Interleukin-6 and tumour necrosis factor-α cooperatively promote cell cycle regulators and proliferate rheumatoid arthritis fibroblast-like synovial cells

Kaneshiro, Kenta ; Sakai, Yoshitada ; Suzuki, Kohjin ; Uchida, Koto ; Tateishi, Koji ; Terashima, Yasuhiro ; Kawasaki, Yoshiko ; Shibanuma,…

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IL-6 and TNF-α cooperatively promote cell cycle regulators and proliferate Rheumatoid Arthritis fibroblast-like synovial cell.

Kenta Kaneshiro[1], Yoshitada Sakai[2], Kohjin Suzuki[1], Koto Uchida[1], Koji Tateishi[3], Yasuhiro Terashima[3], Yoshiko Kawasaki[4], Nao Shibanuma[5], Kohsuke Yoshida[1] and Akira Hashiramoto[1].

[1] Department of Biophysics, Kobe University Graduate School of Health Sciences, Kobe, Japan

[2] Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

[3] Department of Orthopedics, Kohnan Kakogawa Hospital, Kakogawa, Japan

[4] Department of Rheumatology, Kobe Kaisei Hospital, Kobe, Japan

[5] Department of Orthopedic Surgery, Kobe Kaisei Hospital, Kobe, Japan

Running head: IL-6 and TNF-α proliferate of RA-FLS

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Corresponding Author

Akira Hashiramoto,

Postal address: 654-0142

E-mail: hash@ med.kobe-u.ac.jp

Phone: +81-78-796-4544

Abstract

To elucidate the roles of IL-6 and TNF-α in cell cycle regulation and proliferation of rheumatoid arthritis fibroblast-like synovial cell (RA-FLS).

Under stimulation of IL-6/soluble IL-6 receptor (sIL-6R) and TNF-α, we examined the expression of cell cycle regulators ($p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$, CDK4, CDK6, Cyclin D, Cyclin E, pRB) by qPCR, western blotting and immunofluorescence staining. With or without 10% of FBS, the expression of pRB was examined by western blotting. DNA synthesis and cellular viabilities were examined by BrdU assay and WST-8 assay, respectively. After transfected with siRNA/$p16^{INK4a}$, siRNA/$p21^{Cip1}$, siRNA/$p27^{Kip1}$, siRNA/*CDK4* or siRNA/*CDK6*, RA-FLS was successively stimulated with or without IL-6/sIL-6R or TNF-α to determine the cellular viabilities.

IL-6/sIL-6R significantly decreased the expression of $p16^{INK4a}$, whereas increased $p21^{Cip1}$, *Cycin E1*, CYCLIN D and pRB. TNF-α decreased the expression of *CDK4*, whereas significantly increased the expressions of $p27^{Kip1}$, *CDK6*, *Cyclin E1/E2*, CYCLIN D, CYCLIN E, pRB and phosphorylared pRB (phospho-pRB). By immunofluorescence staining, CYCLIN D and phospho-pRB were simultaneously stained in the single cell. In serum free culture, the expression of pRB was apparently decreased. DNA synthesis and cellular viabilities were significantly increased by IL-6/sIL-6R and TNF-α. Silencing of CDK6 attenuated the cellular viabilities induced by IL-6 and TNF-α.

Results indicate that IL-6 and TNF-α interact with each other in regulating the cell cycle and accelerate the proliferation of RA-FLS.

Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory disease characterized by a tumor-like synovial overgrowth, leading to joint destructions (1). Cytokines are known to be pathogenic factors that are the primary target for current RA treatment, indeed, pro-inflammatory cytokines IL-6 and TNF-α are elevated in sera and synovial fluid of RA patients (2, 3).

IL-6 possesses pleiotropic functions associated with RA. Bindings of IL-6 to two types of IL-6 receptor (IL-6R), such as membrane bound IL-6R (mbIL-6R) and soluble IL-6R (sIL-6R), trigger IL-6 signals. Either soluble or membrane-bound, the IL-6/IL-6R complex induces the formation of a gp130 homodimer to promote an intracellular signaling cascade (4-6). The IL-6/IL-6R complex induces C-reactive protein, serum amyloid A, hepcidin and fibrinogen in hepatocyte. Besides, IL-6 act as a activator for differentiation of naïve $CD4^+$ T cell into Th17 with transforming growth factor-beta, differentiation of progenitor cell into osteoclast and production of matrix metalloproteinases (MMPs) with IL-1 in of RA fibroblast-like synoviocyte (FLS) (PMID: 24524085, PMID: 22870451). Moreover, IL-6 promotes RA-FLS to produce vascular endothelial growth factor and facilitates angiogenesis under co-culture with human umbilical vein endothelial cells (PMID: 19277666). Eventually, these functions of IL-6 results in development of pannus formation. Therefore, tocilizumab binds to IL-6R, blocks IL-6 signaling and thus neutralizes IL-6-associated symptoms in RA therapy (PMID 29725131).

On the other hand, binding of TNF-α and TNF receptor (TNFR) induces IL-6, MMPs, intracellular adhesion molecule, vascular cell adhesion molecule and proliferation of RA –FLS by activating NF-κB (7). As is well known, two subtypes of TNFR such as TNFR1 (p55) and TNFR2 (p75) have a characteristic difference; TNFR1 has death domain, while TNFR2 doesn't (8-10). In addition, TNF-α increases proliferation of macrophages and B-cells, which results in the infiltration of inflammatory cells and RA-FLS into synovium ( ). Thus, TNF inhibitors binds to TNF and neutralize the TNF-associated symptoms in RA.

In case with synovial fluid of RA patients, higher amounts of both sIL-6R and TNF-α exist as compared to those of osteoarthritis (11, 12). Although TNF-α promotes synovial

cell proliferation (7, 13), details of cell growth action, induced by IL-6, have not yet reached a unified view (13, 14). Thus, in this study, we newly examined the effects of IL-6 and TNF-α on cell cycle regulators and reviewed the mechanism of synovial cell proliferation.

The cell cycle is a continuous rotation of series of interphases including G1, S, G2 and M phase to influence DNA replication and mitosis (15). The cell cycle regulators are mainly consisted by Cyclins, Cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs) and Retinoblastoma protein (pRB) (16). CDKIs, intracellular proteins binding to CDKs, contain two families including INK4 family and Cip/Kip family which inhibit the activities of CDKs (17). CYCLIN D binds to activate CDK4/6 with $p21^{Cip1}$/$p27^{Kip1}$, while CYCLIN E binds to activate CDK2. On the other hand, $p16^{INK4a}$ binds to inhibit the activities of CDK4/6, and $p21^{Cip1}$/$p27^{Kip1}$ bind to inhibit the activities of CDK2 (17). Then, activated CDKs phosphorylate serine 795 residue of pRB, resulting in releasing pRB from E2F to express *Cyclin E* gene (18-20). Finally, CYCLIN E/CDK2 complexes drive RA-FLS into S phase entry, leading to DNA synthesis and proliferation (21).

The cell cycle regulators are mandatory factors which controls cell division and proliferation of RA-FLS (22). In the presenting study, we elucidated the crucial mechanism of vigorous and active overgrowth of synovium in relation to cell cycle regulators and pro-inflammatory cytokines.

