IκB kinase ε contributes to the pathogenesis of osteoarthritis by promoting cartilage degradation

内田, 泰輔

Akasaki Yukio (Orcid ID: 0000-0002-3666-3952)
Lotz Martin K. (Orcid ID: 0000-0002-6299-8799)

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Taisuke Uchida[1], Yukio Akasaki[1], Takuya Sueishi[1], Ichiro Kurakazu[1], Masakazu Toya[1], Masanari Kuwahara[1], Ryota Hirose[1], Yuki Hyodo[1], Hidetoshi Tsushima[1], Martin K. Lotz[2], Yasuharu Nakashima[1]

Author affiliations

1 Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

2 Department of Molecular Medicine, The Scripps Research Institute, CA, USA

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[1]Taisuke Uchida, MD, Yukio Akasaki, MD, PhD, Takuya Sueishi, MD, PhD, Ichiro Kurakazu, MD, PhD, Masakazu Toya, MD, PhD, Masanari Kuwahara, MD, PhD, Ryota Hirose, MD, Yuki Hyodo, MD, Hidetoshi Tsushima, MD, PhD, Yasuharu Nakashima, MD, PhD: Kyushu University, Fukuoka, Japan; [2]Martin K. Lotz, MD: The Scripps

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Corresponding author: Yukio Akasaki

Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka city, Fukuoka, 812-8582, Japan.

E-mail: akasaki.yukio.443@m.kyushu-u.ac.jp

Tel: +81-92-642-5488

Fax: +81-92-642-5507

Abstract

Objective. Nuclear factor kappa B (NF-κB) signaling is an important modulator in osteoarthritis (OA), and IκB kinase ε (IKKε) regulates the NF-κB pathway. This study was undertaken to identify the functional involvement of IKKε and the effectiveness of IKKε inhibition in OA pathogenesis.

Methods. IKKε expression in normal and OA human knee joints was analyzed immunohistochemically. Gain- or loss-of-function experiments were performed using human chondrocytes. OA was surgically induced in mice, which were then treated with intra-articular injection of BAY-985, an IKKε/TBK1 inhibitor, every 5 days for 8 weeks. Mice were subsequently subjected to histologic examination.

Results. IKKε protein expression was increased in human OA cartilage. In vitro, expression levels of OA-related factors were downregulated by knockdown of IKKε in human OA chondrocytes using siRNA or BAY-985. Conversely, IKKε overexpression significantly increased the expression of OA-related catabolic mediators. In western blot analysis, IKKε overexpression increased the phosphorylation of IκBα and p65 in human chondrocytes. In vivo, intra-articular injection of BAY-985 in mice attenuated OA-related cartilage degradation and hyperalgesia via NF-κB signaling.

Conclusion. These results suggest that IKKε regulates cartilage degradation through a

catabolic response mediated by NF-κB signaling, and is a potential target for OA treatment. Furthermore, BAY-985 may serve a major compound in drugs developed for OA.

Introduction

Osteoarthritis (OA), the most prevalent joint disease, is a degenerative condition triggered and driven, in part, by the innate immune system and local production of inflammatory mediators through the Toll-like receptors (TLRs) and/or Interleukin-1 receptor (IL-1R) pathway (1, 2). Downstream enzymes such as MMP13, ADAMTS4, and NOS2 are directly involved in OA cartilage degradation (3-5). Nuclear factor kappa B (NF-κB), a key transcription factor that mediates the aforementioned inflammatory signaling, has long been recognized as a pathogenic factor, and therefore it and its upstream regulators, co-factors, and downstream effectors are all potential cellular targets for the treatment of OA (6-8).

The IκB kinase (IKK) family consists of IKKα, IKKβ, IKKγ, and IKKε/TBK1, all of which are upstream regulators of NF-κB (9, 10). When IKK complex (IKKα/β/γ) is activated by extracellular stimuli, IκB proteins are phosphorylated and degraded. Following this activity, NF-κB dimers are released, then migrate to the nucleus, leading to specific gene transactivation (11). The canonical pathway involves NF-κB dimers composed of the p65 and p50 subunits and requires the IKK complex, which does not include IKKε/TBK1. A number of previous studies have extensively investigated the roles of the canonical IKKα/β/γ in OA pathogenesis and the pharmacological inhibitory effects

of those IKKs on OA treatment (12-15). In a mouse OA model, low doses of IKKα/β inhibitor, BMS-345541, mitigated OA development by suppressing matrix-degrading enzymes, whereas high doses of inhibitor induced chondrocyte apoptosis (15). A phase 2a human study using the compound SAR113945, a specific inhibitor of the canonical IKK complex, showed that a statistically significant difference for the Western Ontario and McMaster Universities Arthritis Index (WOMAC) pain subscore in a subgroup of patients with effusion at baseline, but failed to show any effects in the overall group (14).

