

Interleukin-18 binding protein regulates the apoptosis and necroptosis of fibroblast-like synoviocytes and chondrocytes in rheumatoid arthritis

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Abstract

Objective

Interleukin (IL)-18 plays a pro-inflammatory role in rheumatoid arthritis (RA), and its soluble inhibitor IL-18 binding protein (IL-18BP) has a potential therapeutic role. We investigated the role of IL-18BP on the joint destruction process of RA by accessing the effects of IL-18BP on fibroblast-like synoviocytes (FLSs) and chondrocytes.

Methods

Peripheral blood mononuclear cells (PBMCs) from patients with RA and healthy controls were cultured under T cell proliferative conditions with 10, 50, or 100 ng/mL of IL-18BP. After three days of culture, flow cytometry for CD4⁺ T cells was performed using various IL-18BP concentrations. The apoptosis and necroptosis of FLSs and chondrocytes were measured by flow cytometry using annexin V and propidium iodide (PI) and western blot under TNF- α stimulation with IL-18BP (10, 50, and 100 ng/mL).

Results

Differentiation of CD4⁺ IL-17A⁺ and CD4⁺ IL-4⁺ cells decreased and that of CD4⁺ CD25^{high} Foxp3⁺ and CD4⁺ interferon (IFN)- γ ⁺ cells increased on addition of IL-18BP to cultured RA patient-driven PBMCs. RA-FLS migration ability was not suppressed by IL-18BP after 12 or 24 h. IL-18BP increased annexin V⁺ FLS level and reduced annexin V⁺ chondrocyte level in a dose-dependent manner, whereas PI⁺ annexin V FLS and chondrocyte levels were suppressed by 50, 100 ng/mL IL-18BP in culture.

Conclusion

The administration of IL-18BP regulated the type 17 helper T cell/ regulatory T cell imbalance and attenuated the production of pro-inflammatory cytokines. IL-18BP further increased FLS apoptosis and decreased the necroptosis of FLS/chondrocytes and apoptosis of chondrocytes suggesting the joint preservative potential of IL-18BP.

Key words

interleukin-18 binding protein, rheumatoid arthritis, fibroblast-like synoviocyte, chondrocyte, apoptosis, necroptosis

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Received on December 6, 2022; accepted
 in revised form on March 24, 2023.

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Introduction

Synovial hypertrophy (pannus) and small joint destruction are the specific clinical features associated with rheumatoid arthritis (RA), and prevention of these irreversible articular destructions is the main treatment goal in RA (1). Increased chronic inflammation in RA is caused by various activated immune cells including T and B cells and synovium forming cells, which can cause pain, morning stiffness, and swelling of affected joints (1, 2). This joint destruction is irreversible, and uncontrolled inflammation in patients with RA eventually develops into cartilage erosion and deformity of the joint (3, 4). Increased amount and activation of fibroblast-like synoviocytes (FLSs) of pannus secretes matrix metalloproteinases (MMPs), pro-inflammatory cytokines, and the expression of receptor activator of nuclear factors B ligand (RANKL), all of which cause the erosion of cartilage and adjacent cortical bones (3, 5). MMP produced by FLS degrades cartilage collagen, and MMP-3 levels are positively correlated with RA disease activity (6). The preservation of chondrocytes and concomitantly reducing FLS may be an attractive treatment modality in RA. The cell death can be divided into physiologic cell death (apoptosis) and pathologic cell death (necroptosis) (7). The pathologic cell death induces inflammation response in adjacent tissues (7), therefore, impact of issued medication on mode of cell death should be also revealed.

Interleukin (IL)-18 was identified in 1995, and was first reported as playing a role in interferon (IFN)- γ production and type 1 helper T cell (Th1) development (8). mRNA levels of IL-18 are increased in RA synovium (9) and the serum and synovial fluid of patients with RA (10, 11). The IL-18 binding protein (IL-18BP) is the naturally secreted soluble inhibitor of IL-18, which interrupts the function of IL-18 (12). Administration of IL-18 augmented arthritis in collagen-induced arthritis (CIA) mice (9), whereas suppression of IL-18 with its soluble inhibitor, IL-18BP, inhibited arthritis in CIA mice (13, 14). We previously showed that IL-18BP could reduce RANKL and

IL-17A-mediated osteoclastogenesis *in vitro* (10). Taken together, these findings suggest that IL-18 plays a pathologic role in RA and that IL-18BP has potential as an anti-arthritis agent for RA. However, the direct role of IL-18BP on RA-FLSs or chondrocytes has not yet been elucidated.

The cluster of differentiation (CD) 4⁺ helper T cells are composed of several subtypes (15). The type 17 helper T cell (Th17) population is increased in the serum and synovium of patients with RA (16, 17), and these cells can produce various pro-inflammatory cytokines such as IL-17A, IL-6, and tumour necrosis factor (TNF)- α . These cytokines were shown to mediate FLS proliferation and activation (18, 19). The regulatory T cell (Treg) population is decreased in RA (16, 17), which is notable since Tregs play an anti-inflammatory role in RA (20). Responder to a TNF inhibitor showed a decrease in Th17s and an increase in Tregs (21), which implies that the regulation of the Th17/Treg imbalance could predict treatment response in RA (22). Furthermore, reducing pro-inflammatory cytokines such as IL-17A, IL-6, and TNF- α can reduce not only inflammatory response, but also the abnormally increased osteoclastogenesis present in RA (23).

In this study, we investigated the regulatory role of IL-18BP on helper T cell differentiation and cytokine production in peripheral blood mononuclear cells (PBMCs) from patients with RA under T cell proliferative conditions. We also examined the effects of IL-18BP on the apoptosis and necroptosis of RA-FLSs and chondrocytes.