Material and Methods

Cell Culture

Synovial tissues were obtained during joint surgery from patients with RA fulfilling the American College of Rheumatology (ACR) classification criteria (23) (Supplemental Table I). Tissues were minced and stirred with 2 mg/mL collagenase (Wako, Tokyo, Japan) in serum-free DMEM (Nissui, Tokyo, Japan) at 37℃ for 1 hour. Primary cultured RA-FLS were established and maintained in DMEM supplemented with 10% heat-inactivated FBS (GE Healthcare, Little Chalfont, UK), 2mM L-glutamine (Life Technologies, Carlsbad, CA, USA) and 1% penicillin–streptomycin (Life Technologies) at 37℃. RA-FLS of passages 3–6 were used in the experiment.

The study was approved by the ethics committee of Kobe University (approval #579) and Kobe Kaisei Hospital (approval #0072), and written informed consent was obtained from each patient before study enrolment, according to the Declaration of Helsinki.

Antibodies and Agents

Anti-Cyclin D antibodies (#06-137) were obtained from Merck Millipore (Burlington, MA, USA). Anti-Cyclin E antibodies (sc-198), anti-actin antibodies (sc-1616), anti-Histone H2B antibodies (sc-10808) were obtained from Santa cruz (Dallas, TX, USA). Anti-pRB antibodies (ab181616) were obtained from Abcam (Cambridge, UK). Anti-phoshpho-pRB (Ser795) antibodies (R6878 and #9301) were obtained from Sigma Aldrich (St. Louis, MO, USA) and Cell signaling technology (Danvers, MA, USA). Anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific F(ab')$_2$ fragment (NA9340) and anti-mouse IgG, Horseradish Peroxidase-Linked Species-Specific F(ab')$_2$ fragment (NA9310) were obtained from GE Healthcare.

Alexa Fluor 594 conjugated anti-mouse IgG antibodies (#8890) and Alexa Fluor 488 conjugated anti-rabbit antibodies (#4412) were Cell signaling technology. IL-6 and sIL-6R were from Peprotech (Rocky Hill, NJ, USA), TNF-α from R&D systems (McKinley, NE, USA).

RNA extraction, Reverse transcription and Quantitative polymerase chain reaction (qPCR)

RA-FLS were cultured (8.0×10^4 cells/well) with or without IL-6 (100ng/ml)/sIL-6R

(100ng/ml) or TNF-α (10ng/ml) in 6 well Microplate (IWAKI, Shizuoka, Japan) for 0~32hours or 72 hours. Total RNA was extracted by RNeasy Mini Kit and QIAshredder (Qiagen, Hilden, Germany). Reverse transcription was performed with ReverTraAce ® (TOYOBO, Osaka, Japan). qPCR was performed and analyzed on StepOnePlus Real-Time PCRSystems (Applied Biosystems, Foster City, CA, USA). The TaqMan probes were: Hs00427621_m1 for TATA box binding protein (*Tbp*), Hs00923894_m1 for *CDKN2A*, Hs00355782_m1 for *CDKN1A*, Hs01597588_m1 for *CDKN1B*, Hs00364847_m1 for *CDK4*, Hs01026371_m1 for *CDK6*, Hs01026536_m1 for *CCNE1*, Hs00180319_m1 for *CCNE2*, respectively.

Western Blotting

RA-FLS were cultured (4.0×10^5 cells/well) with or without 10% FBS in 100mm/Tissue culture dish (IWAKI) for 48 hours. RA-FLS were cultured (4.0×10^5 cells/well) with or without IL-6/ sIL-6R (100ng/ml) or TNF-α (10ng/ml) in 100mm/Tissue culture dish for 48 or 72 hours. RA-FLS were lysed with RIPA buffer [50mmol/l Tris-HCl pH8.0, 150mmol/l, 0.5w/v% Na deoxycholate, 0.1w/v% sodium dodecyl sulfate, 1.0w/v% NP-40] and subjected to SDS-PAGE. Then, samples were transferred to PVDF membrane (Merck Millipore), probed with antibodies and developed by an immunostar® LD (ECL: Wako).

For separated collection of nuclear proteins, RA-FLS were cultured (1.2×10^6 cells/well) with or without IL-6/sIL-6R (100ng/ml) or TNF-α (10ng/ml) in 150mm/Tissue culture dish (IWAKI) for 48 hours and nuclear extracts were isolated using nuclear/cytosol fractionation kit (PromoKine, San Diego, CA, USA).

In Cell ELISA

RA-FLS were cultured (3.0×10^3 cells/well) with or without IL-6/sIL-6R or TNF-α for 48 or 72 hours, then protein expressions of CYCLIN D and CYCLIN E were measured by the In Cell ELISA Kit (BIOO Scientific, Cat. No. 3440-02, Austin, TX, USA).

Immunofluorescence staining

RA-FLS were cultured (6.0×10^3 cells/well) with or without IL-6/sIL-6R (100ng/ml) or TNF-α (10ng/ml) in Lab-Tek Chamber Slide w/Cover Glass Slide Sterile (Thermo fisher

scientific, Waltham, MA, USA) for 48 hours. RA-FLS were fixed with 4% Paraformaldehyde (Wako) for 15 min. and blocked by using Blocking One Histo (Nacalai tesque, Kyoto, Japan) for 1hour. Primary antibodies for CYCLIN D or phospho-pRB (Ser795) were incubated overnight at 4℃. Alexa Fluor594 and Alexa Fluor 488 conjugated antibodies were incubated for 1hour at room temperature. Then, DAPI staining (Sigma Aldrich) was performed for 5 minutes at room temperature.

Cell viability assay

RA-FLS were cultured (3.0×10^3 cells/well) with or without IL-6/sIL-6R (100ng/ml) or TNF-α (10ng/ml) in 96 well Microplate (IWAKI) for 72hours. The cell viabilities were measured by using Cell-counting kit 8 (Dojindo, Kumamoto, Japan) after 72hours' stimulation. DNA synthesis of the RA-FLS was determined by labeling DNA with BrdU for 24 hours after 48 hours' stimulation, according to the manufacturer's instructions (Cat. no. 1 647 229, Roche, Basel Switzerland). The results were expressed as percentage relative to unstimulated RA-FLS cultures.

RNA interference

Silencer® Select Negative Control Small Interfering (si) RNA (Cat. no. 4390843), siRNA/$p16^{INK4a}$ (s218), siRNA/$p21^{Cip1}$ (s417), siRNA/$p27^{Kip1}$ (s2837), siRNA/*CDK4* (s2822) and siRNA/*CDK6* (s53) were obtained from Thermo Fisher Scientific. After introducing siRNAs into RA-FLS for 48 hours using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies), RA-FLS were stimulated with or without IL-6/sIL-6R or TNF-α for 72 hours to measure cell viabilities using Cell Couting Kit-8.

Statistical Analysis

The Student's t-test and the Bonferroni correction were used to compare differences between two experimental groups, and Dunnett's test were used to compare differences between the control and others. All statistical tests were two-sided and $p < 0.05$ was considered statistically significant.

The statistical analyses were performed by EZR software, version 1.36, based on R and R commander (24).

