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The clock gene Bmal1 controls inflammatory mediators in rheumatoid arthritis fibroblast-like synoviocytes

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ABSTRACT

Object: To clarify the involvement of clock genes in the production of inflammatory mediators from RA-FLS, we examined the role of Bmal1, one of the master clock genes.
Methods: RA-FLSs were stimulated with IL-1β (0, 20 ng/mL), IL-6 (0, 20 ng/mL), IL-17 (0, 20 ng/mL), TNF-α (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) to examine the expression of Bmal1, MMP-3, CCL2, IL-6, IL-7 and IL-15 by qPCR and immunofluorescence staining. After silencing Bmal1, RA-FLSs were stimulated with IL-1β (0, 20 ng/mL), TNF-α (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) to examine the expressions of inflammatory mediators; MMP-3, CCL2, IL-6 and IL-15 by qPCR, ELISA and immunofluorescence staining.
Results: Bmal1 expressions were increased by IL-1β, TNF-α and IFN-γ stimulations. Under stimulations with TNF-α, IL-1β, and IFN-γ, mRNA and protein expressions of MMP-3, CCL2 and IL-6 were suppressed by siBmal1.
Conclusion: Results indicate that Bmal1 contributes the production of MMP-3, CCL2, and IL-6 from RA-FLS, implying Bmal1 is involved in the pathogenesis of RA by regulating the inflammation.

1. Introduction

Most living organisms on the earth possess circadian rhythms regulated by clock genes, thereby controlling important internal functions of the human body including body temperature, hormone secretion, and sleep-wake cycle. The major clock genes are composed of Brain and muscle Arnt-like protein 1 (Bmal1), Circadian locomotor output cycles kaput (Clock), Period (Per), and Cryptochrome (Cry). To form a transcriptional-translational feedback loop and regulate each other's expression, clock genes further cooperate with other clock-regulated genes; D site of the albumin promoter binding protein (Dbp), hepatic leukemia factor (Hlf), thyrotroph embryonic factor (Tef), E4-binding protein 4 (E4bp4), Retinoic-acid-receptor related orphan receptor a (Rora), and Rev-erba [1,2].

Rheumatoid arthritis (RA) is an autoimmune polyarthritis, and RA patients exhibit characteristic diurnal variations in disease symptoms, such as 'morning stiffness' of joints and increased cytokine production in the midnight [3]. Indeed, we reported that expressions of circadian clock genes were correlated with the disease activity of RA [4,5].

The pathogenesis of RA is characterized by tumor-like proliferation of RA fibroblast-like synoviocyte (RA-FLS), which also produce inflammatory mediators such as matrix metalloproteinases (MMPs), chemokines and cytokines that promote joint destruction [6–9].

Interleukin (IL)-1β and tumor necrosis factor (TNF)-α stimulate osteoclastogenesis by increasing MMP-1, MMP-3, MMP-9, MMP-13 production in RA-FLS, indirectly promoting joint destruction [10]. In cultured chondrocytes, RA-FLS and collagen-induced arthritis (CIA), IL-1β and TNF-α have been reported to regulate MMP gene expression through intracellular signaling pathways [11]. Furthermore, IL-1β and TNF-α increase Bmal1 expression in RA-FLS [12–14].

Chemokines induce osteoclastogenesis of RA joints, migration of effector T cells into synovial tissues and angiogenesis essential for

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tumor-like proliferation of RA-FLS [15]. C–C motif ligand 2 (CCL2), a representative chemokine produced from RA-FLS, triggers various signaling pathways and is involved in the migration of inflammatory cells to the synovium [16]. Both IL-1β and TNF-α induce CCL2 production from FLSs, and interferon (IFN) synergistically increases CCL2 production by IL-1β [17–19].

IL-6 produces MMPs, differentiates lymphocytes, and affects infiltrations of inflammatory cells and the angiogenesis [20,21]. Concentrations of IL-6 in synovial fluids of RA patients are increased compared with those of osteoarthritis (OA) patients [22], and IL-6 and TNF-α cooperatively proliferate RA-FLS through modulating cell cycle regulators, as we reported [23].

Cytokines of the IL-17 family, including L-17A and IL-17F, act synergistically with TNF, IL-1, and IL-6 to induce the production of inflammatory cytokines, chemokines such as C-X-C motif chemokine ligand 1 (CXCL1), and MMPs from RA-FLS. IL-7 also directly stimulates the production of TNF-α from macrophages and osteoclastogenic cytokines from T cells [24–26]. IL-15, another member of γ-chain cytokine, is also increased by stimulations of TNF-α and IL-1β [27,28]. IL-15 binds to IL-15 receptor (IL-15R) expressed on RA-FLS, subsequently induces proliferation of RA-FLS, since blockade of the IL-15 pathway using either an IL-15R antagonist or neutralizing anti IL-15 antibodies reduces expressions of apoptotic proteins Bcl-xL and Bcl-2 [28].