Methods

Patients

Peripheral blood was obtained from nine patients with RA. Inclusion criteria for patients with RA were fulfillment of the 2010 classification criteria for RA (24), and over 18 years of age. The demographic, laboratory, disease activity score-28 joints (DAS28), and current medication information were collected when the blood samples were obtained. The synovium and cartilage samples were obtained from patients with RA and osteoarthritis (OA) who

Funding: this research was supported by a grant from the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology, Republic of Korea (NRF-2021R1A2C1010075).

Competing interests: none declared.

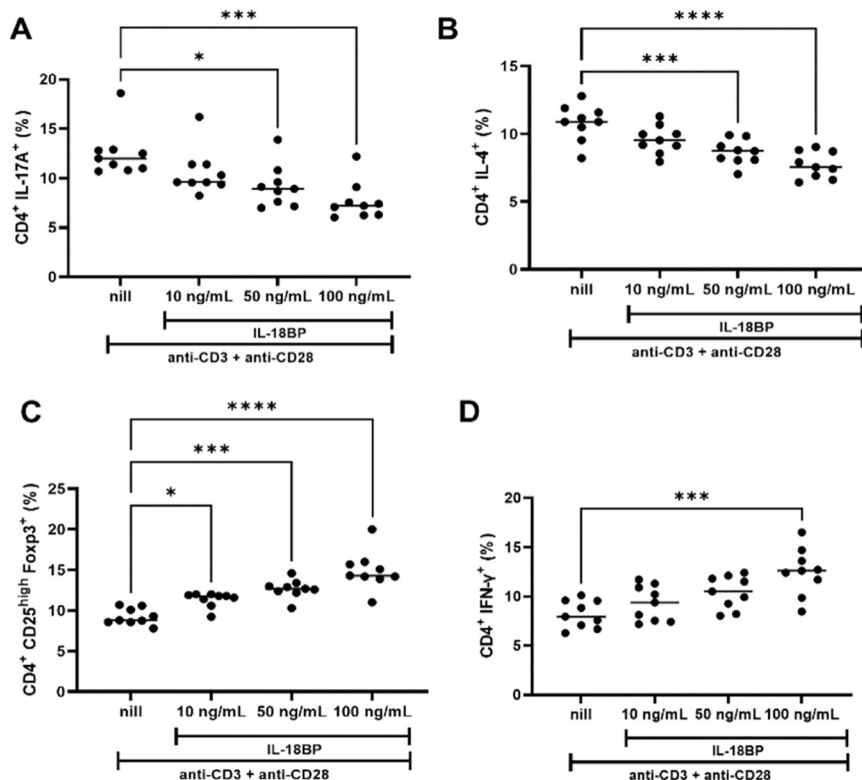


Fig. 1. CD4⁺ T cell differentiation of RA-PBMCs measured by flow cytometry under T cell proliferative condition. PBMCs were obtained from a total 9 RA patients, and PBMCs (1×10^6) was cultured in anti-CD3 antibody (1 μ g/mL) preincubated plate. Then, anti-CD28 (1 μ g/mL) with 0, 10, 50, 100 ng/mL of IL-18BP were added, then cultured for 72 hrs. Then percentage of (A) CD4⁺ IL-17A⁺, (B) CD4⁺ IL-4⁺, (C) CD4⁺ CD25^{high} Foxp3⁺, and (D) CD4⁺ IFN-γ⁺ T cell were measured by flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

underwent total knee replacement or total hip replacement surgery, then FLSs and chondrocytes were isolated as previously described and stored with liquified nitrogen (25, 26). This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Before enrolment, we explained the purpose and procedure of the study to all participants and ensured they fully understood it. Then informed consent was obtained from all participants. The experimental protocol was approved by the Institutional Review Board of Konkuk University Medical Center (KUMC 2021-12-034).

In vitro culture of PBMCs under T cell proliferative conditions with IL-18BP

PBMCs from patients with RA ($n=9$) and healthy control (HC, $n=7$) were obtained using the standard Ficoll-Paque density gradient method (GE Healthcare Biosciences, Uppsala, Sweden). CD4⁺ cells and CD14⁺ cells isolated from PBMC of RA patients with easysep human CD4 Tcell isolation kit (STEMCELL no. 117952) or easysep human CD14 positive selection kit