Results

The expressions of CDKIs and CDK4/6 mRNA were modulated by IL-6/sIL-6R and TNF-α for 0~32 hours' stimulation.

Under stimulations of IL-6/sIL-6R or TNF-α for 0~32hours, we first examined time-dependent expressions of $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$, *CDK4* and *CDK6* mRNA in RA-FLS. As shown in Fig. 1, stimulations of IL-6/sIL-6R decreased expressions of $p16^{INK4a}$ at 16 ($p<0.05$), 24 ($p=0.06$) and 32 hours ($p<0.01$), while increased $p21^{Cip1}$ at 8 hours ($p<0.05$). Stimulations of TNF-α increased expressions of $p27^{Kip1}$ at 8 hours ($p<0.05$) and 24 hours ($p=0.09$). Although TNF-α significantly increased expressions of *CDK6* at 16, 24 and 32 hour ($p<0.05$), *CDK4* was decreased at 8 hours' stimulation by TNF-α ($p<0.05$).

The expression of CYCLIN D/pRB protein and the phosphorylation of pRB protein were induced by IL-6/sIL-6R or TNF-α for 48hours' stimulation.

We next studied the expressions of CYCLIN D/pRB and the phosphorylation of pRB in RA-FLS in the presence or absence of IL-6/sIL-6R or TNF-α for 48 hours.

In serum free condition, the inherent expression of pRB was decreased and thus shrinking features were observed in RA-FLS (Fig. 2A). The expression of pRB apparently increased by solo stimulation of TNF-α while it was slightly induced by IL-6/sIL-6R, and the combination of those significantly enhanced phosphorylation of pRB (Fig. 2B). IL-6/sIL-6R also effected slightly but significantly on the expression of CYCLIN D, and it was apparently significant by TNF-α (Fig. 2B and C). When we studied the expression of CYCLIN D and phospho-pRB by immunofluorescence staining, they were simultaneously expressed in each cell and IL-6/sIL-6R and TNF-α stimulation suppressed the shrinkage of RA-FLS (Fig. 2D).

The expressions of Cyclin E1/E2 mRNA, and CYCLIN E protein were modulated by IL-6/sIL-6R or TNF-α for 72hours' stimulation.

To further track the temporal flow of cell cycle progression, mRNA expressions of *Cyclin E1*/*E*2 were examined until 72 hours. As shown in Fig. 3A, *Cyclin E1* was significantly increased by both IL-6/sIL-6R and TNF-α. In case with *Cyclin E2*, solo stimulation of IL-6/sIL-6R did not affect the expression of *Cyclin E2* while TNF-α and co-stimulation of IL-6/sIL-6R and TNF-α did significantly increased that. Finally, the

entire protein expression of CYCLIN E was increased by TNF-α, and IL-6/sIL-6R apparently enhanced that (Fig. 3BC).

The cell viabilities of RA-FLS were increased by IL-6/sIL-6R and TNF-α stimulation for 72hours, which was canceled by siRNA/CDK6.

The incorporation of BrdU was significantly increased by TNF-α and co-stimulation of both IL-6/sIL-6R and TNF-α, and solo stimulation of IL-6/sIL-6R appeared to increase it about 7-fold as compared to unstimulated controls though not statistically significant (p=0.16) (Fig. 4A). Meanwhile, the cellular viabilities were increased by both of IL-6/sIL-6R and TNF-α (Fig. 4B).

We then examined effects of IL-6/sIL-6 and TNF-α on cellular viabilities under conditions of RNA interference using $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$, *CDK4* and *CDK6* siRNA.

By RNA interference, mRNA expressions of $p16^{INK4a}$, $p21^{Cip1}$, $p27^{Kip1}$, *CDK4*, *CDK6* were decreased (Supplemental Fig. 1). siRNA/$p21^{Cip1}$ significantly increased the cellular viabilities even in unstimulated conditions, and this was reproduced by IL-6/sIL-6R or TNF-α-stimulation. siRNA/*CDK4* increased the viabilities of unstimulated RA-FLS, however, siRNA/$p16^{INK4a}$ and siRNA/$p27^{Kip1}$ did not affect that. Under solo or co-stimulation of IL-6/sIL-6R and TNF-α, siRNA/*CDK6* attenuated the viabilities of RA-FLS (Fig. 5).

Discussion

Although serum levels of IL-6 and TNF-α positively correlated in RA (PMID 8457224), it has remained unclear how they cooperated to promote cell cycle mechanism, and we presented here that an aspect of the RA pathogenesis was expected to be formed by crosstalk of IL-6 and TNF-α, in terms of cell cycle progression and proliferation of RA-FLS.

At first, Cytokine-derived actions on CDKs/CDKIs were reported to express various features depending on cell types and their conditions. For example, TNF-α signaling induces $p27^{Kip1}$ expression in smooth muscle cells through FoxO1 (25), and IL-6 down-regulates $p16^{INK4a}$ expression in human breast stromal fibroblasts (26). Further, the knockdown of $p16^{INK4a}$ up-regulates proliferation of glioma cell (27), while $p21^{Cip1}$ inhibits proliferation in RA-FLS (28). With reference to the reported mechanisms described above, we confirmed in RA-FLS that IL-6 stimulations decreased expressions of $p16^{INK4a}$ but increased $p21^{Cip1}$, TNF-α increased $p27^{Kip1}$ and *CDK6* but decreased *CDK4*, and they synergistically induced CYCLIN D and pRB (Fig. 1 and 2). Interestingly, IL-6 and TNF-α independently initiated the mRNA expressions of *CDK6* and *CDKIs* (Fig. 1), and then they cooperated with each other for the induction of CYCLIN D and pRB in the latter phase of cell cycle regulation (Fig. 2). As reported, CDK inhibitor improves the arthritis score of CIA mice (29) and overexpression of Cyclin D induces proliferation in human glioblastoma cells (30). Since the lesser expression of pRB in G0 phase became higher in G0/G1 transition (31), in our observations, actions of pRB appeared to force RA-FLS to be pro-proliferative due to cytokine's stimulation.

The expression profile of *Cyclin E1/E2* mRNA at 72 hours was consistent with that of CYCLIN D protein (Fig. 3A). It should be noted that TNF-α act more strongly in inducing CYCLIN E to drive the cell cycle of RA-FLS into S phase (Fig. 3B and C). Previous report that knockdown of Cyclin E inhibited tumor development of mouse breast cancer was supportive to our observations (32). In consequence, DNA synthesis and proliferation of RA-FLS were regulated by both IL-6 and TNF-α, and it was dominantly controlled by TNF-α (Fig. 4). Finally, as shown Fig. 5, we revealed that cytokines-related proliferation of RA-FLS was invoked by the increased expression of *CDK6*; a coactivator and recruiter of NF-κB subunit p65 into nucleus (33).

Since RA-FLS was reported to be driven their 'tumor-like' proliferation by various

pathogenic factors including oncogenes, growth factors or transcriptional factors (34-37), we confirmed the relation between another human-derived cells and cytokine/cell cycle regulation in the same environments as this presenting study.