As described above, RA-FLS is widely known to produce a variety of inflammatory mediators, however, the involvement of clock genes remains unclear. In this study, we examined the roles of Bmal1 on productions of inflammatory mediators in RA-FLS to reveal the relationship between clock gene Bmal1 and the pathogenesis of RA.

2. Materials and methods

2.1. Cell culture

Synovial tissues were obtained during joint surgery from patients with RA, diagnosed in according to American College of Rheumatology (ACR) classification criteria [29]. Tissues were minced and stirred with 2 mg/mL collagenase (Wako, Tokyo, Japan) in serum-free Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) at 37 °C for 1 h. Primary cultured fibroblast-like synovial cell lines were established and maintained in DMEM including 10 % heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Life Technologies), 7.5 % Sodium Bicarbonate (Life Technologies), in a humidified incubator at 37 °C in the presence of 5 % CO_2. Cells from passages 3 to 7 were used in the entire experiments.

This study was approved by the ethics committee of Kobe University (approval #579) and Kobe Kaisei Hospital (approval #0017), and written informed consent was obtained from each patient, according to the Declaration of Helsinki.

2.2. Synchronization of RA-FLS by serum shock

RA-FLS were incubated in DMEM containing 50 % horse serum for 2 h to synchronize expressions of clock genes [30].

2.3. RNA interference

Bmal1 small interfering (si) RNA (s1616) (siBmal1) and Silencer® Select Negative Control siRNA (4390843) (siNC) were purchased by Thermo Fisher Scientific. siRNAs were transfected into RA-FLS by using Lipofectamine™ RNAiMAX (Life Technologies) for 48 h. After that, RA-FLS were synchronized by serum shock and then incubated with TNF-α (0, 20 ng/mL), IL-1β (0, 20 ng/mL) and IFN-γ (0, 20 ng/mL) for 16, 24 and 32 h.

2.4. RNA extraction, Reverse transcription

After serum shock, RA-FLSs ($8.0x10^4$ cells/well) were cultured in 6 well Microplates (IWAKI) and stimulated with IL-1β (0, 20 ng/mL), IL-6 (0, 20 ng/mL), IL-17 (0, 20 ng/mL), TNF-α (0, 20 ng/mL) and IFN-γ (0, 20 ng/mL). Total RNA was extracted every 8 h until 32 h after cytokine stimulation by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Reverse transcription was performed with ReverTraAce ® (TOYOBO, Osaka, Japan).

2.5. Quantitative polymerase chain reaction (qPCR)

Synthesized complementary DNA was analyzed by qPCR on StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The TaqMan probes were: Hs00427620_m1 for Tbp, Hs00154147_m1 for Bmal1, Hs01007553_m1 for Per2, Hs00172734_m1 for Cry1, Hs00901393_m1 for Cry2, Hs00231857_m1 for Clock, Hs00609747_m1 for Dbp, Hs01115720_m1 for Tef, Hs00171406_m1 for Hlf, Hs00536545_m1 for Rora, Hs00253876_m1 for Rev-erba, Hs00968305_m1 for MMP-3, Hs00234140_m1 for CCL2, Hs00174131_m1 for IL-6, Hs00174202_m1 for IL-7, Hs01003716_m1 for IL-15. The values were represented relative to Tbp.

2.6. Enzyme linked immunosorbent assay (ELISA) for cytokines in culture supernatant

RA-FLSs ($8.0x10^4$ cells/well) were cultured in 6 well Microplates (IWAKI), transfected with siBmal1 and then stimulated with IL-1β (0, 20 ng/mL), TNF-α (0, 20 ng/mL) and IFN-γ (0, 20 ng/mL) for 32 h. The productions of MMP-3, CCL2 and IL-6 in the culture supernatant were analyzed by ELISA (R&D systems).