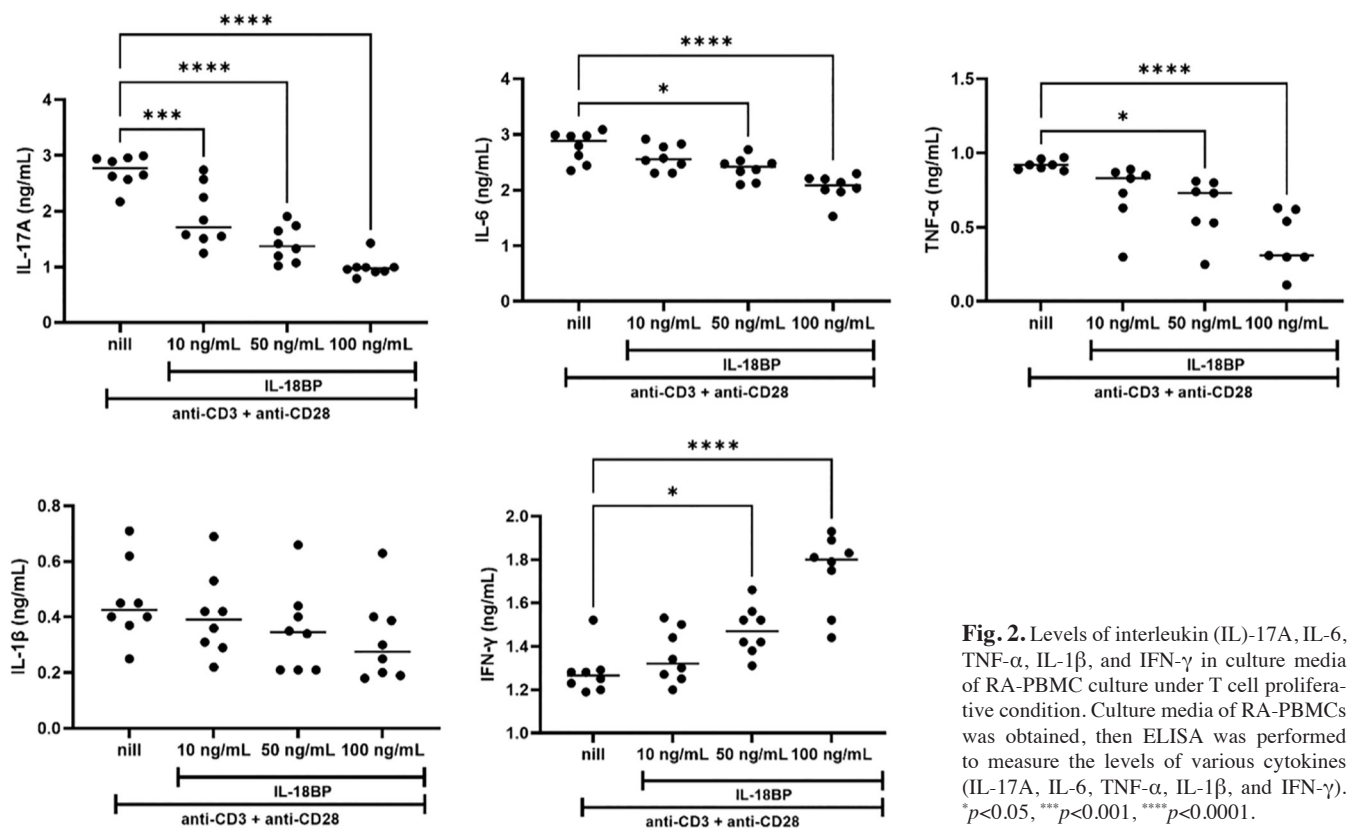


Fig. 2. Levels of interleukin (IL)-17A, IL-6, TNF-α, IL-1β, and IFN-γ in culture media of RA-PBMC culture under T cell proliferative condition. Culture media of RA-PBMCs was obtained, then ELISA was performed to measure the levels of various cytokines (IL-17A, IL-6, TNF-α, IL-1β, and IFN-γ). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(STEMCELL no. 17858). Cells (PB-MCs from RA patients or HC, CD4⁺ cells/CD14⁺ cells from RA patients) were cultured in RPMI-1640 medium (Gibco BRL, Carlsbad, CA, USA) containing penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (Gibco BRL) that had been inactivated by heating to 55°C for 30 min. Cell suspensions were dispensed into 48-well plates (Eppendorf). The cell culture plate was pre-incubated with an anti-CD3 antibody (1 µg/mL) for 1 h at 37°C. Then, PBMCs from RA patients or HC, CD4⁺ cells/CD14⁺ cells from RA patients (1×10^6) were plated in 48-well plates (Nunc) with an anti-CD28 antibody (1 µg/mL; R&D Systems) and 0, 10, 50, or 100 ng/mL of IL-18BP (R&D Systems) and cultured for 72 h. Floating cells and culture media were then harvested for flow cytometry and enzyme-linked immunosorbent assay (ELISA).

ELISA of the culture media from PBMC culture to determine cytokine levels

Culture media of RA-PBMCs, HC-PBMCs, RA-CD4⁺ cells, and RA-CD14⁺ cells with various concentrations of IL-18BP (0, 10, 50, or 100 ng/mL) was collected after 72 h. The levels of IL-17A, IL-6, TNF-α, IL-1β, and IFN-γ (R&D Systems, Minneapolis, MN, USA) in the culture media were measured by ELISA according to the manufacturer's protocol. The detection levels of cytokines ranged from 10 to 2,000 pg/mL. A standard curve was drawn by plotting the optical density against the log of the concentration of recombinant cytokines, and the curve was used to determine cytokines' concentrations in the test samples.

Migration test for RA-FLS under IL-18BP treatment

RA-FLSs were seeded in culture inserts (Catalog number: 80209, Ibidi, GmbH, Martinsried, Germany) at a density of 3×10^4 cells per well. After allowing the cells to attach overnight, the RA-FLSs were starved in serum-free Dulbecco's Modified Eagle Medium (DMEM) to stop cell proliferation (27). They were then provided with fresh DMEM me-

dium and IL-18BP was added at various concentrations (0, 10, 50, or 100 ng/mL). The light microscope pictures were taken at ×100 magnification 12 and 24 h later and the empty space was quantified by using ImageJ software.

In vitro RA-FLS culture with TNF-α stimulation and IL-18BP

RA-FLSs and OA-FLSs (1.5×10^5 cells/mL) were plated onto 12-well culture plates with serum-free DMEM for 24 h. After 24 h of starvation, culture media was changed to 10% FBS-added DMEM with TNF-α 30 ng/mL. IL-18BP was added at concentrations of 0, 10, 50, or 100 ng/mL. RA-FLSs were harvested 72 h after media change.

