m1 for *IL-1β*, Mm00446190_m1 for *IL-6*, Mm00439618_m1 for *IL-17A*, Mm00443258_m1 for *TNF*, Mm01168134_m1 for *IFN-γ*, Mm01290062_m1 for *GM-CSF* and Mm99999915_g1 for *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Expression levels were normalized to *GAPDH*.

2.5.2. Expressions of clock genes

Bmal1, Cry1, Per2, E4-binding protein 4 (E4bp4), and *Retinoic-acid-receptor-related orphan receptor α (Rora)*, in isolated splenic lymphocytes were analyzed using TaqMan® Array plates by comparative ΔΔCt method. Expression levels were compared with 18 s ribosomal RNA of control mice.

2.6. Western blotting

Hepatic tissues were lysed with RIPA buffer and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), probed with anti-STAT3 (phospho Y705) antibody (Abcam, Cambridge, UK), anti-STAT3 (Cell signaling technology), anti-mouse IgG Ab (GE Healthcare), or anti-rabbit IgG Ab (GE Healthcare). ImmunoStar® LD (Wako) was used for detection.

2.7. Statistical analyses

Student *t* test was used to compare differences between two experimental groups. Tukey HSD test was used to compare differences between more than three experimental groups and considered a probability of $< 5\%$ ($p < 0.05$) to be statistically significant. The statistical analyses were performed by EZR, based on R and R commander [28].