IKKε, also referred to as IKKi or IKBKE, is a non-canonical member of the IKK family and was first discovered as a kinase induced by lipopolysaccharide (LPS) binding to TLR4 in mouse macrophage cell lines (16). TANK binding kinase 1 (TBK1) is a homologous protein of IKKε that can bind each other to orchestrate downstream pathways (17). Subsequently, IKKε has been reported to play an essential role in regulating inflammation, innate immunity, and rheumatoid arthritis (9, 18, 19). The primary function of IKKε/TBK1, like other IKKs, is considered to be the activation of NF-κB through IκB phosphorylation (20). On the other hand, overexpression of IKKε was reported to induce NF-κB–independent stimulation of the IL-6 gene promoter via activation of the transcription factor C/EBP-β, thus leading to the growth of prostate cancer (21). In human chondrocytes from OA cartilage, IKKε gene was constitutively

expressed and upregulated by IL1β/ TNFα signaling, however, its pathogenic impact on OA development remains uninvestigated (22). IKKε inhibition was reported to attenuate neuropathic pain in mice (23). Importantly, a recent study discovered that a novel compound named BAY-985 potently and selectively inhibited IKKε/TBK1 (24).

This study aimed to investigate the functional involvement of IKKε in human chondrocytes and to evaluate the therapeutic effect of intra-articularly administered BAY-985 on cartilage degradation in a murine OA model. It was hypothesized that IKKε plays a crucial role in OA pathogenesis by regulating cellular signaling involving NF-κB and C/EBPβ. Identifying the mechanisms whereby IKKε regulates OA progression may lead to the development of a new disease-modifying drug.

Materials and Methods

Clinical samples

Human knee joints from individuals ages 18–65 years were obtained at autopsy with the approval of the Scripps Human Subjects Committee, or from patients undergoing total knee arthroplasty with the approval of the Ethics Committee of Kyushu University Hospital. The entire femoral condyles of normal knee joints were harvested from five donors (mean [± SD] age 36.4 ± 10.8 years [range 18–45 years]; OA grade I) with no

history of joint disease. Human OA joints were obtained from five donors (mean [± SD] age 63.0 ± 8.5 years [range 52–75 years]; OA grade III–IV). Written informed consent was obtained from all subjects.

Immunohistochemistry

Knee joints were fixed in 4% paraformaldehyde (PFA) (Wako Pure Chemical Industries) for 2 days, decalcified with KCX (FALMA) for 2 days, and embedded in paraffin. Samples were cut into 4-μm-thick sections, deparaffinized, and rehydrated; antigen retrieval was performed by incubation overnight with ethylenediaminetetraacetic acid (EDTA) (1 mM) at pH 8.0. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase in methanol for 30 minutes. For the blocking procedure, each specimen was placed in normal horse serum (Vectastain Universal Elite ABC kit; Vector Laboratories) for 30 minutes, then incubated for 1 hour at room temperature with primary anti-IKKε antibody (SC376114, Santa Cruz Biotechnology). Sections were incubated with biotinylated secondary antibodies for 30 minutes, followed by incubation with streptavidin–peroxidase complex (Vectastain Universal Elite ABC kit) for 30 minutes. Antibody complexes were visualized using the diaminobenzidine substrate system (Wako Pure Chemical Industries) and counterstained with hematoxylin. The percentage of cells

positive for IKKε in hematoxylin-stained cells was systematically determined based on three images per section using BZ-II Analyzer software (Keyence).

Cell isolation from human articular cartilage and human chondrocyte culture

Human articular knee cartilage was obtained aseptically from OA patients undergoing knee replacement surgery. All patients provided informed consent, and the protocol was approved by the Ethics Committee of Kyushu University Hospital. The cartilage fragments were digested with 2 mg/ml of collagenase for 12 hours. After digestion, chondrocytes were isolated and cultured in 10-cm dishes in Dulbecco's modified Eagle's medium/F-12 with 10% fetal bovine serum, then used at the time of sub-confluence (25). Human normal chondrocytes were purchased from Lonza. To evaluate the gene expression of IKKε, cells were treated with 10 μg/ml lipopolysaccharide (LPS), 1 ng/ml interleukin1β (IL1β), and 10 ng/ml tumor necrosis factor α (TNFα) for 6 hours.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and reverse-transcribed into cDNA using PrimeScript RT Reagent (Takara Bio). qRT-PCR was

performed on a CFX Connect Real-Time System (Bio-Rad) using TB Green Premix EX TaqII (Takara Bio). GAPDH was used as an internal control to normalize the levels of other target genes across samples. The sequences of the forward and reverse primers are provided in Table S1.