In vitro chondrocyte culture with fibronectin fragment (FN-f) + TNF-α stimulation and IL-18BP

RA-chondrocytes and OA-chondrocytes (1.5×10^5 cells/mL) were seeded in 12-well culture plates with serum-free DMEM/F12 (11320-033, Gibco) for 24 h. Then the culture media of the chondrocytes were replaced with 10% FBS containing DMEM/F12 with TNF-α 30 ng/mL + 5 µg/mL fibronectin fragment (FN-f) (28), and IL-18BP (0, 10, 50, or 100 ng/mL) was added. After 72 h of culture, the RA-chondrocytes were harvested for flow cytometry and western blot.

Flow cytometry

To quantify IL-17A⁺, IL-4⁺, IFN-γ⁺, and CD25⁺ forkhead box P3 (FOXP3)⁺ cells in CD4⁺ T cells, harvested PBMCs were immunostained using a PerCP-conjugated anti-CD4 antibody (BD Biosciences, San Jose, CA, USA), then fixed and permeabilised using a Cytotfix/Cytoperm Plus kit (BD Biosciences). As per the manufacturer's instructions, PBMCs were stained with phycoerythrin-conjugated anti-IL-17A (eBiosciences, San Diego, CA, USA), APC-conjugated anti-IL-4 (BD Bioscience), FITC-conjugated anti-IFN-γ (BD Bioscience), or allophycocyanin-conjugated anti-CD25 (BD Biosciences) with phycoerythrin-conjugated anti-FOXP3 (BioLegend, San Diego, CA, USA) antibodies. For the apoptosis and necroptosis analysis, an apoptosis

staining kit (640914, Biolegend) with anti-annexin V and anti-propidium iodide (PI) was used, according to the manufacturer's protocol. Cells were detected using a FACSCalibur flow cytometer (BD Pharmingen, Franklin Lakes, NJ, USA).

Western blot

After 72 hrs, RA-FLSs (stimulated with TNF-α 30 ng/mL) and RA-chondrocytes (stimulated with TNF-α 30 ng/mL + FN-f 5 µg/mL) with various concentration of IL-18BP were harvested. Protein concentrations were measured by Bradford method (P7200-050, Genedepot, TX, USA). The protein levels of caspase-3, caspase-7, MLKL, pMLKL, RIP3, p-RIP3, and β-actin (all from Cell signaling) were detected using Chemi Doc system (LAS-400) and Hyperfilm-enhanced chemiluminescence (ECL) reagents (no. W3653-020, Genedepot, TX, USA). Band density was measured using the ImageJ software.

Statistical analysis

The data are presented as mean ± standard error of the mean. One-way analysis of variance with *post-hoc* Bonferroni's multiple comparison test was used. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

The effect of IL-18BP on T cell differentiation and cytokine production

The detailed information of enrolled RA patients are summarised in Supplementary Table S1. Under T cell proliferation conditions (anti-CD3 and anti-CD28 Ab stimulation) of RA-PBMCs, CD4⁺ T cell differentiation was assessed by flow cytometry. The differentiation of CD4⁺ IL-17A⁺ and CD4⁺ IL-4⁺ T cell populations was decreased under the 50 and 100 ng/mL IL-18BP condition (Fig. 1A-B). The frequency of CD4⁺ CD25^{high} Foxp3⁺ T cells was increased in a dose-dependent manner in the 10, 50 and 100 ng/mL conditions, and CD4⁺ IFN-γ⁺ T cell increased under the 100 ng/mL IL-18BP