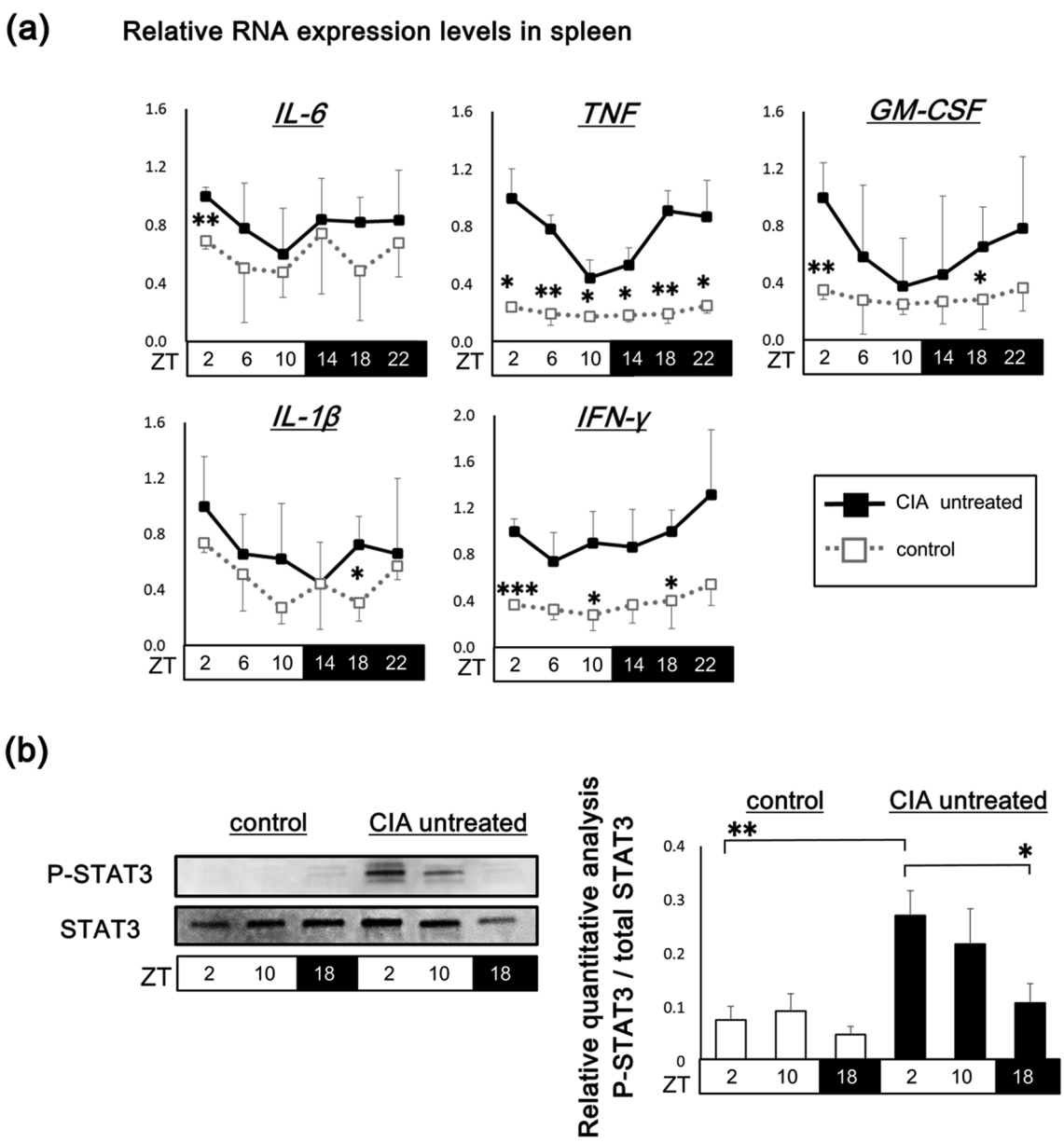

Fig. 1. Cytokines expressions and Phosphorylation of STAT3 of CIA untreated and control mice. **(a)** Cytokines expressions in spleen of CIA untreated and control mice. Splenic lymphocytes were isolated at ZT2, 6, 10, 14, 18 and 22 on day21. Expression levels were normalized to *GAPDH* and are represented relative to values of ZT2. *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$. Values shown were means ± SEM ($n = 3$). **(b)** Phosphorylation of STAT3 in liver of CIA untreated and control mice. Hepatic proteins from mice at ZT2, 10, and 18 on day21 were examined. *: $p < 0.05$, **: $p < 0.005$. Values of quantitative analysis were means ± SEM ($n = 3$). P-STAT3: phospho-STAT3.

3. Results

3.1. Circadian variations of cytokine productions in CIA mice

In untreated CIA mice, the expressions of *IL-6, TNF, GM-CSF* and *IL-1β* reached maximum levels at ZT2 and that of *IFN-γ* at ZT22 (Fig. 1a). The lowest expression of all cytokines accumulated in ZT10 ~ 14 time zone, representing overall diurnal variations, whereas that were not observed in expressions of *TNF, IFN-γ,* and *GM-CSF* in control group (Fig. 1a). In liver of CIA mice, phosphorylation of STAT3 also exhibited diurnal variations in which the strongest phosphorylation was detected at ZT2 in contrast with control group. (Fig. 1b).

3.2. Inhibition of phosphorylation of STAT3 by Baricitinib

Phosphorylation levels of STAT3 were normalized to total STAT3 and are represented relative to ZT2 of each groups. In untreated group, STAT3 was significantly phosphorylated from ZT22 ~ 10, especially peaked at ZT2 (Fig. 2a; left). In ZT0 treated group, phosphorylation of STAT3 were completely suppressed throughout the observation period (Fig. 2a; middle) whereas they were detected at ZT2 ~ 6 in ZT12 treated group (Fig. 2a; right).

When phosphorylation levels of STAT3 were compared at 2 time points, 2hrs after Baricitinib administration, STAT3 in CIA untreated group was significantly phosphorylated than that in control group.