siRNA transfection

Human OA chondrocytes were seeded in 12-well plates at a density of 0.75×10^5 cells/well. After 1 day, they were transfected with small interfering RNAs (siRNAs) (10 nM) targeting IKKε (siIKKε; Santa Cruz Biotechnology), TBK1 (siTBK1; Santa Cruz Biotechnology), or p65 (sip65; Santa Cruz Biotechnology) using RNAiMAX (Thermo Fisher Scientific). The control group was transfected with control siRNA (siCtrl; Santa Cruz Biotechnology). Two days after transfection, cells were stimulated with or without LPS (10 μg/ml) or IL-1b (1 ng/ml) for 6 hours. The transfection efficiency was evaluated by qRT-PCR.

BAY-985 treatment of human normal and OA chondrocytes

Human normal and OA chondrocytes were treated for 48 hours with 1 μM, 5 μM, or 10 μM BAY-985 (MedChem Express), which was previously identified as an IKKε/TBK1

inhibitor (24). After BAY-985 treatment for 48 hours, cells were stimulated with LPS (10 μg/ml) for 6 hours.

IKKε overexpression

Recombinant adenoviral vectors encoding constitutively active IKKε or control Ad-GFP were purchased from SignaGen Laboratories. Human normal chondrocytes were infected with adenovirus using Lipofectamine 3000 (Thermo Fisher Scientific) at 15 multiplicities of infection with or without BAY-985 (10 μM) (26). Forty-eight hours after infection, cells were stimulated with LPS (10 μg/ml) for 6 hours.

Western blotting

Whole-cell lysates were extracted from human chondrocytes using LIPA lysis buffer (Sigma-Aldrich) with protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich). Cell lysates were electrophoresed in 4–12% gradient polyacrylamide gels (Thermo Fisher Scientific), and the resolved proteins were transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with blocking buffer (Takara Bio), washed in Tris-buffered saline with Tween (TBST), and incubated with primary antibodies (all from Cell Signaling Technology) against IKKε

(1:500; #2905), p-p65 (1:500; #3033), p65 (1:500; #8242), p-IκBα (1:500; #9246), IκBα (1:500; #4814), p-C/EBPβ (1:500; #3084), C/EBPβ LAP (1:500; #3087), and GAPDH (1:1000; #5174) diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (TOYOBO). After washing in TBST, secondary anti–rabbit IgG antibodies (1:1000; #7074, Cell Signaling Technology) or anti-mouse IgG antibodies (1:1000; sc-516102, Santa Cruz Biotechnology) were added. Immunoreactivity was detected with ECL Prime (Amersham Biosciences) and photographed using an Ez Capture MG (ATTO). Band densities were calculated using CS Analyzer 3.0 (ATTO) (25).

Mouse OA model

All animal experiments were approved by the Animal Experiment Committee of Kyushu University, and were performed according to the rules of our institution. Male C57Black6/J mice were obtained from SLC Japan. Mice were housed three to five mice in a cage and were able to freely access food and water in the Research Center for Human Disease Modeling of our institution. The center was maintained in a specific pathogen-free condition and on a 12 hours light/dark cycle at all times (27). OA was induced by destabilization of the medial meniscus (DMM), and the medial collateral ligaments (MCL) of 12-week-old mice were transected as previously described (28). Sham surgery

was performed in a separate group of mice using the same approach without DMM or MCL transection. The mice were euthanized 4 or 8 weeks after the operation, and subjected to histologic analysis. The group size is nine mice for 4 and 8 weeks after the operation in each group. Mice were randomly divided into each group based on a random number table. Group sizes were decided following power analysis; a minimum of 9 mice per group were required to detect a minimum 30% difference in means of Osteoarthritis Research Society International (OARSI) histopathology grading between application of BAY-985 and saline after the operation (power = 0.8, $\alpha = 0.05$), based on our previous study (26, 27). Knee sections in the medial sagittal plane were stained with Safranin O–fast green. OA severity was quantified by OARSI histopathology grading (on a scale of 0–6) (29) by two independent observers in a blinded manner, and averaged to minimize observer bias. The scores of the medial femur and tibia were added (total score of 0-12). Cartilage thickness was measured for the greatest perpendicular distance of medial tibial cartilage thickness by image J software (30). Osteophyte maturity and size were evaluated by examining anteromedial tibial regions in each mouse and were scored by two observers using a previously described scoring system (on a scale of 0–3) (31). Synovial inflammation was also scored by two observers using a previously described scoring system (on a scale of 0–3) (26, 32).