In Human embryonic kidney (HEK) 293 cells (JCRB9068, Lot: 10172008, NIBIOHN, Osaka, Japan), the expression of *CDK6* mRNA was decreased by IL-6/sIL-6R, while those of *CDK4* mRNA, CYCLIN D, pRB and phosphorylation of pRB were not modulated by IL-6/sIL-6R or TNF-α s(Supplemental Fig. 2A and B). The mRNA expression of *Cyclin E1* was increased by TNF-α and *Cyclin E2* was decreased by co-stimulation of IL-6/sIL-6R and TNF-α, while protein expression of CYCLIN E was changed by neither IL-6/sIL-6R nor TNF-α (Supplemental Fig. 2C). Finally, cellular viabilities was decreased by TNF-α and co-stimulation of IL-6/sIL-6R and TNF-α, whereas it was not changed by solo stimulation of IL-6/sIL-6R (Supplemental Fig. 2D). These results indicated that IL-6 and TNF-α might induce apoptosis but not proliferation of HEK293, unlike RA-FLS. Notably, major differences were the expressions of CDK6, Cyclin D, Cyclin E and pRB as shown in Fig. 1, 2, 3, 5 and Supplemental Fig. 2A, B, C.

Although RA-FLS contains both TNFR1 and TNFR2 (8), ‘death signal’ originated from TNFR1 is suppressed (38) and NF-κB signaling pathway is highly activated (7). Thus, RA-FLS expresses the vigorous proliferation and the resistance for apoptosis in its nature (39). In this point of view, excessive or dominant signal transduction, originated from TNF-α, highlights the unique character of RA-FLS, rather than those from IL-6 as shown in this study.

In summary, as shown in Fig. 6, our results show CDK6 and CDKIs are the initiation factors proceeding signal transductions due to pro-inflammatory cytokines in RA-FLS. These cell cycle regulators cooperatively affected the same target, CYCLIN D, at first, and the outcome of those were dominantly modulated by TNF-α in points of accelerating DNA synthesis and proliferation in RA-FLS, depending on CYCLIN D, pRB and CYCLIN E. Indeed, co-treatments of both cytokine and cell cycle inhibitor were more effective as compared to those of solo-treatments in CIA mice (29), although a single use of biologic cytokine inhibitor is permitted in the real clinical field. Moreover, our results and previous reports also imply that the phosphorylation of pRB is essential for proliferation of RA-FLS. Further study is required in inhibiting the phosphorylation of pRB as another therapeutic target with genomic editing.

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Conflict of interest

None

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Figure

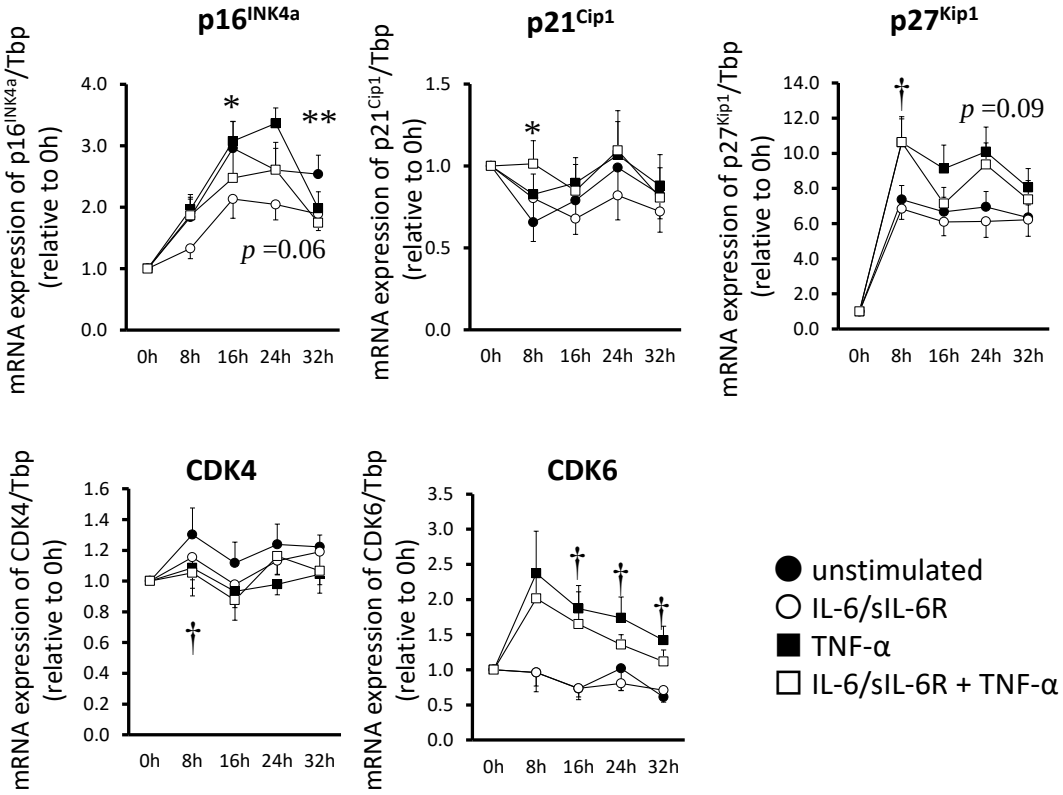


Figure 1

The mRNA expressions of CDKIs and CDKs under 0~32hours' stimulation of IL-6/sIL-6R and TNF-α. * stands for unstimulated *vs.* IL-6/sIL-6R. † stands for unstimulated *vs.* TNF-α. *p <0.05, **p <0.01, †p <0.05. The values shown are expressed as means ± SEM (n=7).

Stimulations of IL-6/sIL-6R decreased expressions of *p16^{INK4a}*, but increased those of *p21^{Cip1}*. TNF-α also increased *p27^{kip1}* and *CDK6*, whereas decreased *CDK4*.