2.7. Antibodies and agents

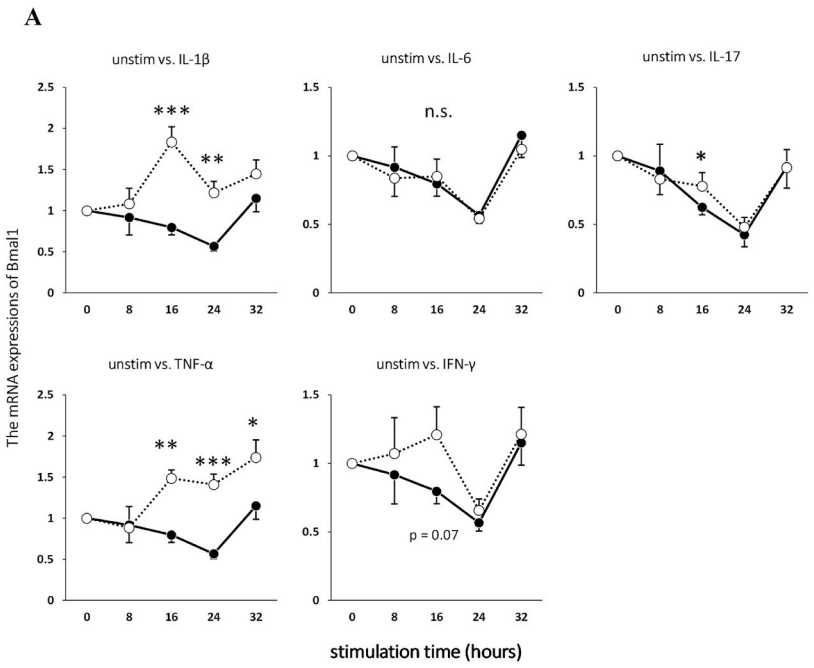
Anti-BMAL1 antibodies (NBP2-02544) were purchased by Novus Biologicals a biotechne brand (Centennial, USA). Anti-MMP-3 antibodies (ab52915) were obtained from abcam (Cambridge, UK). Anti-CCL2 antibodies (A51548-100) were obtained from EPIGENTEK (Farmingdale, NY, USA). Anti-IL-6 antibodies (21865-1-AP) were obtained from proteintech (Rosemont, IL). Anti-Rabbit IgG (H + L), F (ab') 2 Fragment (Alexa Fluor® 488 conjugate) (#4412) and anti-Mouse IgG (H + L), F (ab') 2 Fragment (Alexa Fluor® 594 conjugate) (#8890) were obtained from Cell Signaling Technology (Danvers, MA, USA). IL-1β, IFN-γ, IL-6, and IL-17 were obtained from Peprotech (Rocky Hill, NJ, USA), and TNF-α was obtained from R&D Systems (McKinley, NE, USA).

2.8. Immunofluorescence

RA-FLSs ($1.0x10^4$ cells/well) were cultured in Lab-Tek Chamber Slide (Thermo Fischer scientific), transfected with or without siBmal1, and then stimulated with IL-1β (0, 20 ng/mL), IL-6 (0, 20 ng/mL), IL-17 (0, 20 ng/mL), TNF-α (0, 20 ng/mL) and IFN-γ (0, 20 ng/mL) for 32 h. After that, RA-FLSs were fixed by incubation with cold 100 % methanol for 15 min at 4 °C. The fixed cells were incubated with Blocking One Histo (Nacalai, Japan) for 1 h at room temperature, then incubated with the primary antibodies of MMP-3, CCL2 and IL-6 at 4 °C overnight. The cells were incubated with secondary antibodies for 1 h at room temperature. The nuclei were stained with 4', 6-diamidino-2-phenylinndole (DAPI; Sigma Aldrich). Images were captured using a Carl Zeiss Axio Vert.A1 microscope (Carl Zeiss, Oberkohen, Germany) equipped with the software AxioVision 4.8 and an objective lens (40x magnification, 0.4 numerical aperture).

2.9. Statistical analysis

Statistical significance was determined by the Student's t-test and



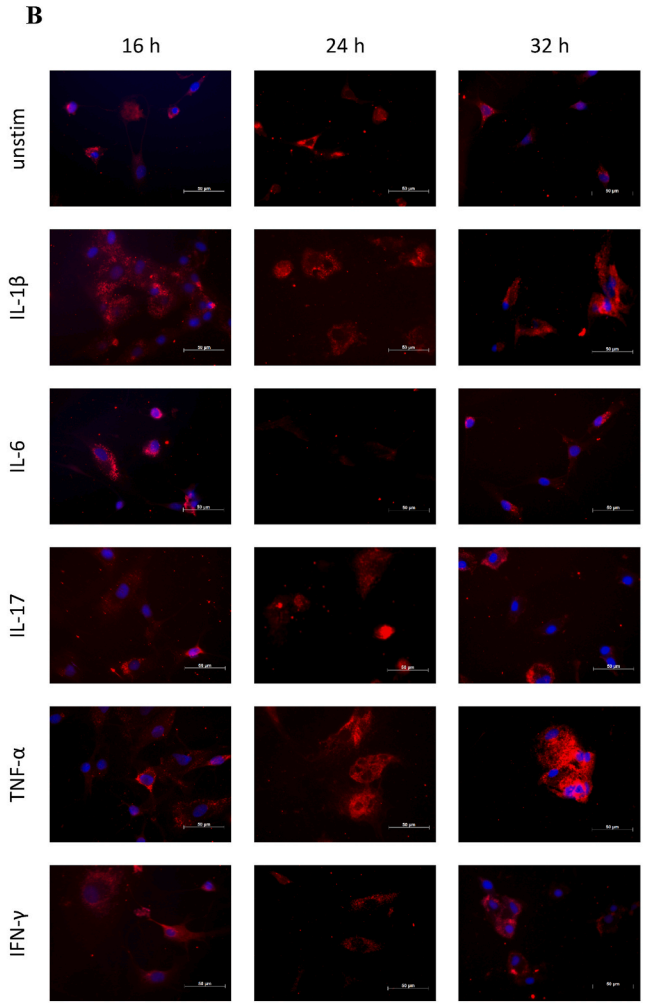


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Fig. 1. The expressions of Bmal1 in RA-FLSs by cytokine stimulation.
(a) Relative mRNA expression levels of Bmal1 in RA-FLSs incubated with TNF-α (0, 20 ng/mL), IL-1β (0, 20 ng/mL), IL-6 (0, 20 ng/mL), IL-17 (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 0, 8, 16, 24, 32 h. Each value was represented relative to unstimulated. ●(solid line), unstim; ○(dotted line), cytokine stimulation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *v.s.* Control, n = 6, means ± standard error of the mean (SEM).
(b) Immunofluorescence images of Bmal1 on RA-FLSs incubated with TNF-α (0, 20 ng/mL), IL-1β (0, 20 ng/mL), IL-6 (0, 20 ng/mL), IL-17 (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 16, 24, 32 h (400 ×, scale bar = 50 μm).