Application of BAY-985 in the OA model

DMM+MCL transection or sham operations were performed on the left knee joints of mice. The injection solution comprised 10 mM BAY-985 stock solution (MedChemExpress) diluted with saline. Ten microliters of either 10 μM BAY-985 or saline as vehicle was injected into the intra-articular spaces of each mouse knee joint starting from the day of surgery every 5 days for 4 or 8 weeks. Mice were euthanized 4 or 8 weeks after the operation.

Immunofluorescence

Sections were stained with primary antibody against IKKε (SC376114; Santa Cruz Biotechnology), phospho-IκBα (NB100-81987; Novus Biologicals), IL6 (#12912; Cell Signaling Technology), and MMP13 (ab39012; Abcam) at room temperature for 1 hour, then incubated with Alexa Fluor–conjugated secondary antibodies (Thermo Fisher Scientific). The numbers of IKKε–, phospho-IκBα–, MMP13-, and IL6–positive cells in the mouse model were quantified by counting immunopositive cells in sagittal sections of the knee joint at 100× magnification (IKK; n = 5 per group, p- IκBα, MMP13-, and IL6; n = 9 per group). The percentages of positively stained cells per section were counted

using the BZ-II Analyzer software program.

Knee hyperalgesia

Knee hyperalgesia was measured using a Pressure Application Measurement (PAM) device (Bioseb) by adapting previously described methods (33). Mice were manually restrained, and the hind paw was lightly pinned with a finger to hold the knee in flexion at a similar angle for each mouse. The PAM transducer was pressed against the medial side of the knee while the operator's thumb lightly held the lateral side of the knee. The operator increased the amount of force at a constant rate. When the mouse tried to withdraw its knee, the force was recorded. Three measurements were obtained per knee, and the withdrawal force data were averaged. Knee hyperalgesia was assessed before surgery and 2, 4, 6, and 8 weeks after sham or DMM+MCL transection surgery.

Statistical analysis

All experiments were repeated at least four times. Data are presented as means ± SD. Data normality was assessed by the Shapiro–Wilk test. When the distribution was normal, statistically significant differences between groups were determined by Student's t-test or the Tukey–Kramer test. When the distribution was not normal, the Wilcoxon test or Steel–

Dwass test was used. All data analyses were performed using JMP 14 statistical software (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

Results

IKKε expression is higher in OA cartilage and is increased by pro-inflammatory cytokines.

We initially performed immunohistochemical analysis to compare the expression of IKKε in human normal and OA cartilage. OA cartilage showed a significantly higher percentage of IKKε-positive cells than normal cartilage (Figure 1A). In addition, the gene expression of *IKKε* was significantly higher in human OA knee chondrocytes than in normal chondrocytes (Figure 1B). Finally, LPS, IL1β, and TNFα significantly increased the gene expression of *IKKε* in human normal and OA chondrocytes (Figure 1C).

IKKε knockdown with siRNA decreases *IL6, MMP13, ADAMTS4,* and *NOS2* gene expression in human OA chondrocytes.

To explore the involvement of IKKε in OA pathogenesis, we analyzed the effects of IKKε knockdown on inflammatory and catabolic gene expression in siIKKε-transfected human OA chondrocytes stimulated with LPS or IL1β. IKKε knockdown by siRNA significantly

attenuated the gene expression of *IL6, MMP13, and ADAMTS4* compared to siCtrl in the absence of LPS and IL1β. The expression of *IL6, MMP13, ADAMTS4,* and *NOS2* was significantly decreased by siIKKε in the presence of LPS (Figure 2A). Similar to LPS, in the presence of IL1β, siIKKε significantly attenuated the gene expression of IL6, MMP13, and ADAMTS4 compared to siCtrl (Figure S1). In contrast, NOS2 gene expression increased by siIKKε under IL1β stimulation.

TBK1 knockdown with siRNA increases *IKKε*, *MMP13*, *ADAMTS5,* and *NOS2* gene expressions in human OA chondrocytes, and then siIKKε decreases *IL6* and *MMP13* gene expressions in siTBK1-transfected cells.

To explore the functional involvement of TBK1 in OA pathogenesis, we analyzed the effect of TBK1 knockdown on inflammatory and catabolic gene expression in human OA chondrocytes stimulated with LPS. TBK1 knockdown by siRNA significantly increased the gene expression of *IKKε*, *MMP13*, *ADAMTS5*, and *NOS2* (Figure 2B). To eliminate the effect of IKKε upregulation, siIKKε/siTBK1-transfected chondrocytes were generated. The siIKKε significantly decreased the expression of *IL6* and *MMP13* in siTBK1-transfected chondrocytes (Figure 2B).

Effects of the IKKε/TBK1 inhibitor BAY985 in human normal and OA chondrocytes.