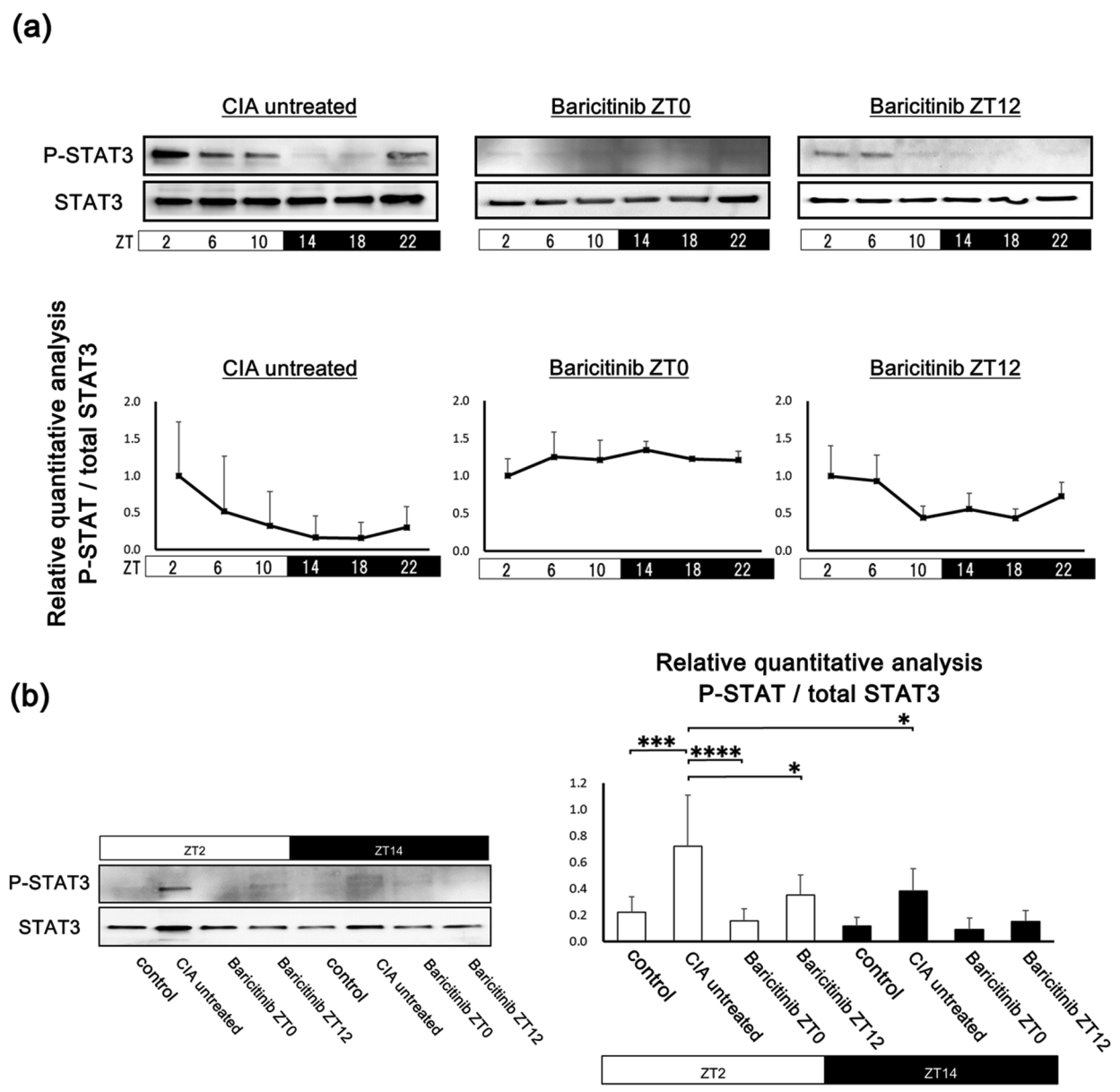

Fig. 2. Suppression of STAT3 phosphorylation by Baricitinib in each groups. **(a)** Phosphorylation of STAT3 in liver. Hepatic proteins from mice at ZT2, 6, 10, 14, 18 and 22 on day21. Values of quantitative analysis were means ± SEM (n = 3 ~ 5). P-STAT3: phospho-STAT3. **(b)** Comparison of Phospho-STAT3 (P-STAT3) in liver at ZT2 and ZT14. *: $p < 0.05$, ***: $p < 0.0005$, ****: $p < 0.0005$. Values of quantitative analysis were means ± SEM (n = 6 ~ 8).

Phosphorylation levels in ZT0/ZT12 treated group were lower than CIA untreated group. By contrast, at ZT14, phospho-STAT3 in CIA untreated group was lower than that at ZT2, representing the circadian manifestation of joint inflammation in mice (Fig. 2b).

3.3. Impact of Baricitinib chronotherapy on production of IL-6 and TNF-α in splenic RNA and sera

To evaluate the effects of Baricitinib treatment on inflammatory cytokines, we first evaluated RNA expressions levels of *IL-6* and *TNF* in spleen. In all experimental groups, productions of both cytokines showed fluctuations in 24hrs period. Especially in ZT0 treated group, relative *IL-6* expression level was significantly decreased as compared to CIA untreated group at ZT2 (Fig. 3a).

Second, in CIA untreated mice, IL-6 in sera was significantly elevated as compared to control group at ZT2 ~ 6 ($p = 0.000, 0.006, 0.011$). In ZT0 treated group, IL-6 was significantly decreased as compared to CIA untreated group at ZT2, 6 and 12. Low concentration of IL-6 was also determined at ZT4 ($p = 0.057$). In ZT12 treated group, IL-6 was decreased as compared to CIA untreated group at ZT2 and ZT18 (Fig. 3b; left).

Third, TNF-α in sera of CIA untreated group was significantly higher than control group at all time points. In ZT0 treated group, TNF-α was significantly decreased as compared to CIA untreated group at ZT2, 6 and 22. Low concentration of TNF-α was also determined at ZT4 ($p = 0.086$). In ZT12 treated group, TNF-α was decreased as compared to CIA untreated group at ZT14. At ZT6, TNF-α of ZT0 treated group was lower than that of ZT12 treated group, in contrast, that of ZT12 treated group was lower than ZT0 treated group at ZT14 (Fig. 3b; right).

3.4. RNA expression levels of cytokines in spleen at ZT2/ZT14

At ZT2, the expressions of *IL-6*, *IFN-γ*, *TNF* and *GM-CSF* in ZT0 treated group were significantly decreased as compared to untreated mice, whereas those of ZT12 group were not. The expressions of *IL-1β*,