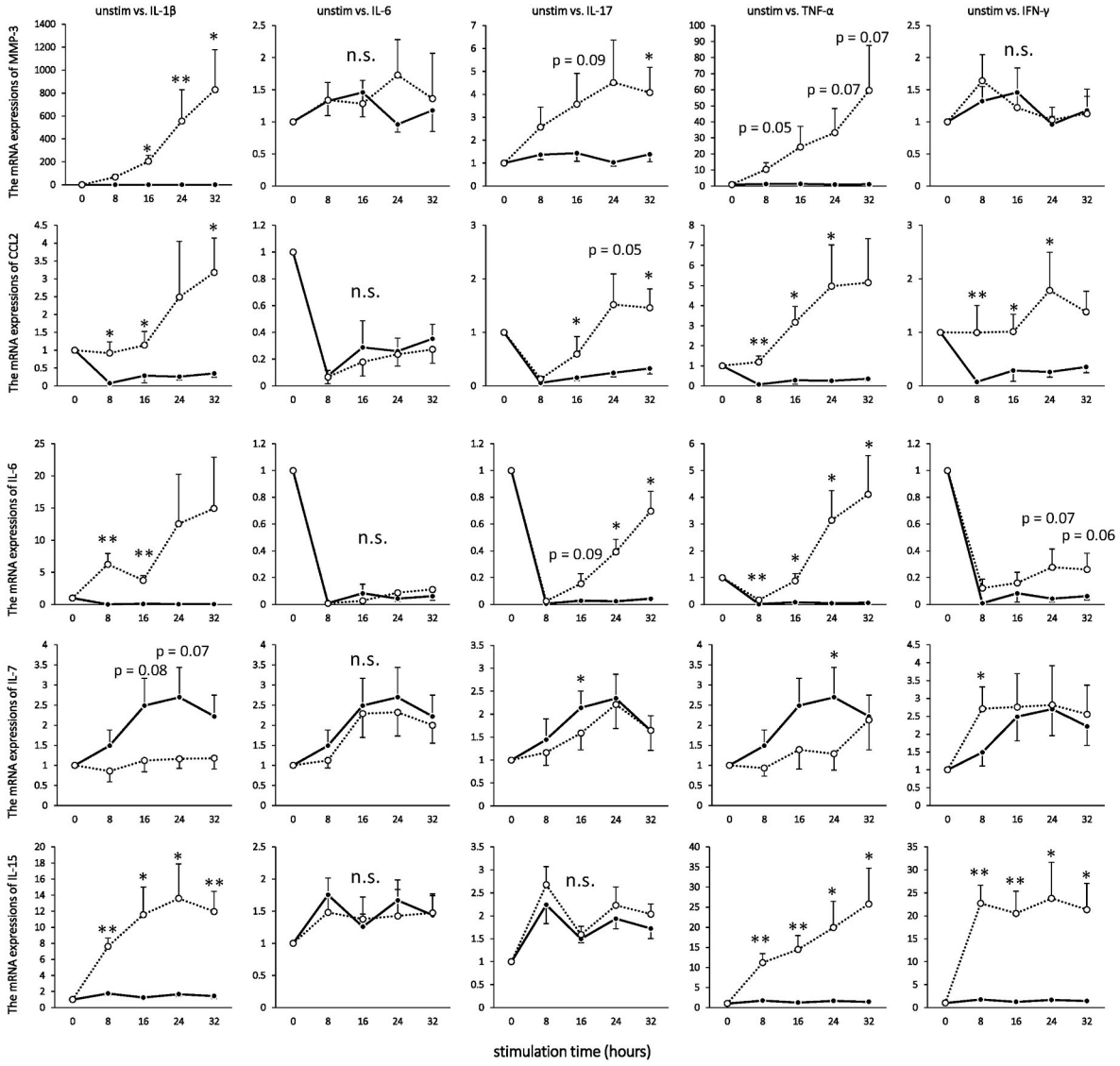

Fig. 2. Relative mRNA expression of MMP-3, CCL2, IL-6/7/15 in RA-FLSs incubated with TNF-α (20 ng/mL), IL-1β (20 ng/mL), IL-6 (0, 20 ng/mL), IL-17 (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 0, 8, 16, 24, 32 h. Each value was represented relative to unstimulated. ●(solid line), unstim; ○(dotted line), cytokine stimulation. *$p < 0.05$, *v.s.* Control, n = 6, means ± SEM.