BAY-985 was used to analyze the effects of IKKε/TBK1 kinase activity inhibition in human normal and OA chondrocytes. LPS-induced gene expression of *IL6, MMP13, ADAMTS4,* and *NOS2* was significantly suppressed in a dose-dependent manner by 48 hours of pretreatment with BAY-985 (Figure 2C, D). BAY-985 most significantly attenuated the LPS-induced gene expression *of IL6, MMP13, ADAMTS4,* and *NOS2* at a concentration of 10 μM. At a concentration of 1 μM, BAY-985 significantly attenuated the expressions of *MMP13* and *ADAMTS4* in normal chondrocytes rather than OA chondrocytes.

BAY-985 restores the effect of IKKε overexpression in human normal chondrocytes.

Transfection with recombinant adenoviral vector in human normal chondrocytes increased IKKε protein expression (Figure 3A). IKKε overexpression significantly increased the expression of *IL6, MMP13, ADAMTS4,* and *NOS2* in the presence of LPS, and these increases were significantly suppressed down to the baseline by BAY-985 treatment (Figure 3B).

IKKε overexpression activates NF-κB signaling and BAY-985 reverses this effect.

IKKε overexpression in human normal chondrocytes significantly increased the phosphorylation of IκBα at $Ser^{32/36}$ compared to GFP control at 20 minutes after LPS stimulation, and the elevation was reversed by BAY-985 (Figure 4A). Furthermore, IKKε overexpression significantly increased the phosphorylation of p65. BAY-985 reversed this p-p65 elevation. On the other hand, total p65, IκBα, p-C/EBPβ, and total C/EBPβ levels were not affected by IKKε overexpression (Figure 4A).

p65 knockdown reverses the IKKε overexpression–mediated inflammatory response.

To explore the involvement of p65 downstream of IKKε, the effect of p65 knockdown was examined using sip65-transfected human normal chondrocytes stimulated with LPS. p65 knockdown significantly reversed the gene expression of *IL6, MMP13, ADAMTS4,* and *NOS2*, which was increased by IKKε overexpression (Figure 4B).

Cartilage degradation in mice with OA is delayed by intra-articular injection of BAY-985.

We examined the expression of IKKε in the cartilage of mice knee. Immunofluorescence showed that the percentage of IKKε-positive cells was higher in DMM+MCL transection mice than in sham-operated mice at 4 and 8 weeks after surgery (Figure 5A). We next

examined the effects of BAY-985 on cartilage degradation in the OA mouse model. BAY-985 solution or saline was injected into the knee joint space of DMM+MCL transection and sham-operated mice starting from the day of surgery every 5 days for 4 or 8 weeks. To assess the effects of intra-articular injection of BAY-985 in the early stage of OA, we examined histology at 4 weeks after surgery. OARSI score increased after DMM+MCL transection with no significant difference between vehicle and BAY-985 (Figure 5B). There were no significant differences in cartilage thickness, synovitis score, osteophyte maturity, and osteophyte size between sham and OA surgery. We evaluated the immunofluorescence of phospho-IκBα, Il6, and Mmp13 4 weeks after surgery. Compared to vehicle, BAY-985 significantly decreased the number of cells expressing p-IκBα, Il6, and Mmp13 in articular cartilage (Figure 5C, D).

Histologic analysis 8 weeks after surgery revealed that mouse knee joints treated with 10 μM of BAY-985 had a significantly lower OARSI score than those treated with vehicle control (Figure 6A). Cartilage thickness significantly decreased after DMM+MCL transection in vehicle control but not in BAY-985-treated group. The osteophyte maturity score increased after DMM+MCL transection, but with no significant difference between BAY-985 and vehicle control. On the other hand, DMM+MCL transection had no significant effect on osteophyte size and synovitis (Figure 6A). We examined knee

hyperalgesia before surgery and 2, 4, 6, and 8 weeks after surgery using the PAM device. Compared to vehicle, BAY-985 significantly decreased knee hyperalgesia at all time points (Figure 6B).

Discussion

This is the first study to characterize IKKε expression in human cartilage tissue and its OA-related functional involvement in chondrocytes. IKKε protein was more highly expressed in human cartilage in OA patients than in healthy controls. IKKε gene expression in human normal and OA chondrocytes was upregulated by pro-inflammatory factors such as LPS, IL1β, and TNFα, suggesting that IKKε is deeply involved in human OA pathogenesis. LPS-mediated catabolic factors, including IL6, MMP13, ADAMTS4, and NOS2, were downregulated by the loss of IKKε function. Conversely, gain of IKKε function increased the expression of these catabolic factors, and BAY-985, a selective inhibitor of IKKε/TBK1, reversed this effect. High expression of IKKε was observed in peripheral blood lymphocytes and other immune cells such as those in lymphoid tissues and the pancreas (16). IKKε has been reported to exacerbate the pathology of several kinds of cancers and obesity-related metabolic dysfunctions (9, 34). In a mouse model of collagen □–induced rheumatoid arthritis, IKKε increased nociceptive and inflammatory

responses (18). Taken together, IKKε is deeply involved in the pathogenesis of a variety of diseases.