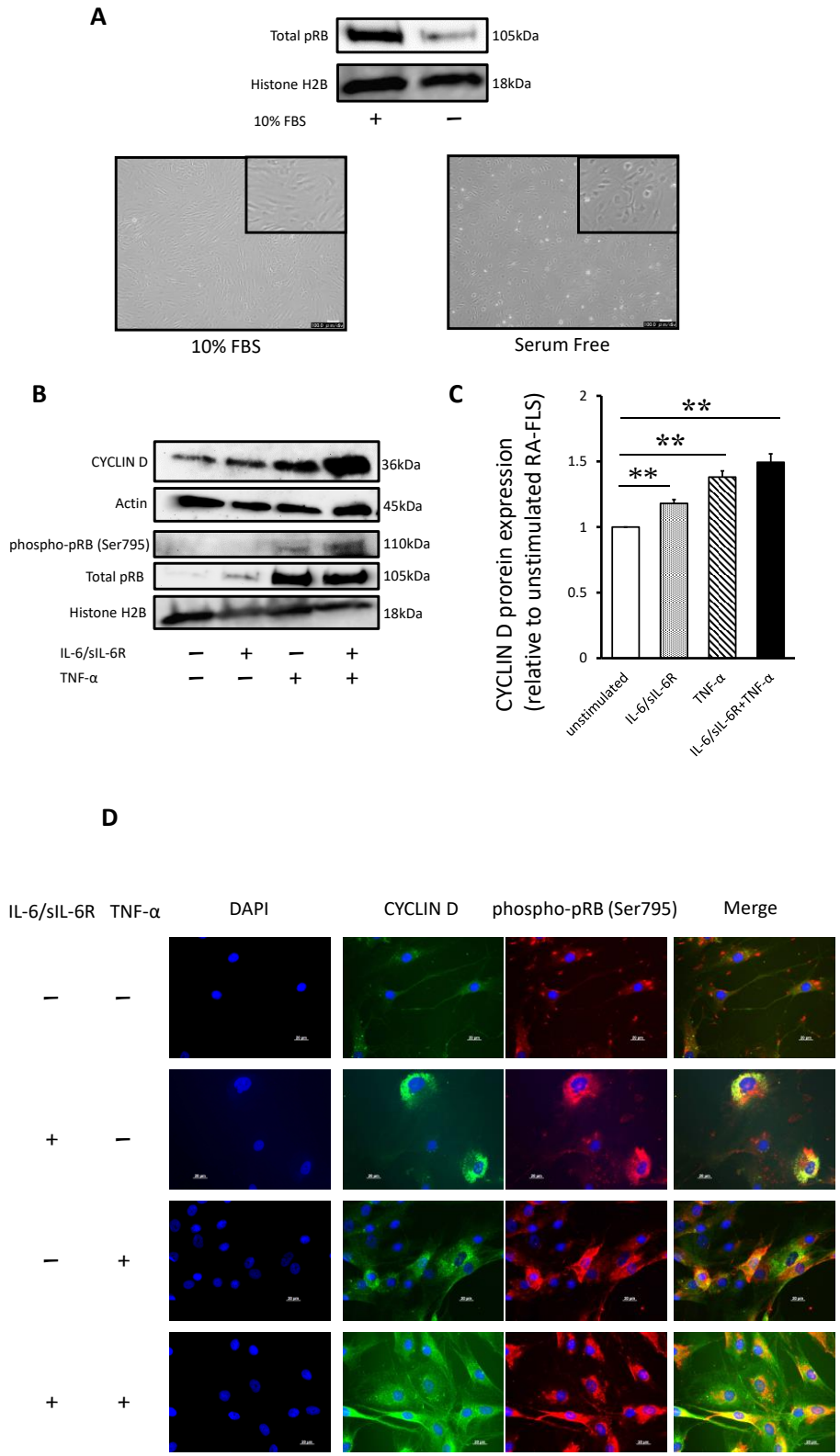

A
Total pRB
105kDa
Histone H2B
18kDa
10% FBS
+
−
10% FBS
Serum Free
B
CYCLIN D
36kDa
Actin
45kDa
phospho-pRB (Ser795)
110kDa
Total pRB
105kDa
Histone H2B
18kDa
IL-6/sIL-6R
TNF-α
C
CYCLIN D prorein expression
(relative to unstimulated RA-FLS)
unstimulated
IL-6/sIL-6R
TNF-α
IL-6/sIL-6R+TNF-α
D
IL-6/sIL-6R
TNF-α
DAPI
CYCLIN D
phospho-pRB (Ser795)
Merge

Figure 2

The protein expressions of CYCLIN D, pRB and phospho-pRB (Ser795) under 48hours' stimulation of IL-6/sIL-6R and TNF-α in western blotting, ELISA and immunocytochemistry. $^{**}p < 0.01$. The values shown are expressed as means ± SEM (n=7).

(A) The protein expression of pRB and the morphological change in RA-FLS with or without FBS. In serum free medium, protein expression of pRB were decreased and shrinking features of RA-FLS were observed.

(B) The protein expressions of CYCLIN D and pRB for 48 hours' stimulation. IL-6/sIL-6R slightly up-regulated CYCLIN D expression and TNF-α highly induced that, while co-stimulation of those significantly up-regulated that as compared to sole stimulation of TNF-α or IL-6/sIL-6R. The phosphorylation of pRB was increased by TNF-α and co-stimulation of IL-6/sIL-6R and TNF-α, as compared to sole stimulation of IL-6/sIL-6R.

(C) The In Cell ELISA analysis of CYCLIN D.

(D) Immunocytochemistry of CYCLIN D and phospho-pRB (Ser795), and morphological changes of RA-FLS. Either IL-6/sIL-6R or TNF-α solely induced CYCLIN D expressions and phospho-pRB in nuclei, and synergistic effects of those were observed in co-stimulation of L-6/sIL-6R and TNF-α. CYCLIN D and phospho-pRB (Ser795) were simultaneously stained in the single cell. To compare with unstimulated RA-FLS, IL-6/sIL-6R and TNF-α stimulation suppressed the shrinkage of cells. Magnification is ×400.