Holm correction. $p < 0.05$ was considered to be significant.

3. Results

3.1. mRNA expression of Bmal1 in RA-FLSs was increased by cytokine stimulations

First, we analyzed the mRNA expressions of the clock genes in RA-FLSs after IL-1β, IL-6, IL-17, TNF-α or IFN-γ stimulations by qPCR. The expression of Bmal1 was significantly increased by IL-1β, TNF-α and IFN-γ (Fig. 1A). Furthermore, the protein expression of BMALl1 in RA-FLS was significantly increased after IL-1β, TNF-α and IFN-γ stimulations (Fig. 1B).

3.2. mRNA expressions of inflammatory mediators in RA-FLSs were increased by cytokines

Secondly, we analyzed the mRNA expressions of MMP-3, CCL2, IL-6, IL-7, IL-15 in RA-FLSs after IL-1β, IL-6, IL-17, TNF-α or IFN-γ stimulations by qPCR. As shown in Fig. 2, the expression of MMP-3 was significantly upregulated by IL-1β, IL-17 and TNF-α. The expression of CCL2 was significantly upregulated by IL-1β, TNF-α and IFN-γ. The expression of IL-6 was significantly upregulated by IL-1β, IL-17 and TNF-α. The expression of IL-15 was significantly upregulated by IL-1β, TNF-α and IFN-γ, respectively. In contrast, IL-7 was downregulated by IL-1β, IL-17 and TNF-α.