In contrast to siIKKε, siTBK1, a homologous protein of IKKε, increased MMP13 and NOS2 gene expressions with accompanying IKKε upregulation in human OA chondrocytes. Subsequently, siIKKε significantly decreased the expression of IL6 and MMP13 in siTBK1-transfected cells. These results suggest that at least, upregulated IKKε but not TBK1 plays a pathogenic role in OA-related inflammatory and catabolic gene expression in human chondrocytes. Although the interaction and feedback between IKKε and TBK1 in chondrocytes remain unclear, the effects of BAY-985 in this study would be due to primarily IKKε inhibition.

Further mechanistic analyses demonstrated the effect of IKKε on the regulation of NF-κB but not C/EBPβ in human chondrocytes. In particular, overexpression of IKKε increased both IκBα and p65 phosphorylation, which induce NF-κB dimer migration to the nucleus. Validating this finding, the effect of IKKε overexpression on the gene expression of catabolic factors was restored by p65 knockdown using siRNA. NF-κB signaling is closely associated with OA development (6-8), although targeted therapeutic strategies to inhibit this signaling have not yet been established. Previous studies showed that use of an adenoviral vector or peptide nanoparticle to deliver siRNA targeting NF-

κB into knee joints suppressed cartilage degradation in rat and mouse OA models (35, 36). However, safety concerns remain even if the transfection efficacy of viral-based vectors is excellent (37). Intra-articular administration of decoy oligodeoxynucleotides that inhibit NF-κB activation was previously shown to suppress OA progression (38). However, the half-life of free oligodeoxynucleotides was found to be too short to achieve a sufficient therapeutic effect (39).

In a surgically induced OA mouse model, intra-articular administration of BAY-985 elicited decreases in IκBα phosphorylation and catabolic factor expressions, followed by a protective effect against cartilage degradation. These results indicate that IKKε inhibition by BAY-985 attenuated cartilage degradation by suppressing the catabolic response via NF-κB signaling. At 4 weeks, DMM+MCL transection exacerbated OARSI score with no significant difference between vehicle and BAY-985, although the score change might be too small to detect the effect of BAY-985 in the early stage of OA. Possibly, BAY-985 did not prevent the onset of cartilage injury caused by mechanical stress, but rather the subsequent cartilage degradation caused by biochemical stress from 4 to 8 weeks after surgery in this OA model.

There was a discrepancy between histology and hyperalgesia in the BAY-985 efficacy. Hyperalgesia started to decrease at 2 weeks after surgery by BAY-985 treatment, whereas

there were no significant histological changes in cartilage, synovium, and osteophyte at least 4 weeks after surgery. A histological study also reported that the DMM+MCL transection model showed only a little cartilage degradation at 2-4 weeks (28). It is possible that the effect of BAY-985 on hyperalgesia was not based on the histological change in joint tissues. This discrepancy may be explained by the effect of IKKε inhibition on neuropathic pain. A previous study reported that genetic depletion or pharmacological inhibition of IKKε led to a significant reduction of mechanical hyperalgesia mediated by reduced activation of NF-κB in mice (23). Therefore, BAY-985 injection might decrease hyperalgesia as early as 2 weeks after OA surgery, due to the pain suppression effect of IKKε inhibition.

A previous study showed that homozygous knockout of p65 caused OA development by inducing chondrocyte apoptosis, whereas p65 hetero knockout slowed OA development by suppressing matrix-degrading enzymes (7). IKKα, IKKβ, IKKγ, or TBK1 knockout mice die embryonically or shortly after birth (40-44). By contrast, IKKε knockout mice are viable and fertile, meaning that IKKε does not completely inhibit NF-κB signaling at the level of life-threatening (45). Furthermore, IκBα degradation in IKKε-deficient mice was shown to be unaltered after stimulation with TNF, IL1, LPS, or polyI:C (10). Therefore, an IKKε inhibitor might be safer and have advantages over IKKα/β/γ

inhibitors as a therapeutic target for OA. In this study, the 5-day injection windows were able to gain these positive effects, but optimal dosing intervals need to be determined in the future clinical safety and efficacy trials.

This study had several limitations. First, IKKε inhibition had inconsistent effects on NOS2 gene expression in a siRNA experiment. In the presence of LPS, the gene expression of NOS2 was decreased, while in the absence of LPS, it was unchanged. In the presence of IL1β, NOS2 gene expression increased by siIKKε. Second, BAY-985 is an ATP-competitive kinase inhibitor of IKKε/TBK1, therefore, it is difficult to elucidate how much of its effects could be due to IKKε or TBK1 inhibition. Third, we should have used IKKε knockout mice to further elucidate the involvement of IKKε in OA pathogenesis.