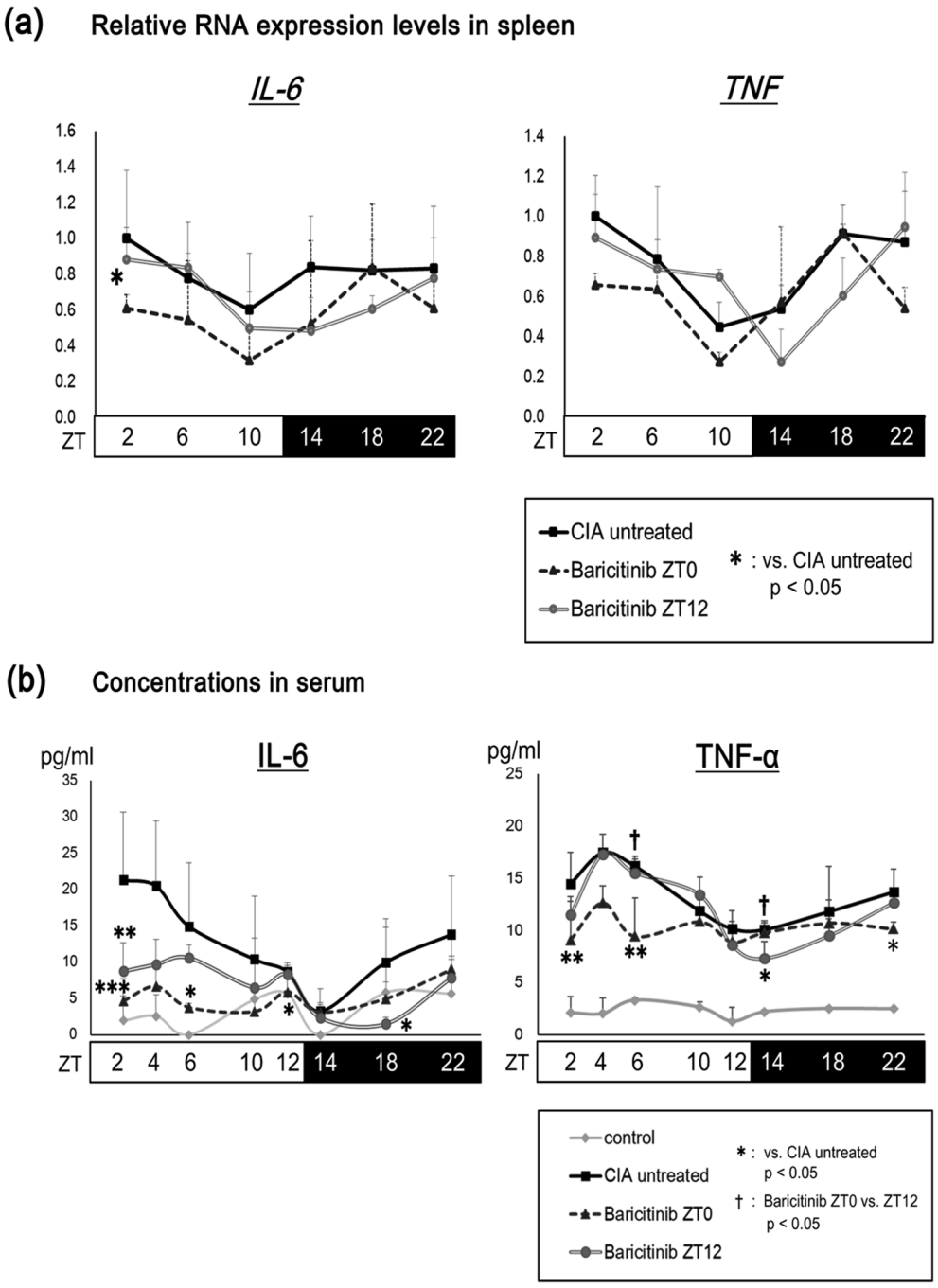

Fig. 3. Impact of Baricitinib chronotherapy on production of IL-6 and TNF-α in splenic RNA and sera. **(a)** Relative mRNA expression levels of *IL-6* and *TNF* in spleen at ZT2, 6, 10, 14, 18 and 22 on day21. * $p < 0.05$. Values shown were means ± SEM (n = 3). **(b)** Concentrations of IL-6 and TNF-α in sera at ZT2, 4, 6, 10, 12, 14, 18 and 22 on day21. *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$, †: $p < 0.05$. Values shown were means ± SEM (n = 3 ~ 9).

IL-17A in lymphocytes were significantly decreased by Baricitinib treatment as compared to untreated group (Fig. 4a). By contrast, at ZT14, significant differences were not detected in every groups (Fig. 4b).

3.5. Suppression of arthritis by chronotherapy; Baricitinib administration at ZT2 or ZT12

Arthritis score of both Baricitinib treated groups significantly decreased from day3 as compared to the untreated group, while arthritis score of ZT0 treated group was significantly suppressed as compared to ZT12 treated group from day12 (Fig. 5a). In HE staining, joint destructions with synovial hyperplasia and neovascularization were observed in untreated group, and synovial hyperplasia were also observed in ZT12 treated group, though the joint structure appeared almost normal in ZT0 treated group (Fig. 5b; first row, x200). In addition, toluidine blue showed normal cartilage component with thickness in ZT0 treated group as compared to those of destructed or thinned in untreated group or ZT12 treated group (Fig. 5b; second row, x200). Synovail cells facing the joint cavity and infiltrated inflammatory lymphocytes in lining layer expressed IL-6 and TNF-α in untreated group or ZT12 treated group, while it was suppressed in ZT0 treated