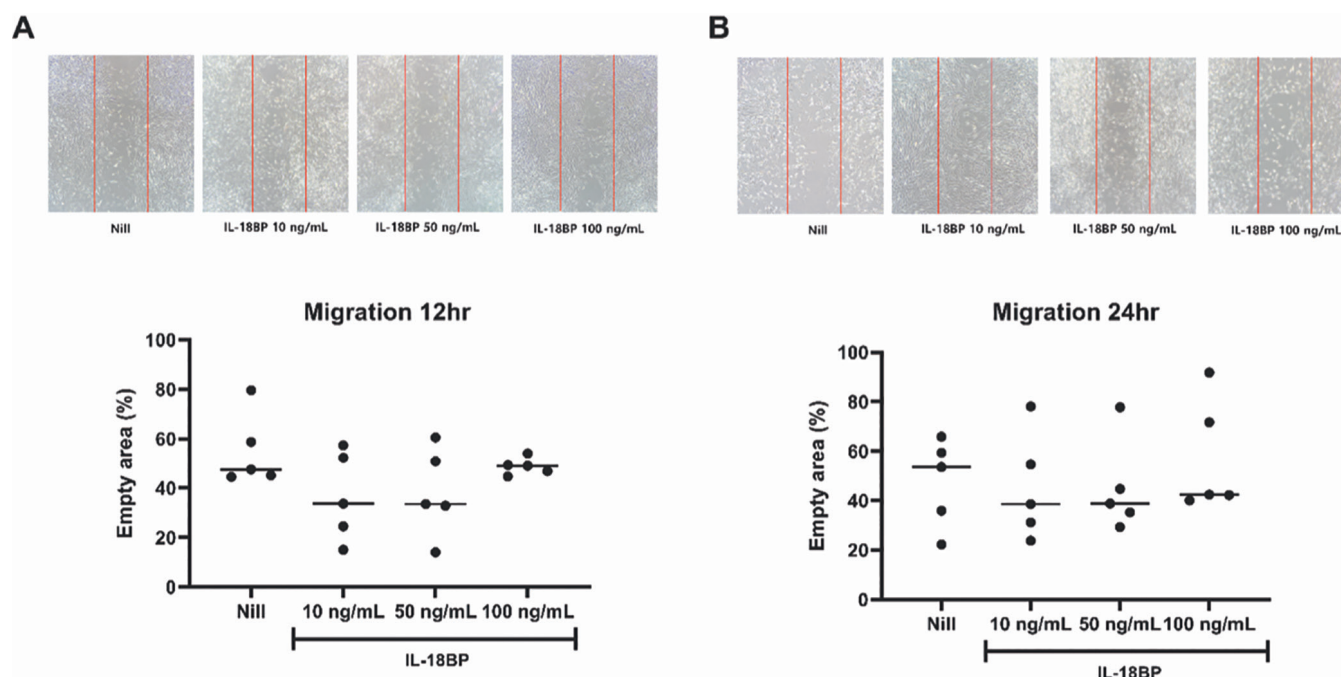


Fig. 3. Effect of IL-18BP on RA-FLS migration. RA-FLS (5×10^6 , $n=5$) was plated in 12-well with IL-18BP (0, 10, 50, and 100 ng/mL), and cultured for (A) 12 and (B) 24 hrs. The empty area between 500 μ m was measured, which indicate larger empty space means lower migration activity. Presented with representative pictures of migration (distance between two red lines is 500 μ m).

condition (Fig. 1C-D). Similar with results from RA-PBMCs, the addition of IL-18BP decreased CD4⁺ IL-17A⁺ and CD4⁺ IL-4⁺ T cell populations, whereas CD4⁺ CD25^{high} Foxp3⁺ T cell and CD4⁺ IFN- γ ⁺ T cell population increased by IL-18BP addition in HC-PBMCs (Suppl. Fig. S1A-D). The cytokine levels in the culture media were measured by ELISA. The production of IL-17A were decreased with 10, 50, and 100 ng/mL of IL-18BP, and IL-6 and TNF- α levels were suppressed in a dose-dependent manner following the administration of 50 and 100 ng/mL IL-18BP. IFN- γ was increased following the administration of 50 and 100 ng/mL IL-18BP (Fig. 2). The cytokine levels of culture media from HC-PBMCs also showed decrease of IL-17A, IL-6, TNF- α , whereas IFN- γ level increased by IL-18BP addition. Furthermore, level of IL-1 β significantly decreased in culture media of HC-PBMC by adding IL-18BP (Suppl. Fig. S2). Furthermore, CD4⁺ cells (CD4⁺ T cells) and CD14⁺ cells (monocytes) were isolated from RA-PBMCs, and same experiment condition was applied (anti-CD3 and anti-CD28 Ab stimulation with 0, 10, 50, and 100 ng/mL IL-18BP). Then, cytokine levels of CD4⁺ cells and CD14⁺

cells culture media were measured. In both CD4⁺ cells and CD14⁺ cells culture media, the levels of IL-17A, IL-6, TNF- α , and IL-1 β decreased, whereas IFN- γ level increased by IL-18BP addition (Suppl. Fig. S3-S4).

Effects of IL-18BP on RA-FLS migration

RA synovitis is characterised by hypertrophy and invasiveness which causes joint destruction, therefore inhibiting RA-FLS migration is another potential therapeutic target. The migration of RA-FLS ($n=5$) was not significantly suppressed with IL-18BP treatment after both 12 and 24 h of culture time (Fig. 3A-B).

Modulatory effect of IL-18BP on RA-FLS cell death (apoptosis and necroptosis)

The RA-FLSs ($n=11$) and OA-FLSs ($n=5$) were cultured with 30 ng/mL TNF- α for 72 h, and the populations of annexin V⁺ (apoptosis) and PI⁺ annexin V⁺ (necroptosis) were measured by flow cytometry. Annexin V⁺ RA-FLSs were increased on administration of 50 and 100 ng/mL of IL-18BP, whereas the PI⁺ annexin V population was decreased on the administration of 50

and 100 ng/mL of IL-18BP (Fig. 4A). In western blot, the apoptosis markers (caspase-3 and -7) were increased in 10, 50, and 100 ng/mL and 50, 100 ng/mL of IL-18BP, respectively. In aspect of necroptosis marker, the levels of MLKL and p-MLKL decreased in 100 ng/mL of IL-18BP, and RIP3 and p-RIP3 decreased in 50 and 100 ng/mL of IL-18BP (Fig. 4B). Similar with RA-FLSs, the Annexin V⁺ FLSSs increased in IL-18BP 100 ng/mL added condition, and PI⁺ annexin V population decreased in 10, 50, and 100 ng/mL IL-18BP added condition in OA-FLSs experiment (Suppl. Fig. S5).

Effects of IL-18BP on RA-chondrocyte cell death (apoptosis and necroptosis)

In culture media of RA-chondrocytes ($n=6$) and OA-chondrocytes ($n=4$), 5 μ g/mL FN-f + 30 ng/mL TNF- α were added with 0, 10, 50, or 100 ng/mL IL-18BP. After 72 h of culture, flow cytometry was performed to measure apoptosis and necroptosis of chondrocytes. In RA-chondrocyte experiment, annexin V⁺ chondrocytes decreased in a dose-dependent manner with 10, 50, and 100 ng/mL IL-18BP administration. The percentage of PI⁺ annexin V⁺ chondro-