In conclusion, our results demonstrated that IKKε was highly expressed in OA cartilage and that IKKε knockdown suppressed catabolic responses mediated by NF-κB signaling both in vivo and in vitro. IKKε is a positive regulator of inflammatory responses in OA, and therefore BAY-985 may be a potent compound in OA treatment.

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Author Contribution

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Akasaki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Uchida, Akasaki, Sueishi, Tsushima, Lotz, Nakashima.

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Analysis and interpretation of data. Uchida, Akasaki, Sueishi, Kurakazu, Toya, Kuwahara, Hirose, Hyodo.

Reference

1. Lotz M, Loeser RF. Effects of aging on articular cartilage homeostasis. Bone 2012;51:241-48.
2. Berenbaum F. Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!). Osteoarthritis and Cartilage 2013;21:16-21.
3. Hayashida M, Okazaki K, Fukushi J, Sakamoto A, Iwamoto Y. CCAAT/enhancer binding protein beta mediates expression of matrix metalloproteinase 13 in human articular chondrocytes in inflammatory arthritis. Arthritis Rheum 2009;60:708-16.
4. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. Clin Orthop Relat Res 2004:S27-36.
5. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, et al. Purification and cloning of aggrecanase-1: A member of the ADAMTS family of proteins. Science 1999;284:1664-66.
6. Choi, Jo, Park, Kang, Park. NF-B Signaling Pathways in Osteoarthritic Cartilage Destruction. Cells 2019;8:734.
7. Kobayashi H, Chang SH, Mori D, Itoh S, Hirata M, Hosaka Y, et al. Biphasic regulation of chondrocytes by Rela through induction of anti-apoptotic and catabolic target genes. Nature Communications 2016;7:13336.
8. Saito T, Fukai A, Mabuchi A, Ikeda T, Yano F, Ohba S, et al. Transcriptional regulation of endochondral ossification by HIF-2α during skeletal growth and osteoarthritis development. Nature Medicine 2010;16:678-86.
9. Yin M, Wang X, Lu J. Advances in IKBKE as a potential target for cancer therapy. Cancer Medicine 2020;9:247-58.
10. Shin C, Choi D-S. Essential Roles for the Non-Canonical IκB Kinases in Linking Inflammation to Cancer, Obesity, and Diabetes. Cells 2019;8:178.
11. Bhatt D, Ghosh S. Regulation of the NF-kappaB-Mediated Transcription of Inflammatory Genes. Front Immunol 2014;5:71.
12. Olivotto E, Otero M, Marcu KB, Goldring MB. Pathophysiology of osteoarthritis: canonical NF-kappaB/IKKbeta-dependent and kinase-independent effects of IKKalpha in cartilage degradation and chondrocyte differentiation. RMD Open 2015;1:e000061.
13. Culley KL, Lessard SG, Green JD, Quinn J, Chang J, Khilnani T, et al. Inducible knockout of CHUK/IKKα in adult chondrocytes reduces progression of cartilage degradation in a surgical model of osteoarthritis. Scientific Reports 2019;9.
14. Grothe K, Flechsenhar K, Paehler T, Ritzeler O, Beninga J, Saas J, et al. IκB kinase inhibition as a potential treatment of osteoarthritis – results of a clinical proof-of-concept

study. Osteoarthritis and Cartilage 2017;25:46-52.