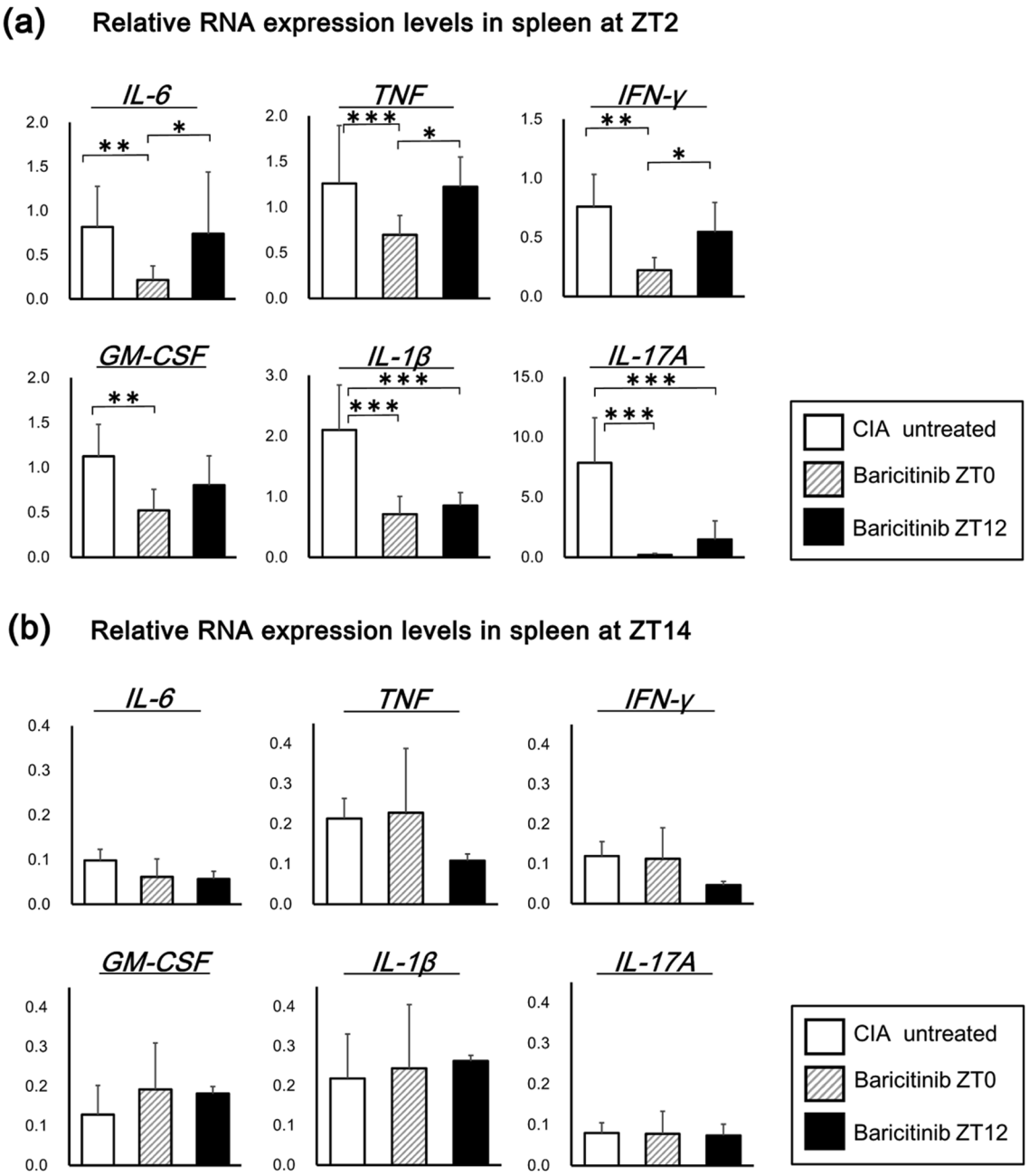

Fig. 4. Cytokines expressions in spleen of mice at ZT2/ZT14. **(a)** Cytokines production in spleen of mice. Splenic lymphocytes were isolated at ZT2 on day21. *: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$. Expression levels were normalized to *GAPDH*. Values shown were means ± SEM ($n = 10 \sim 12$). **(b)** Cytokines production in spleen of mice. Splenic lymphocytes were isolated at ZT14 on day21. Expression levels were normalized to *GAPDH*. Values shown were means ± SEM ($n = 3$).

group (Fig. 5b; third and fourth row, x400).

3.6. Joint distruction factors: MMP-3, RANKL and OPG in sera

MMP-3 in sera of ZT0 treated group significantly decreased as conpared to CIA untreatd group, but that of ZT12 treated group did not. RANKL in sera also significantly decreased in ZT0 treated group as compared to CIA untreated group, but OPG in sera was comparable in all groups. However, RANKL/OPG ratio of ZT0 treated group was significantly decreased as compared to CIA untreated and ZT12 treated group (Fig. 6a). These findings clearly reflected results of both arthritis scores and histological joint destructions in Fig. 5.

3.7. RNA expression levels of clock genes in spleen

In ZT0 treated group, expressions of *Bmal1* were decreased at ZT6 and 10 ($p = 0.087$, $p = 0.057$) and *E4bp4* significantly decreased at ZT14, as compared to CIA untreated group. Also, expressions of *Rora* significantly decreased at ZT2 as compared to ZT12 treated group. In ZT12 treated group, expressions of *Baml1* significantly decreased at ZT6 as compared to CIA untreated group, however, expressions of *Cry1* and *Per2* were comparable in all groups (Fig. 6b).