Based on these results that the expression of Bmal1 and several

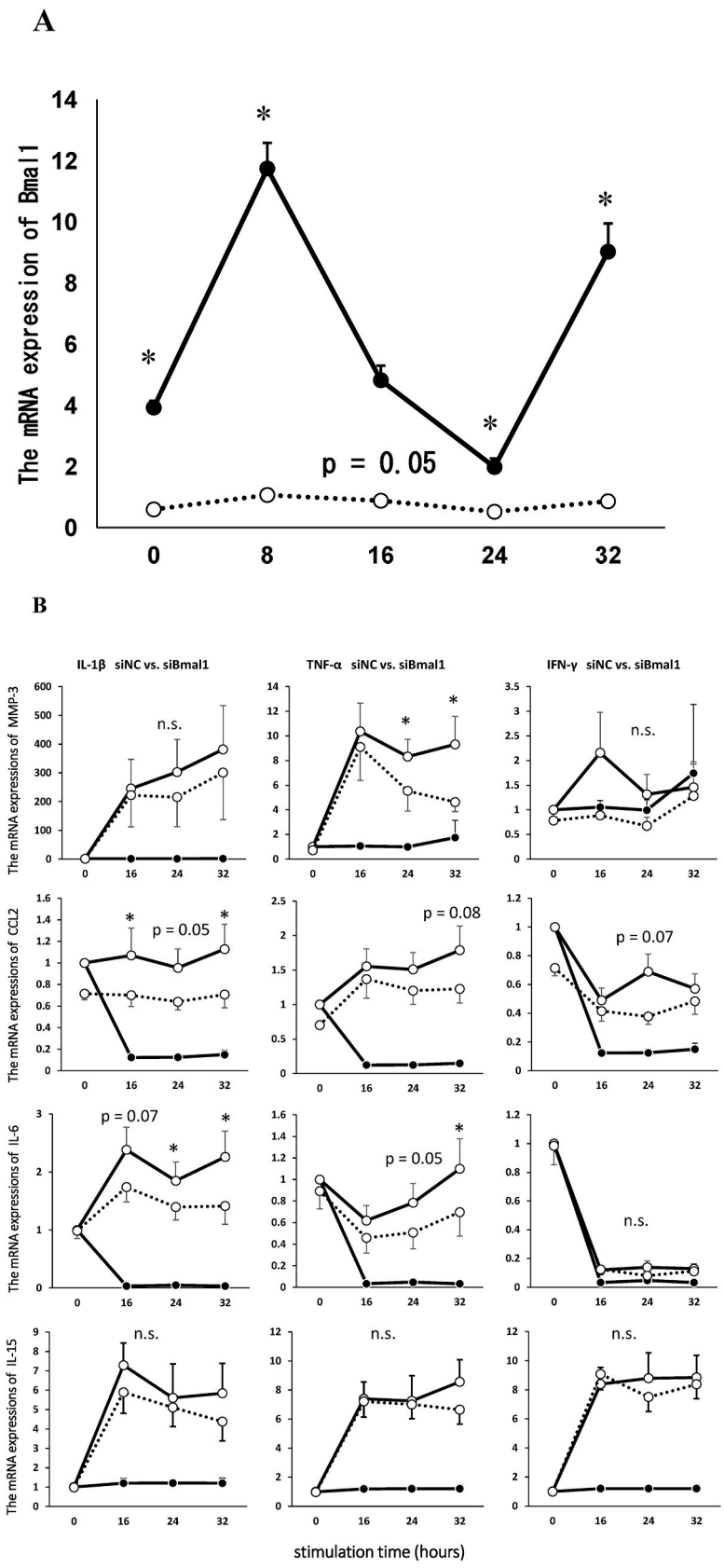

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Fig. 3. The expressions of inflammatory mediators under silencing *Bmal1* in RA-FLSs.
(A) The effect of siBmal1. Each value was represented relative to siNC. *p < 0.05, v.s. siNC, n = 3, means ± SEM. ●(solid line), siNC; ○(dotted line), siBmal1.
(B) Relative mRNA expression of MMP-3, CCL2, IL-6/7/15. RA-FLSs were transfected with siBmal1 and incubated with TNF-α (0, 20 ng/mL), IL-1β (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 0, 16, 24, 32 h. Each value was represented relative to siNC. RA-FLSs were transfected with siBmal1 and incubated with sIL-6R (100 ng/mL) and with TNF-α (0, 20 ng/mL), IL-1β (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 0, 16, 24, 32 h. Each value was represented relative to siNC. *p < 0.05, *v.s.* Control, means ± SEM [n = 7 (*MMP-3*), n = 8 (*CCL2*, *IL-6* and *IL-15*)]. ●(solid line), unstim + siNC; ○(solid line), TNF-α + siNC; ○(dotted line), TNF-α + siBmal1.