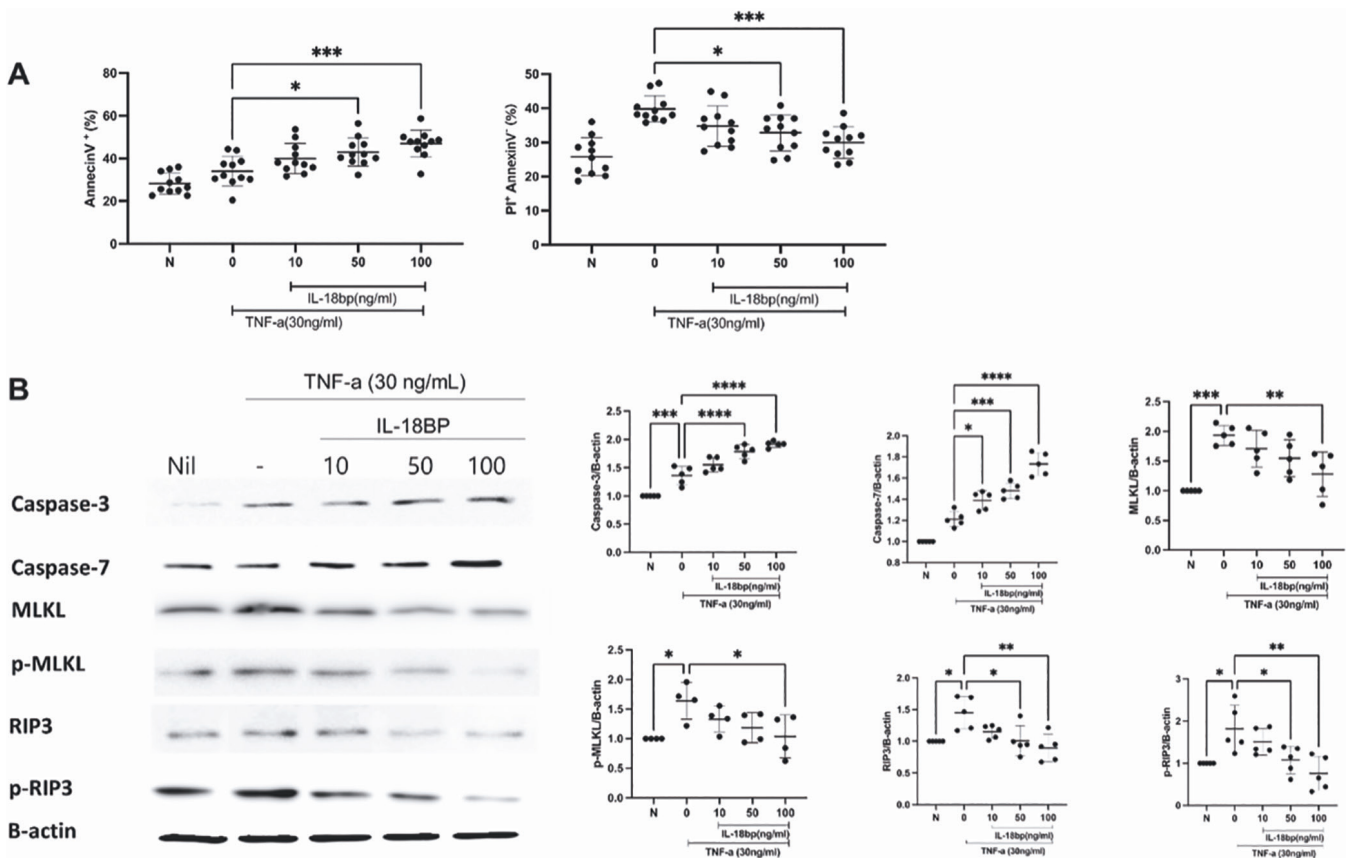


Fig. 4. Apoptosis and necroptosis of RA-FLS under various concentration of IL-18BP (0, 10, 50, 100 ng/mL). RA-FLS (1.5×10^5 , $n=11$) was seeded in 12-well culture plate with TNF- α (30 ng/mL) with 0, 10, 50, 100 ng/mL IL-18BP, then annexin V and propidium iodide (PI) were stained.

A: To determine apoptosis and necroptosis, annexin V⁺ FLS, and PI⁺ annexin V⁺ FLS population were determined by flow cytometry.

B: The apoptosis markers (caspase-3 and -7), necroptosis markers (MLKL, p-MLKL, RIP3, and p-RIP3), and β -actin levels were measured by western blot.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

cytes decreased on treatment with 50 and 100 ng/mL IL-18BP (Fig. 5A). The levels of caspase-3 and caspase-7 were suppressed in 50, 100 ng/mL and 100 ng/mL of IL-18BP, respectively. Also, the levels of MLKL and p-MLKL decreased in 100 ng/mL of IL-18BP, and RIP3 and p-RIP3 decreased in 50, 100 ng/mL and 10, 50, and 100 ng/mL of IL-18BP, respectively (Fig. 5B). In experiment of OA-chondrocytes, the population of annexin V⁺ chondrocytes and PI⁺ annexin V⁺ chondrocytes decreased dose-dependently in IL-18BP added condition (Suppl. Fig. S6).

Discussion

Several studies have shown that IL-18BP has therapeutic potential for the treatment of inflammatory diseases including RA. Treatment with IL-18BP *in vivo* in CIA mice (an RA mouse model) reduced arthritis score and pro-inflammatory cytokine expression in the joints of CIA mice (13, 14). Furthermore, an

in vitro experiment showed that osteoclast differentiation under RANKL or IL-17A stimulated conditions was suppressed by IL-18BP administration, and that Th17 differentiation and related cytokine levels (soluble RANKL, IL-17A) were reduced by IL-18BP treatment under Th17 polarising conditions (10). However, the direct effects of IL-18BP on RA-driven FLSs and chondrocytes have not yet been demonstrated. Thus, in this study, we evaluated the role of IL-18BP on apoptosis and necroptosis on RA-driven FLSs and chondrocytes for the first time. Abnormal synovial hypertrophy and the consequent destruction of cartilage are keystone features of RA, and the prevention of this irreversible joint destruction is the main treatment target in RA research (1). Our results demonstrated that the addition of IL-18BP *in vitro* to cultured RA-driven FLSs and chondrocytes decreased the apoptosis of chondrocytes, whereas increased the apoptosis of FLSs. In addition,

the necroptosis of both RA-FLSs and chondrocytes were suppressed by IL-18BP. These findings illuminate another potential therapeutic mechanism of IL-18BP on RA, which showed direct beneficial effects on critical joint forming cells (FLSs and chondrocytes).