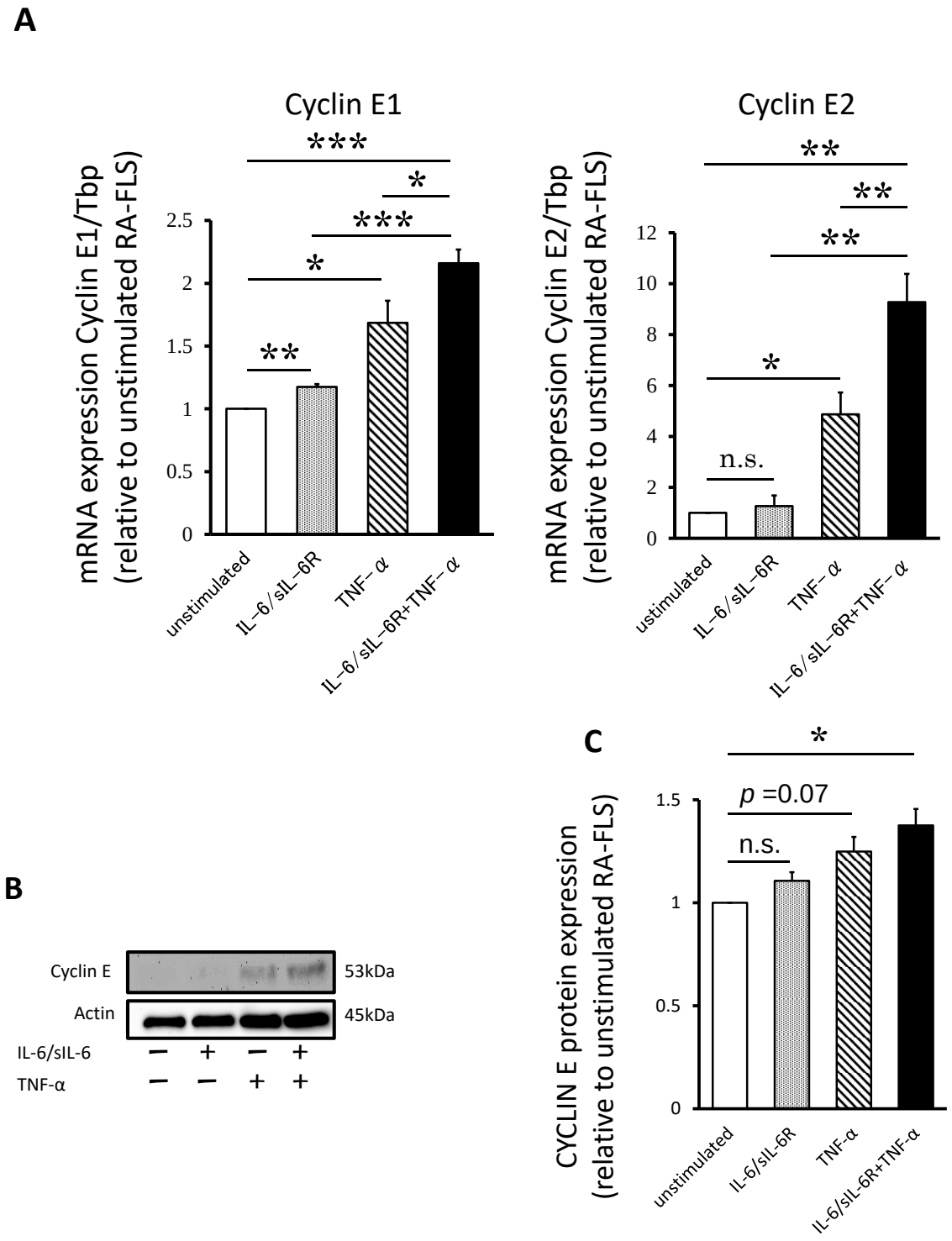


Figure 3

qPCR, western blotting and In Cell ELISA analysis of CYCLIN E under 72 hours' stimulation of IL-6/sIL-6R and TNF-α *p<0.05, **p <0.01, ***p<0.001. The values

shown are expressed as means ± SEM (n=7).

(A) IL-6/sIL-6R increased the mRNA expression of *Cyclin E1*, whereas no changes were observed for *Cyclin E2*. TNF-α showed significant up-regulation on the mRNA expressions of *Cyclin E1*/*E2*, as well as co-stimulation of IL-6/sIL-6R and TNF-α.

(B) The protein expression of CYCLIN E was increased by TNF-α and co-stimulation of IL-6/sIL-6R and TNF-α.

(C) The In Cell ELISA analysis of CYCLIN E. CYCLIN E was significantly increased by co-stimulation of IL-6/sIL-6R and TNF-α (p<0.05), and solo-stimulation of TNF-α also increased CYCLIN E though not statistically significant (p=0.07).

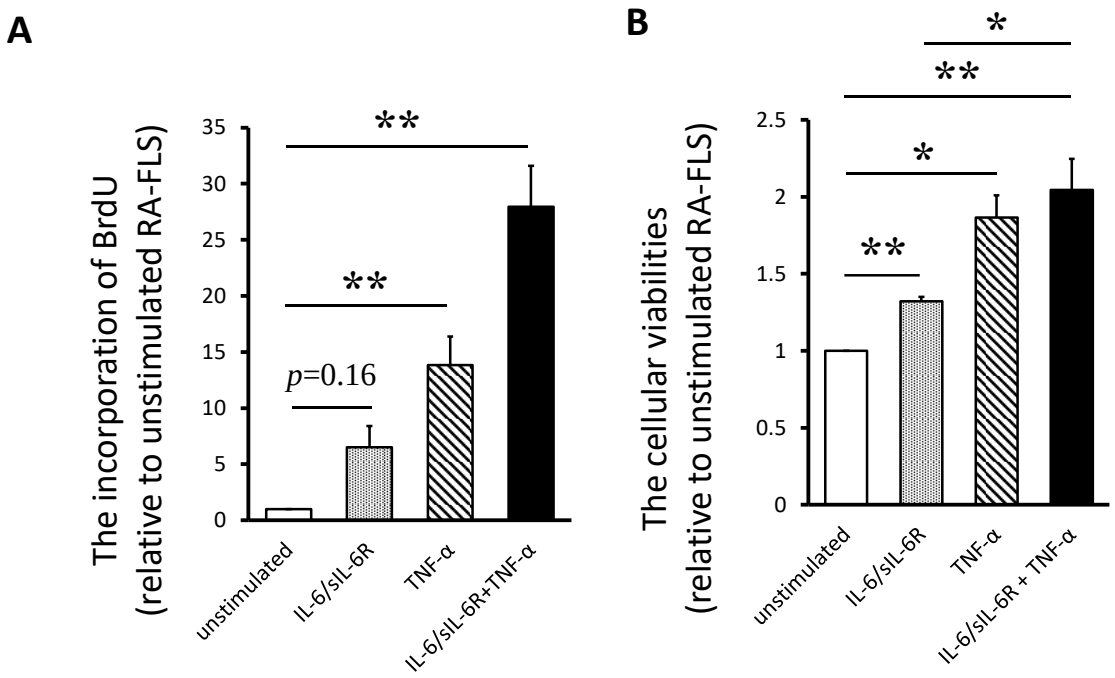


Figure 4

The cellular viabilities of RA-FLS stimulated with or without IL-6/sIL-6R or TNF-α for 72 hours in BrdU assay and WST-8 assay. *p<0.05, **p <0.01. The values shown are expressed as means ± SEM (n=7).

(A) The incorporation of BrdU was increased by TNF-α and co-stimulation of IL-6/sIL-6R and TNF-α.

(B) The cellular viabilities were significantly increased by IL-6/sIL-6R, TNF-α and co-stimulation of those.

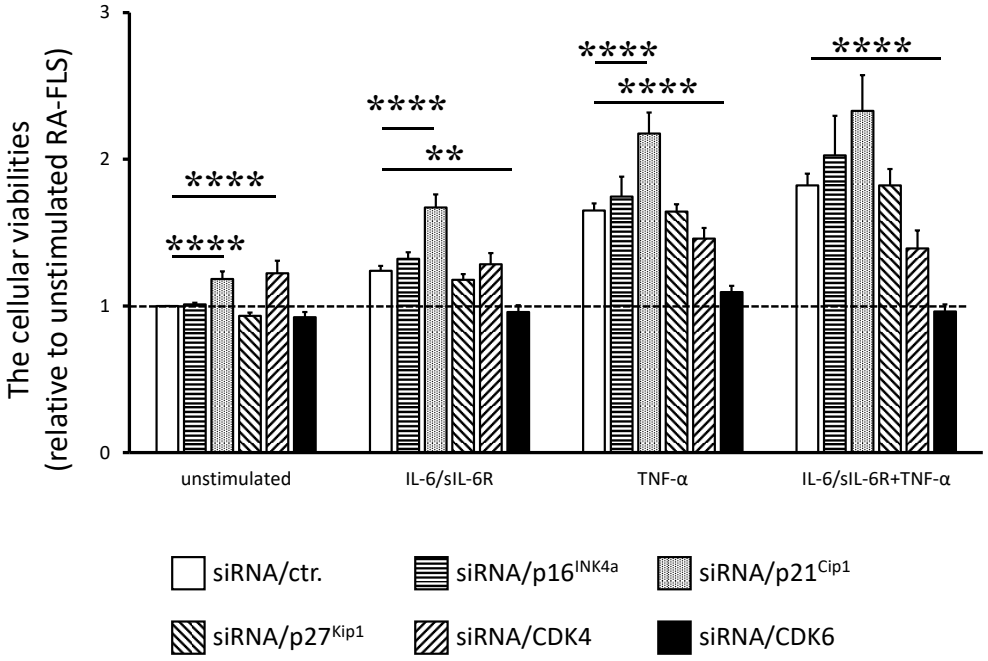


Figure 5

Under the silencing of CDKIs and CDKs, the cellular viabilities of RA-FLS stimulated with or without IL-6/sIL-6R or TNF-α for 72 hours were measured by WST-8 assay. ***p*<0.01, *****p* <0.0001. The values shown are expressed as means ± SEM (n=19 (siRNA/ctr.), n=8 (siRNA/*p16*^{INK4a}, siRNA/*p21*^{Cip1}), n=10 (siRNA/*p27*^{Kip1}), n=7 (siRNA/*CDK4*, siRNA/*CDK6*).