4. Discussions/Conclusions

We examined characteristic circadian rhythms of cytokines in CIA, and proved superior effects of Baricitinib by targeting the period when

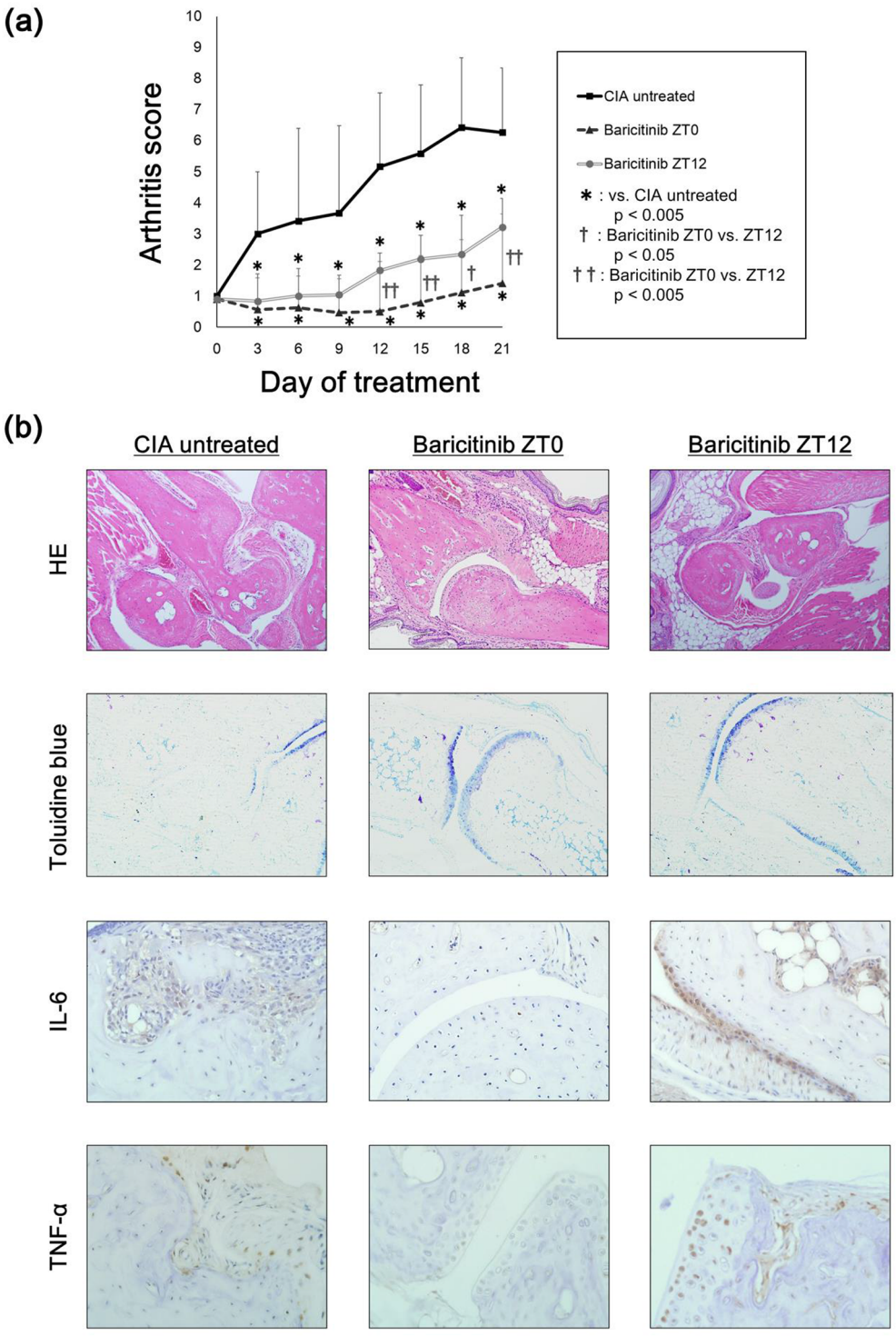

Fig. 5. Arthritis score, pathological findings and immunohistochemistry of cytokines in joint tissue. **(a)** Arthritis scores of CIA mice. *: $p < 0.005$, †: $p < 0.05$, ††: $p < 0.005$. Values shown were means ± SEM ($n = 12 \sim 28$). **(b)** Hematoxylin eosin staining (first row, x200) and toluidine blue staining (second row, x200) of hind limbs. Expressions of IL-6 and TNF-α were examined by Immunohistochemistry (third and fourth row, x400).

cytokine production was increased. JAK inhibitors have shown a promise for curing inflammation related to immune and hematopoietic diseases in the recent years, thus we suppose that chronotherapy further enhances the efficacy of JAK inhibitors.

In CIA mice, various cytokines including *IL-6, TNF, IFN-γ, GM-CSF* and *IL-1β* elevated from ZT22 to ZT2. Similarly, phosphorylation of STAT3 fluctuated from ZT2 to ZT10 (Fig. 1). As reported, diurnal variation is observed the concentration of circulated cytokines in CIA, including IL-1β, IL-6, IFN-γ and TNF-α [6,7]. Signal transmission through JAK/STAT3 pathway is activated by periodically secreted cytokines depending on light environments rearing CIA mice.