The up-regulation of CD4⁺ IL-17A⁺ T cells (Th17) and down-regulation of CD4⁺ CD25^{high} Foxp3⁺ T cells (Treg) are hallmarks of inflammatory diseases including RA (15, 17), and regulating the aforementioned Th17/Treg imbalance has been suggested as a treatment goal in inflammatory diseases (22, 29, 30). Our previous study showed that Th17 decreased and Treg increased on IL-18BP administration under Th17 polarising conditions (10). In present study, we demonstrated that CD4⁺ IL-17A⁺ (Th17) and CD4⁺ IL-4⁺ (Th2) T cell differentiation was suppressed, and in contrast the CD4⁺ CD25^{high} Foxp3⁺ (Treg) and CD4⁺ IFN- γ ⁺ (Th1) populations were increased following IL-18BP

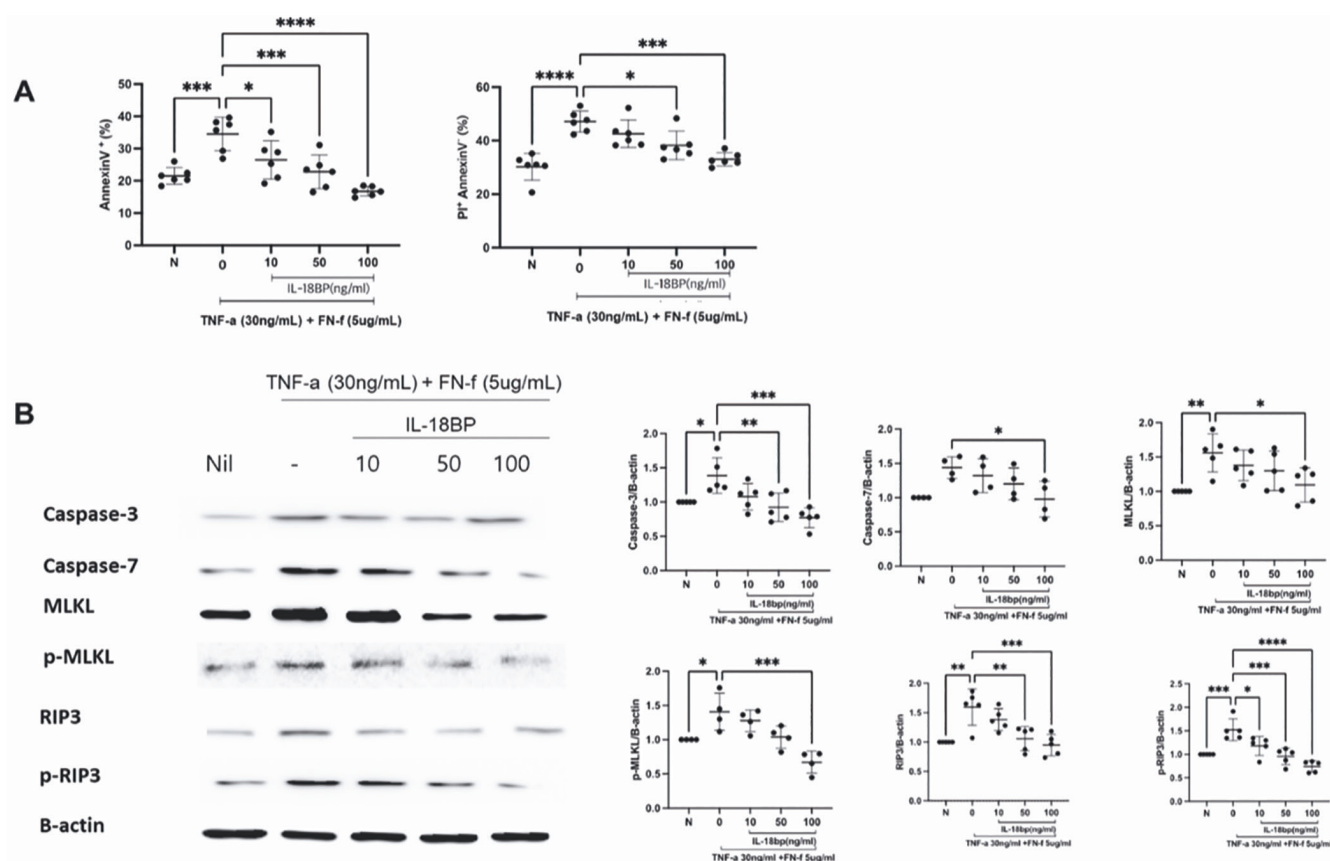


Fig. 5. Effects of IL-18BP on RA-chondrocyte cell death (apoptosis and necroptosis). Chondrocyte (1.5×10^5 , $n=6$) was plated in 12-well, and stimulated with 30 ng/mL TNF- α , 5 μ g/mL fibronectin fragment (FN-f), and 0, 10, 50, 100 ng/mL IL-18BP for 72hrs.

A: The percentage of annexin V⁺ chondrocyte, and PI⁺ annexin V⁺ chondrocytes were measured by flow cytometry.