Viabilities of RA-FLS were significantly suppressed by siRNA/*CDK6*, while siRNA/*p21*^{Cip1} increased that in unstimulated, IL-6/sIL-6R or TNF-α stimulated RA-FLS.

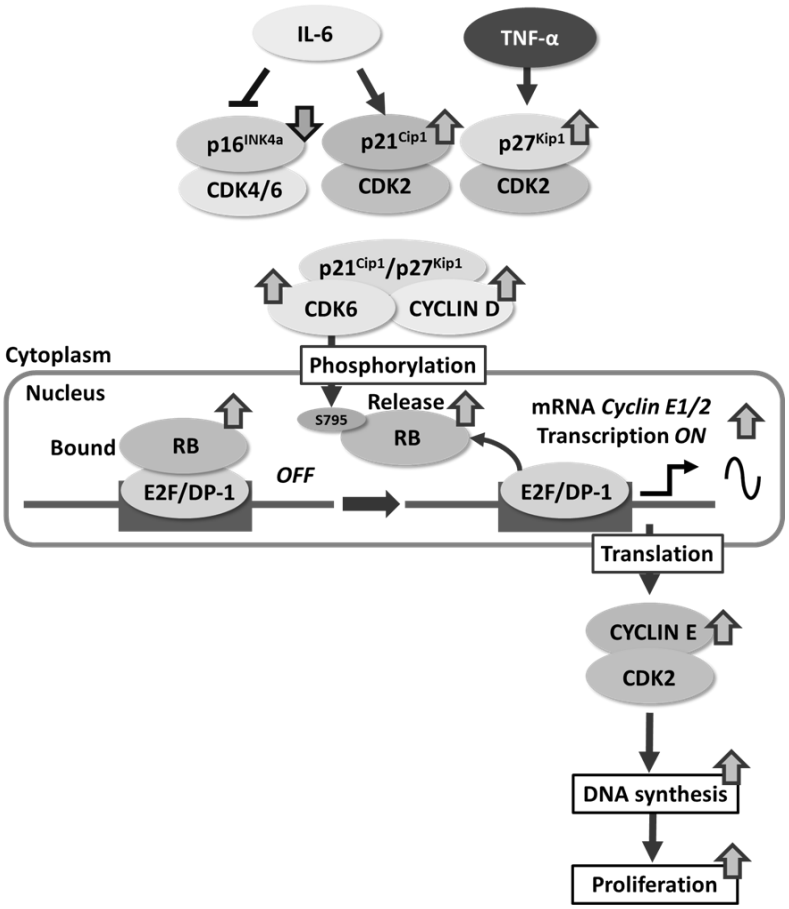
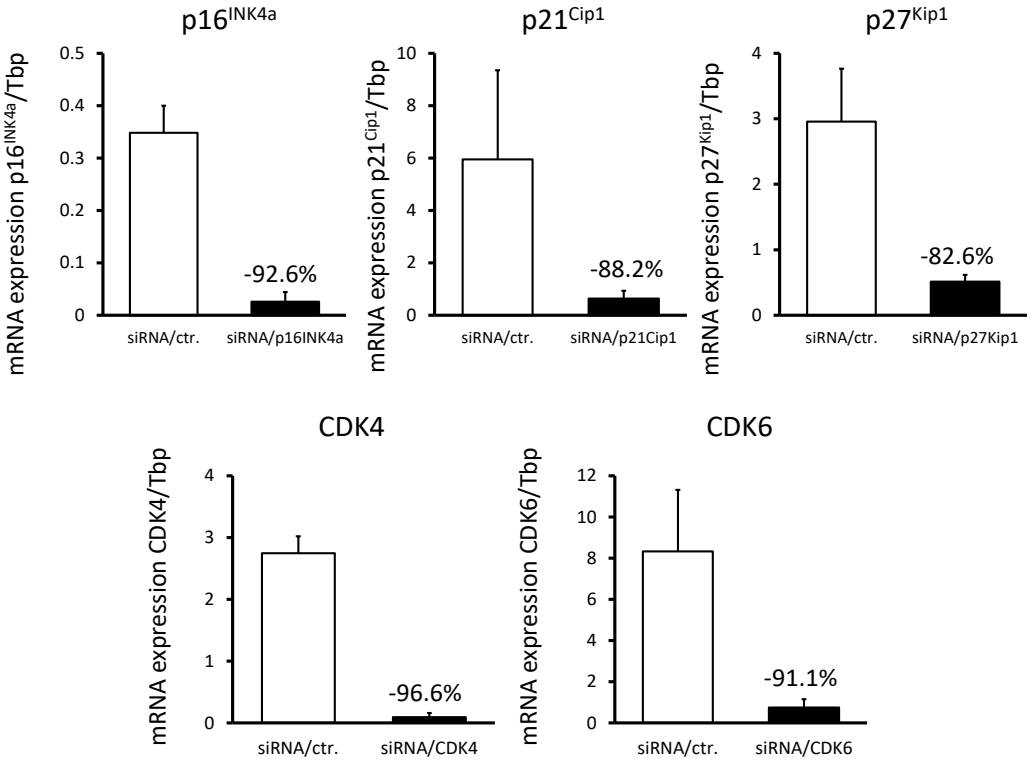