In rats' experiments, an oral dose of 10 mg/kg of Baricitinib is expected to inhibit JAK1/2 signaling, inferred by IL-6 whole blood assay ($\geqq IC_{50}$), until 8hrs after Baricitinib administration. Additionally, Baricitinib treatment at dose of 3 mg/kg inhibits disease scores by 57% during 2 weeks of treatment. Even in mice, 10 mg/kg Baricitinib (twice

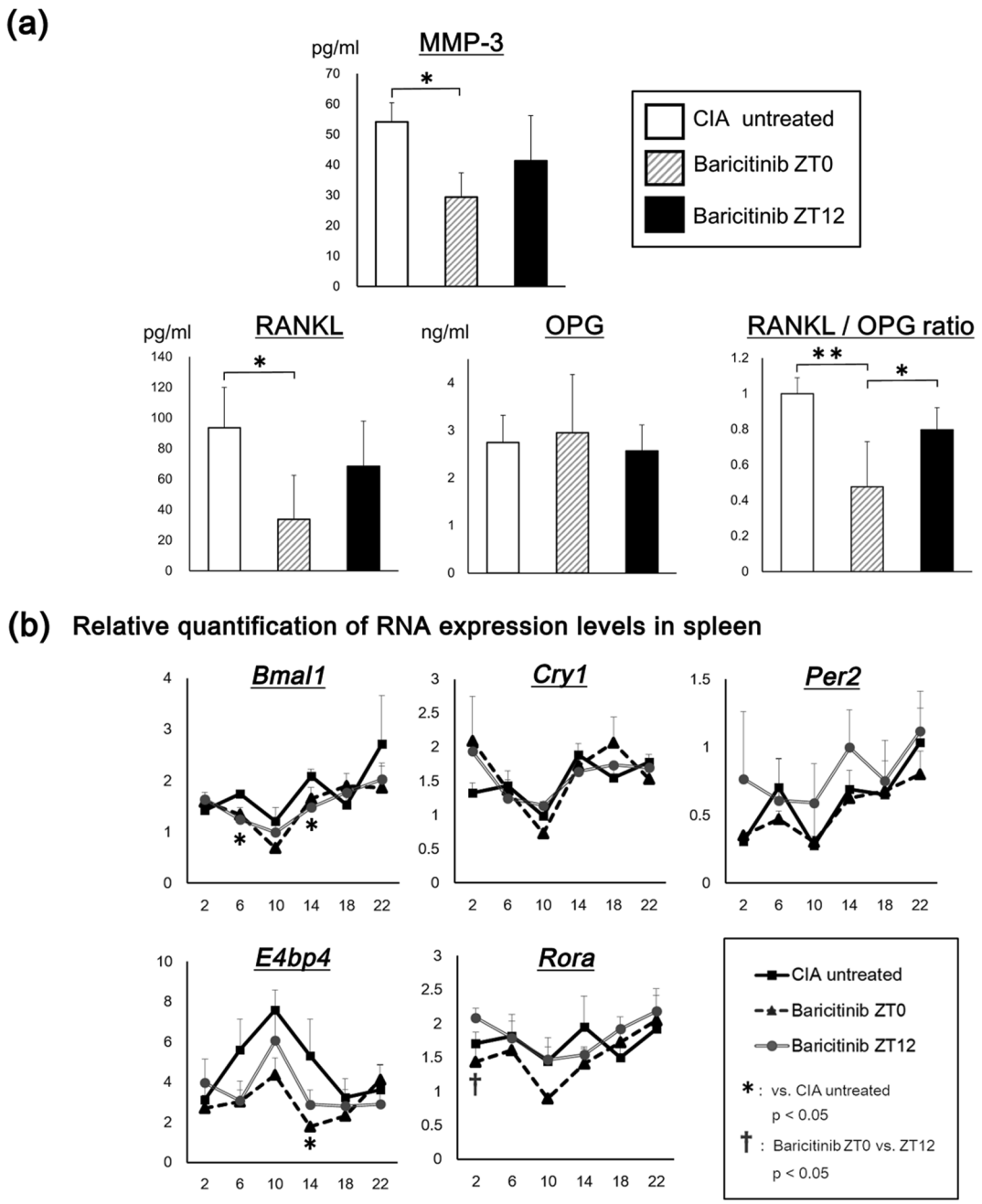

Fig. 6. Joint destruction factors in sera and expressions of clock genes in spleen. **(a)** Concentrations of MMP-3, RANKL and OPG in sera at ZT2 on day 21. *: $p < 0.05$, **: $p < 0.005$. RANKL/OPG ratio was represented relative to values of CIA untreated group. Values shown were means ± SEM (MMP-3: n = 6, RANKL: n = 6, OPG: n = 7, RANKL/OPG ratio: n = 6). **(b)** Relative quantification of clock genes in spleen at ZT2, 6, 10, 14, 18 and 22 on day21. *: $p < 0.05$, †: $p < 0.05$. Expression levels were normalized to 18 s ribosomal RNA. Values shown were means ± SEM (n = 3).

per day) mitigated arthritis scores by 56% [26]. In our study, the arthritis score of Baricitinib treated groups was significantly decreased as compared to the untreated group (Fig. 5a). As shown in Fig. 2a, phosphorylation of STAT3 was not figured out in ZT0 treated group whereas it was significant in CIA untreated group and lesser extent of that was observed even in ZT12 treated group, reflecting the significant difference of arthritis score between ZT0 and ZT12 treatment in Fig. 5a. Results clearly indicated that administration of Baricitinib at ZT0 more effectively suppressed signal transduction through JAK/STAT pathway, than that at ZT12.