B: The expression levels of apoptosis markers (caspase-3 and -7), necroptosis markers (MLKL, p-MLKL, RIP3, and p-RIP3), and β -actin levels were determined by western blot. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

administration in RA-driven PBMCs under neutral T cell proliferative conditions. IL-18 is also known as an IFN- γ inducing factor (8), and IL-18BP is known to diminish Th1 response (12). IFN- γ induces IL-18BP secretion in PBMCs and RA-FLS (31, 32), which implies that there is a negative feedback loop between IFN- γ and IL-18BP. However, our results showed that IL-18BP augmented Th1 differentiation, and increased levels of IFN- γ . The differentiation toward specific subtypes of helper T cells, Th1/Th2/Th17/Treg, are principally governed by dominantly activated transcriptional factors (15), but it is also affected by the interactions of each subtype of helper T cells. Th1 and Th17 counter-regulate the differentiation of each other (33, 34). IL-17 and STAT3 (Th17-inducing transcriptional factor) can suppress IL-12-dependent Th1 differentiation (35, 36), and IFN- γ inhibits Th17 response through indoleamine-2,3-deoxygenase or as a

suppressor of the cytokine signaling 3 pathway (37, 38). Although in cytokine levels, IFN- γ and IL-18BP may form a negative feedback loop, however, at the transcriptional factor level the counter-regulatory role of Th17 and Th1 may explain the enhanced Th1 response caused by IL-18BP. Increased Th1 response was assumed to be a major pathologic process in RA before the identification of Th17 (39-41). However, Th17 is now believed to play a major role in RA pathogenesis (42). Several studies showed anti-arthritis effects of IFN- γ in CIA mice which implies that IFN- γ may suppress RA progression (38, 43). Also, a positive correlation between serum IL-18 and IL-18BP was only observed in active patients with RA (DAS28 ≥ 3.2) (10), and IFN- γ was found to promote IL-18BP production in RA-FLSs (31). Taken together, the increased IFN- γ and Th1 response by IL-18BP may be the result of them antagonising an inflammatory response of IL-18 in RA. The re-

sults from this study (induction of Th1/Treg response and suppression of Th17) suggest a net anti-inflammatory role of IL-18BP on RA.

Cell death is an active process in living organisms and can be divided into three different pathways: 1) apoptosis (programmed cell death), 2) necroptosis, and 3) pyroptosis (7). Apoptosis is induced by intrinsic and extrinsic pathways mediated by B-cell lymphoma-2 family proteins and tumour necrosis factor receptor (TNFR) 1/Toll-like receptor (TLR), respectively (7). The downstream signals of TNFR/TLR-3 and TNFR/TLR-4 promote receptor-interacting protein kinase (RIPK) 1/3, mixed lineage kinase domain-like (MLKL) activation, and induces necroptosis (7). Necroptosis promotes an inflammatory response by producing necrosome complexes, and is known to be involved in cancer progression/metastasis and the pathogenesis of neurodegenerative disorders (44, 45).

Neutrophils in RA synovial fluid activate necroptosis by activating RIPK1/3 and MLKL (46). The necroptosis-inducing intracellular protein, 14-3-3 η , is increased in RA synovium-driven macrophages compared to osteoarthritis synovium driven macrophages, and TNF- α induces necroptosis of macrophages by modulating intracellular 14-3-3 η protein level (47). Furthermore, modulating necroptosis has been suggested as an innovative treatment modality for RA (48). Inhibition of the necroptosis mediator, RIPK1, suppressed arthritis in RA animal models (49, 50). In addition, IFN- γ deficiency was shown to promote Th17 response and necroptosis in CIA mice via increasing the expression of RIPK1/3 and MLKL (51). Apoptosis is a physiologically programmed cell death process that does not induce an inflammatory response (7, 44). In this study, we found that IL-18BP reduced necroptosis of RA-FLSs and chondrocytes and enhanced IFN- γ expression in *in vitro* culture of RA-PBMCs. Furthermore, apoptosis of RA-FLS was increased, whereas apoptosis of chondrocytes was decreased following IL-18BP administration, which implies that IL-18BP has a potential joint preservative role.

The increased migration and consequent invasiveness of RA-FLSs suggested they would be a treatment target for RA (52), and several agents have been shown to inhibit the migration ability of RA-FLS *in vitro* (52-54). In our study, the IL-18BP administration did not suppress the migration of RA-FLSs. The therapeutic potential of IL-18BP may be through the mediation of the cell death of RA-FLSs/chondrocytes and the regulation of CD4⁺ T cells, but not through suppression of RA-FLS migration.

The control of CD4⁺ T cell, cytokine production, apoptosis and necroptosis of FLS / chondrocytes by IL-18BP were not specific in RA driven cells. The regulatory effects of IL-18BP were consistently shown in HC-PBMCs, and OA driven FLS and chondrocyte. These similar results between RA and HC/OA may arise because the *in vitro* stimulation (anti CD3 + anti CD28 Ab for PBMC culture, TNF- α stimulation in

FLS culture, and TNF- α + FN-f stimulation in chondrocyte culture) was also performed in experiments of HC/OA driven cells. Aforementioned *in vitro* stimulations were done to mimic the inflammatory status of RA. Further study including clinical trial of IL-18BP in RA patients should be performed to clarify the effect of IL-18BP in RA, and reveal whether this agent is specifically beneficial for RA or not.

Taken together, our results showed that IL-18BP restored Th17/Treg imbalance and decreased the production of pro-inflammatory cytokines in RA-driven PBMCs. Furthermore, apoptosis of FLSs was increased, whereas chondrocyte apoptosis was decreased on IL-18BP administration. Also, the inflammatory cell death (necroptosis) of RA-FLSs and chondrocytes were suppressed by IL-18BP. These results suggest that IL-18BP has therapeutic potential for the treatment of RA through the regulation of FLS and chondrocyte apoptosis/necroptosis, which is crucial to the joint destructive process of RA.

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