Figure 6

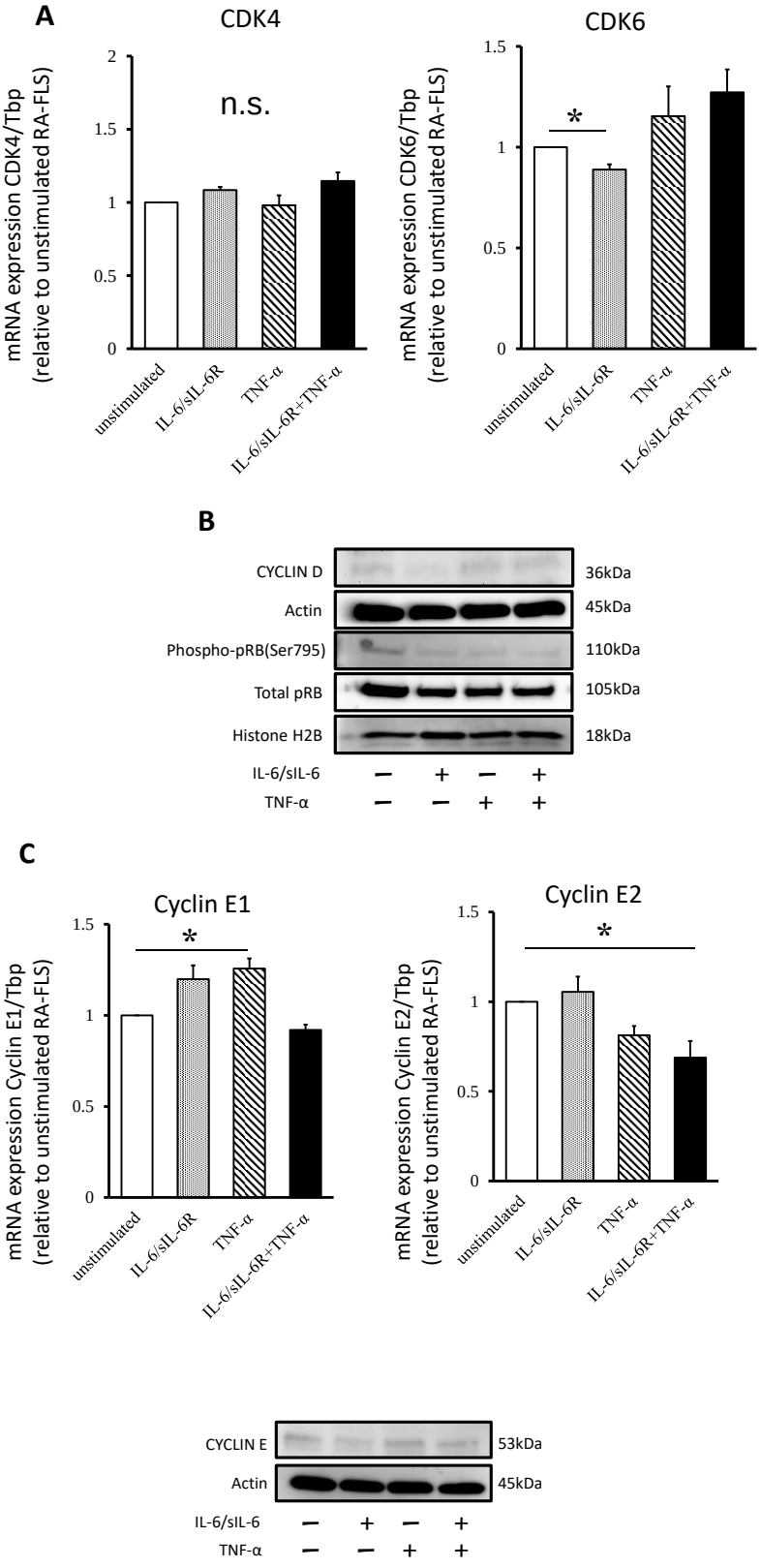
The scheme of the cell cycle modulation by IL-6/sIL-6R and TNF-α. Arrows show how these cytokines acted in this study.

Supplementary table S1. Detailed profile of patients with RA in this study.

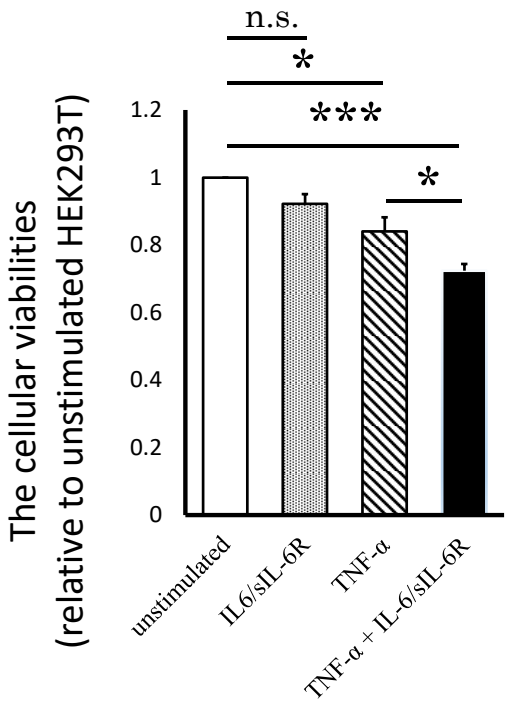
Patient No.	Age	Sex	Tissue	Disease duration (years)	DAS28-ESR	CRP (mg/dL)	RF (IU/mL)	ACPA (U/mL)	MTX (mg/week)	PSL (mg/day)	Other DMARDs
Patient #1	83	F	Knee	18	3.86	2.05	86	128	0	0	None
Patient #2	60	F	Hip	22	3.18	1.00	55	63	6	5	None
Patient #3	55	F	Knee	31	4.08	2.80	36	25	8	0	Tac (1.5mg/day)
Patient #4	80	M	Knee	38	2.52	0.86	62	38	0	10	None
Patient #5	75	F	Knee	28	2.06	0.20	98	122	4	5	None
Patient #6	41	M	Knee	12	5.08	3.20	208	556	8	5	None
Patient #7	83	F	Knee	38	2.38	1.10	38	51	4	5	ADA
Patient #8	62	F	Knee	21	1.68	0.20	41	48	6	0	ETN
Patient #9	42	F	Knee	11	1.83	0.05	51	84	8	2.5	ETN
Patient #10	81	F	Knee	39	2.37	1.08	89	113	4	5	Tac (2mg/day)
Patient #11	60	F	Knee	16	3.09	1.30	42	118	0	2.5	Tac (1.5mg/day) SASP (500mg/day)
Patient #12	49	F	Knee	23	2.91	0.50	63	90	6	0	ADA



Supplementary figure S1. The silencing efficiency of siRNA.


A
CDK4
n.s.
mRNA expression CDK4/Tbp
(relative to unstimulated RA-FLS)
2
1.5
1
0.5
0
unstimulated
IL-6/sIL-6R
TNF-α
IL-6/sIL-6R+TNF-α
CDK6
*
mRNA expression CDK6/Tbp
(relative to unstimulated RA-FLS)
1.5
1
0.5
0
unstimulated
IL-6/sIL-6R
TNF-α
IL-6/sIL-6R+TNF-α
B
CYCLIN D
36kDa
Actin
45kDa
Phospho-pRB(Ser795)
110kDa
Total pRB
105kDa
Histone H2B
18kDa
IL-6/sIL-6
TNF-α
C
Cyclin E1
*
mRNA expression Cyclin E1/Tbp
(relative to unstimulated RA-FLS)
1.5
1
0.5
0
unstimulated
IL-6/sIL-6R
TNF-α
IL-6/sIL-6R+TNF-α
Cyclin E2
*
mRNA expression Cyclin E2/Tbp
(relative to unstimulated RA-FLS)
1.5
1
0.5
0
unstimulated
IL-6/sIL-6R
TNF-α
IL-6/sIL-6R+TNF-α
CYCLIN E
53kDa
Actin
45kDa
IL-6/sIL-6
TNF-α

D



Supplementary figure S2. Expression of CDKIs, CDKs, CYCLIN D, Cyclin E, pRB, phospho-pRB and cell viability under 24, 48, and 72 h stimulation of IL-6/sIL-6R and TNF-α in HEK293.