As shown in Figs. 1, 3 and 4, IL-6 elevate at ZT2 both in spleen and sera of CIA mice, and Baricitinib reduced those in light condition by treatment at ZT0 or those at ZT18 by treatment at ZT12, representing a fair control of Baricitinib for IL-6 production by the suppression of JAK1/2 and STAT3. However, in ZT0 treated group, IL-6 in sera were decreased for longer time than that in ZT12 treated group. It may reflect the effective suppression of signal transduction through JAK/STAT pathway by Baricitinib treatment at ZT0. Moreover, as shown in Fig. 3b, the peak of serum TNF-α levels in light condition was significantly suppressed by Baricitinib treatment at ZT0 whereas treatment at ZT12 reduced that only one time point, at ZT14. Though TNF-α does not directly activate JAK/STAT pathway, it was also reduced by Baricitinib. IL-6 and TNF-α are important cytokines in progression of RA [9–11], thus chronotherapy using Baricitinib would be effective to treat RA.

Expressions of *IFN-γ* and *GM-CSF* were significantly reduced by

administration of Baricitinib at ZT0 (Fig. 4). As reported, a diurnal variation is observed in levels of circulated IFN-γ in CIA mice [6], as well as serum GM-CSF levels in healthy volunteers [29]. In particular, GM-CSF and gp130 are important mediator for arthritic pain [30,31], thus, reduced secretion of GM-CSF or excessive interaction of IL-6 /gp130 may mitigate joint pain in RA. Indeed, recent patient reported outcome demonstrated that Baricitinib showed a rapid effect on patients' joint pain [32,33].

MMP-3 and RANKL clearly reflected results of arthritis score and histological findings (Figs. 5 and 6a). Serum MMP-3 shows a well-known association with disease activity and therapeutic effects of RA [34], and reduction of IL-6, IL-17, and TNF-α ameliorate arthritis by diminishing matrix metalloproteinases such as MMP-3 and RANKL [35]. Moreover, STAT3 activation further induces *RANKL* expression, which promotes osteoclastogenesis and joint destruction [18]. Thus, suppression of STAT3 activation by chronotherapy would reduce RANKL production, and thereby ameliorate arthritis in ZT0 treated group (Figs. 3, 4).

As we previously reported, expressions of clock genes including *Cry1, Per2, E4bp4* and *Rora* were significantly modulated in peripheral blood of RA patients treated with bDMARDs [36]. Although we tested splenic lymphocytes in this study, expressions of *E4bp4* and *Rora* in ZT0 treated group have been modulated as compared to CIA untreated group or ZT12 treated group, and especially, decreased expressions of *E4bp4* over 24hrs time period best reflected the effect of Baricitinib on CIA (Fig. 6b). The relationship between chronotherapy and clock genes needs further validation.

In this study, we examine characteristic diurnal features of cytokines in CIA and propose the optimal administration of Baricitinib by chronotherapy, created based on the circadian rhythm of human. Chronotherapy further enhances the efficacy of drugs, and also enables dose-down of those that take advantage of the increased drug potencies. Thus, the limitation of this study was that effect of drug reduction in the ZT0 treated group and the ZT12 treated group could not be verified since the administration dosage of Baricitinib was single. Lifetime medical expenses for RA patients with advanced joint destruction are required to be high due to drug costs, surgical treatments, rehabilitations or home renovation costs. In this point of view, our presenting results suggest that night time administration of Baricitinib for patients with RA not only provide a superior drug efficacies but also achieve drug dose reduction. To support this, a recent report have shown that reduced dose of Baricitinib has almost equal efficacies comparable to regular dose on long-term disease activities, and the disease control can be quickly recovered by increasing drug dose against relapse of arthritis after reducing dose [37].

Taken together, chronotherapy targeting cytokine secretions is far more effective than previously considered and this study has a social public benefit that can provide optimal treatment of RA to as many patients as possible at a low cost. It is an urgent task imposed on us to conduct the large-scale clinical research and verify therapeutic outcomes of chronotherapy for patients with RA.

Competing financial interests

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CRediT authorship contribution statement

Arisa Yaekura: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Kohsuke Yoshida:** Validation, Methodology, Funding acquisition. **Kanta Morii:** Investigation. **Yuto Oketani:** Investigation. **Ikumi Okumura:** Investigation. **Kenta Kaneshiro:** Investigation. **Nao Shibanuma:** Validation. **Yoshitada Sakai:** Validation. **Akira Hashiramoto:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106549.

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