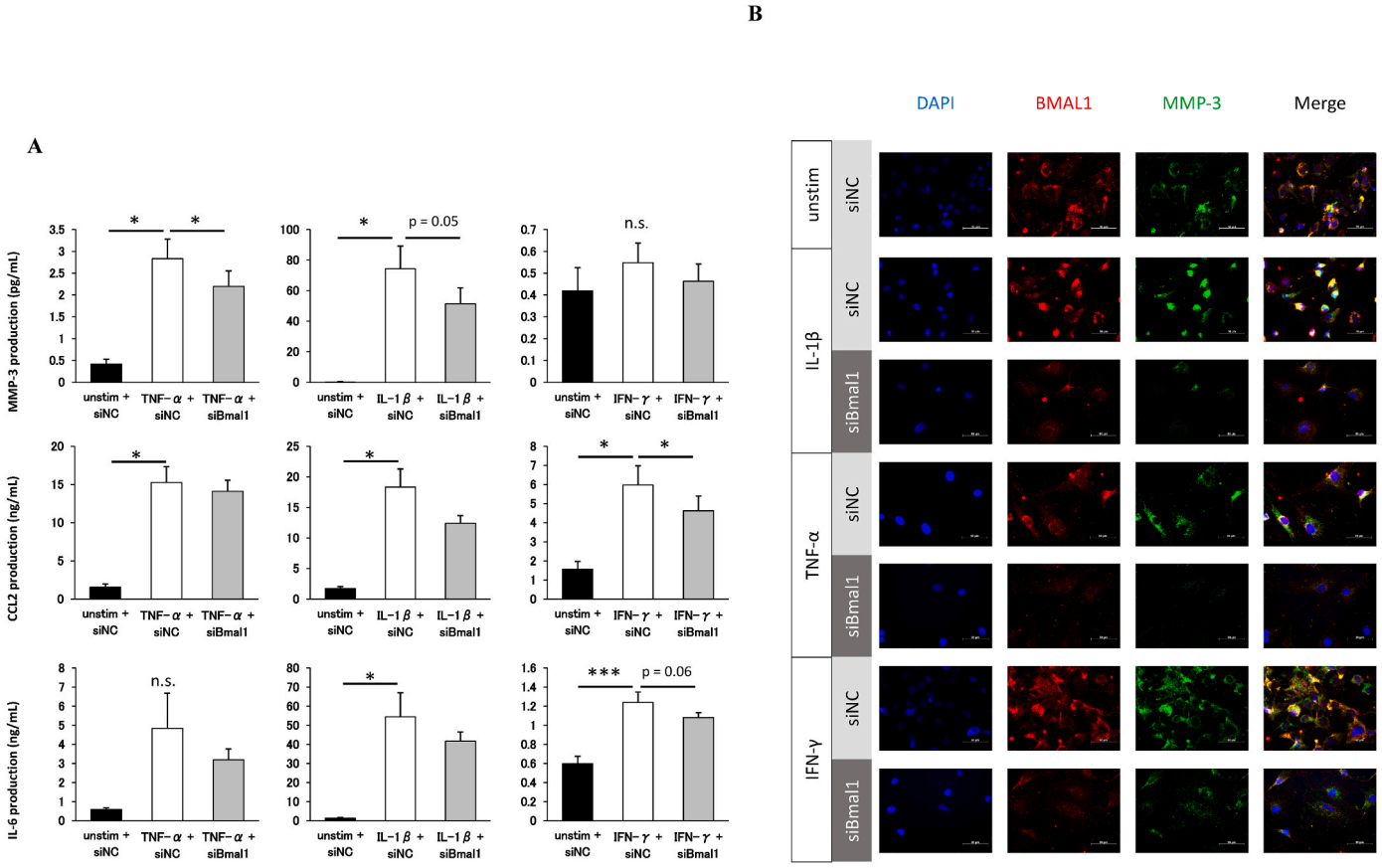

Fig. 4. The productions and the localizations of MMP-3, CCL2 and IL-6 under silencing Bmal1 expression in RA-FLSs.
(A) Concentrations of MMP-3, CCL2 and IL-6 in culture supernatant of RA-FLSs transfected with siBmal1. Cells were incubated with IL-1β (0, 20 ng/mL), TNF-α (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 32 h. Each concentration was represented relative to siNC. *p < 0.05, *v.s.* control, means ± SEM [n = 4 (MMP-3), n = 5 (CCL2, IL-6)]. Black, unstim + siNC; White, cytokine + siNC; Gray, cytokine + siBmal1. **(B)** Immune double staining of Bmal1 (red) and MMP-3, CCL2, IL-6 (green) with DAPI (blue). RA-FLSs, transfected with siBmal1, were incubated with IL-1β (0, 20 ng/mL), TNF-α (0, 20 ng/mL) or IFN-γ (0, 20 ng/mL) for 32 h (400 ×, scale bar = 50 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inflammatory mediators were upregulated by inflammatory cytokines, we next investigated the relationship between Bmal1 and inflammatory mediators such as MMP-3, CCL2, IL-6, and IL-15 using siRNA.

3.3. mRNA expressions of inflammatory mediators in RA-FLSs were decreased by Bmal1 silencing

Prior to the siRNA approach, we examined a knockdown efficiency of siBmal1 by qPCR. RA-FLSs were treated with siNC or siBmal1 for 48 h before RNA extractions. Each of total RNA was extracted every 8 hhours until 32 h. The mRNA expressions of Bmal1 treated with siBmal1 were decreased by approximately 85 % (0 h), 91 % (8 h), 82 % (16 h), 74 % (24 h) and 90 % (32 h) (Fig. 3A).

As shown in Fig. 3B, the expression of MMP-3 by TNF-α was significantly decreased by siBmal1. The expression of CCL2 by IL-1β was significantly decreased by siBmal1. The expression of IL-6 by IL-1β and TNF-α was significantly decreased by siBmal1. The mRNA expression of IL-15 did not decrease by siBmal1.

3.4. Protein expressions of inflammatory mediators in RA-FLSs were decreased by Bmal1 silencing

Finally, we investigated the involvement of Bmal1 in the productions of MMP-3, CCL2 and IL-6 proteins by ELISA. As shown in Fig. 4A, TNF-α-induced MMP-3 production was significantly decreased by siBmal1. IFN-γ-induced CCL2 production was significantly decreased by siBmal1. Both IL-1β-induced MMP-3 production and IFN-γ-induced IL-6 production appeared to decrease by siBmal1 (p = 0.05 and p = 0.06, respectively). In addition, we also analyzed the expression pattern of BMAL1 and MMP-3, CCL2, IL-6 in RA-FLS by immunofluorescence staining, and found that siBmal1 suppressed BMAL1 expressions under all cytokine stimuli. Further, MMP-3, CCL2, and IL-6 expressions were also abolished along with BMAL1, in all observed cells (Fig. 4B).

4. Discussion

We found that Bmal1 controlled the mRNA and protein expressions

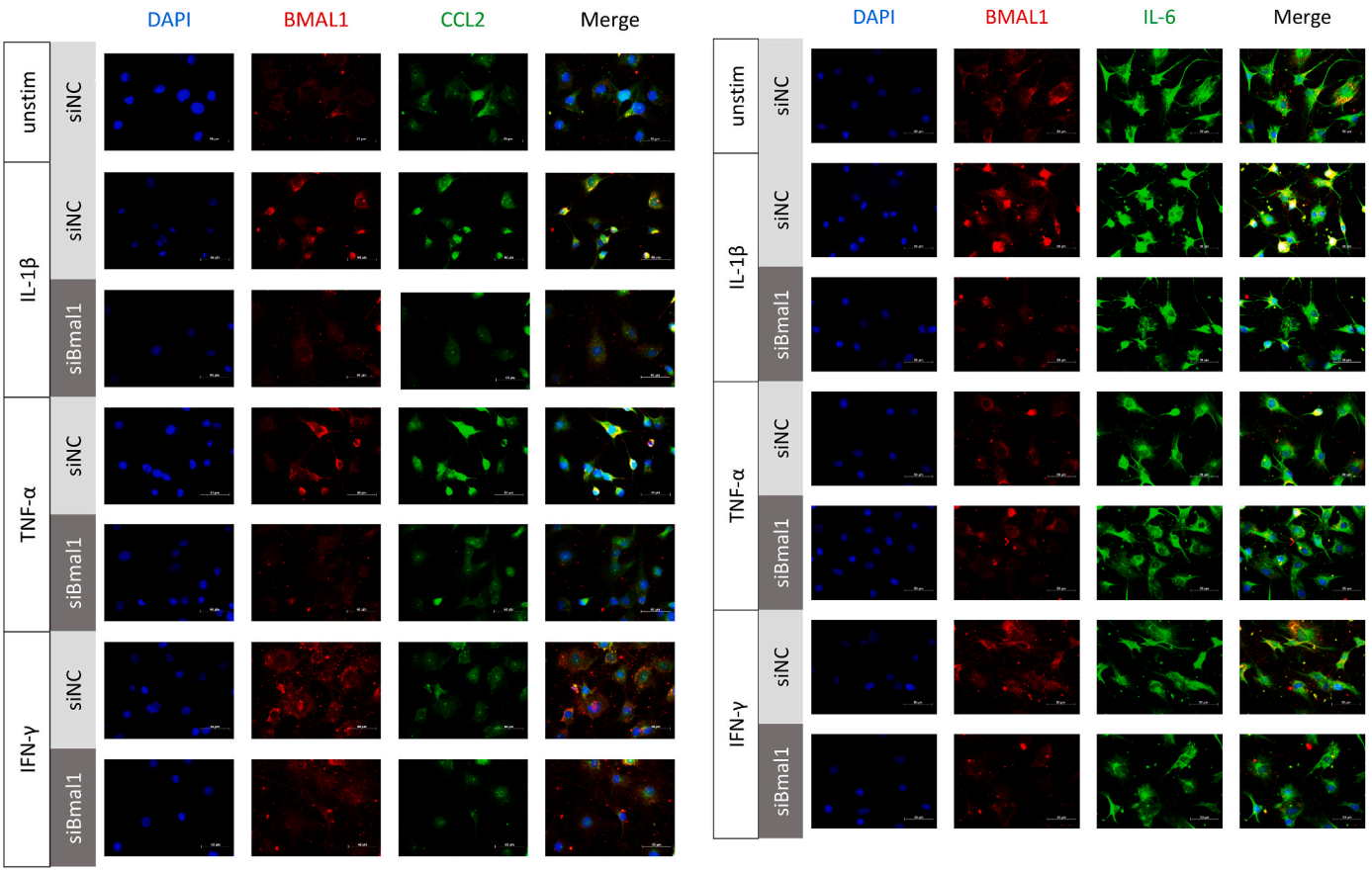

Fig. 4. (*continued*).

of MMP-3, CCL2, IL-6 except for IL-15 in RA-FLS under IL-1β, TNF-α and IFN-γ stimulation.

Since MMP-3, CCL2 and IL-6 are downstream target genes of NF-κB which is a key transcription factor of RA-FLSs [31] and IL-1β, TNF-α, IFN-γ promotes NF-κB transcription activity [32,33], thus it appears that Bmal1 regulate MMP-3, CCL2 and IL-6 by activating the NF-κB signaling pathway in RA-FLS. It has been reported that changes in Bmal expression cause NF-κB-mediated transformation of cellular traits [34,35]. For example, U87MG glioblastoma cells acquired a highly migrative character by silencing Bmal1, which results in upregulation of both AKT phosphorylation and MMP-9 expression, a group of downstream molecules of NF-κB. On the other hand, overexpression of Bmal1 in breast cancer cells enhances cellular migration to further increase their invasive potential. Interestingly, suppression of Bmal1 activated molecules located downstream of NF-κB in glioblastoma cells, whereas in breast cancer cells, enhanced expression of Bmal1 increased mRNA and protein expression of MMP-9, a downstream target gene of NF-κB.

In contrast, reports using biological organ materials from Bmal1 deficient mice expressed different aspects. FLSs isolated from Col6a1-Bmal1−/− mice highly product IL-6 and CCL2 by TNF-α stimulation, while suppress the productions of anti-inflammatory cytokines including IL-10 and IL-13 compared with wild-type mice [36]. Moreover, expressions of MMP-1a/-9/-13/-14, CCL2/28, CCR2 and IL-1β were reported to increase in the myocardium of cardiomyocyte-specific Bmal1 knockout [37]. Taken together, some reasonable differences may exist between the clock genes network controlled within an individual single cell and the function of clock genes affected by the interaction of various biological environments in vivo.

As described in the introduction, IL-7 expression in RA-FLS was reported to increase in RA-FLS under IL-1β and TNF-α stimulation [27], which is not consistent with our result. This discrepancy might results from differences of culture condition and culture time, and a future challenge is to reveal the mechanism of IL-7 expression.

In this study, we revealed a novel role of Bmal1 in producing inflammatory mediators responsible for the pathogenesis of RA. Further study and innovation are required to reveal more detailed features of Bmal1, especially in vivo, to develop a novel therapeutic strategy for RA by targeting Bmal1.

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CRediT authorship contribution statement

Kenta Kaneshiro: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – original draft. **Kanako Nakagawa:** Data curation. **Hikari Tsukamoto:** Data curation. **Genta Matsuoka:** Data curation. **Seitaro Okuno:** Data curation. **Koji Tateishi:** Resources. **Yasuhiro Terashima:** Resources. **Nao Shibanuma:** Resources. **Kohsuke Yoshida:** Data curation, Supervision. **Akira Hashiramoto:** Supervision, Writing – review & editing.

Declaration of competing interest

There is no conflict of interests regarding the publication of this article.

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