15. Murahashi Y, Yano F, Kobayashi H, Makii Y, Iba K, Yamashita T, et al. Intra-articular administration of IkappaBalpha kinase inhibitor suppresses mouse knee osteoarthritis via downregulation of the NF-kappaB/HIF-2alpha axis. Sci Rep 2018;8:16475.
16. Shimada T, Kawai T, Takeda K, Matsumoto M, Inoue J, Tatsumi Y, et al. IKK-i, a novel lipopolysaccharide-inducible kinase that is related to I kappa B kinases. International Immunology 1999;11:1357-62.
17. Pomerantz JL. NF-kappa B activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. The EMBO Journal 1999;18:6694-704.
18. Zhou LF, Zeng W, Sun LC, Wang Y, Jiang F, Li X, et al. IKKepsilon aggravates inflammatory response via activation of NF-kappaB in rheumatoid arthritis. Eur Rev Med Pharmacol Sci 2018;22:2126-33.
19. Verhelst K, Verstrepen L, Carpentier I, Beyaert R. IkappaB kinase epsilon (IKKepsilon): a therapeutic target in inflammation and cancer. Biochem Pharmacol 2013;85:873-80.
20. Shen RR, Hahn WC. Emerging roles for the non-canonical IKKs in cancer. Oncogene 2011;30:631-41.
21. Peant B, Gilbert S, Le Page C, Poisson A, L'Ecuyer E, Boudhraa Z, et al. IkappaB-Kinase-epsilon (IKKepsilon) over-expression promotes the growth of prostate cancer through the C/EBP-beta dependent activation of IL-6 gene expression. Oncotarget 2017;8:14487-501.
22. Wang N, Ahmed S, Haqqi TM. Genomic structure and functional characterization of the promoter region of human IκB kinase-related kinase IKKi/IKKε gene. Gene 2005;353:118-33.
23. Moser CV, Moller M, Fleck SC, Thomas D, Geisslinger G, Niederberger E. Inhibition of the protein kinase IKKepsilon attenuates neuropathic pain in mice. Neuropharmacology 2019;146:198-211.
24. Lefranc J, Schulze VK, Hillig RC, Briem H, Prinz F, Mengel A, et al. Discovery of BAY-985, a Highly Selective TBK1/IKKepsilon Inhibitor. J Med Chem 2020;63:601-12.
25. Kurakazu I, Akasaki Y, Tsushima H, Sueishi T, Toya M, Kuwahara M, et al. TGFβ1 signaling protects chondrocytes against oxidative stress via FOXO1–autophagy axis. Osteoarthritis and Cartilage 2021.
26. Sueishi T, Akasaki Y, Goto N, Kurakazu I, Toya M, Kuwahara M, et al. GRK5 Inhibition Attenuates Cartilage Degradation via Decreased NF-kappaB Signaling. Arthritis Rheumatol 2019.
27. Kuwahara M, Akasaki Y, Kurakazu I, Sueishi T, Toya M, Uchida T, et al. C10orf10/DEPP activates mitochondrial autophagy and maintains chondrocyte viability in the pathogenesis of osteoarthritis. The FASEB Journal 2022;36.

28. Kamekura S, Hoshi K, Shimoaka T, Chung U, Chikuda H, Yamada T, et al. Osteoarthritis development in novel experimental mouse models induced by knee joint instability. Osteoarthritis and Cartilage 2005;13:632-41.
29. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the mouse. Osteoarthritis and Cartilage 2010;18:S17-S23.
30. Joiner DM, Less KD, Van Wieren EM, Hess D, Williams BO. Heterozygosity for an inactivating mutation in low-density lipoprotein-related receptor 6 (Lrp6) increases osteoarthritis severity in mice after ligament and meniscus injury. Osteoarthritis and Cartilage 2013;21:1576-85.
31. Little CB, Barai A, Burkhardt D, Smith SM, Fosang AJ, Werb Z, et al. Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. Arthritis & Rheumatism 2009;60:3723-33.
32. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, et al. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nature Medicine 2009;15:774-80.
33. Miller RE, Ishihara S, Bhattacharyya B, Delaney A, Menichella DM, Miller RJ, et al. Chemogenetic Inhibition of Pain Neurons in a Mouse Model of Osteoarthritis. Arthritis & Rheumatology 2017;69:1429-39.
34. Reilly SM, Chiang SH, Decker SJ, Chang L, Uhm M, Larsen MJ, et al. An inhibitor of the protein kinases TBK1 and IKK-varepsilon improves obesity-related metabolic dysfunctions in mice. Nat Med 2013;19:313-21.
35. Chen LX, Lin L, Wang HJ, Wei XL, Fu X, Zhang JY, et al. Suppression of early experimental osteoarthritis by in vivo delivery of the adenoviral vector-mediated NF-κBp65-specific siRNA. Osteoarthritis and Cartilage 2008;16:174-84.
36. Yan H, Duan X, Pan H, Holguin N, Rai MF, Akk A, et al. Suppression of NF-κB activity via nanoparticle-based siRNA delivery alters early cartilage responses to injury. Proceedings of the National Academy of Sciences 2016;113:E6199-E208.
37. Wang D, Gao G. State-of-the-art human gene therapy: part I. Gene delivery technologies. Discov Med 2014;18:67-77.
38. Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. Osteoarthritis Cartilage 2006;14:839-48.
39. De Stefano D. Oligonucleotides decoy to NF-kappaB: becoming a reality? Discov Med 2011;12:97-105.
40. Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, et al. IKK1-deficient

mice exhibit abnormal development of skin and skeleton. Genes & Development 1999;13:1322-28.

41. Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM. Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science 1999;284:321-5.

42. Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, et al. Embryonic Lethality, Liver Degeneration, and Impaired NF-κB Activation in IKK-β-Deficient Mice. Immunity 1999;10:421-29.

43. Rudolph D, Yeh WC, Wakeham A, Rudolph B, Nallainathan D, Potter J, et al. Severe liver degeneration and lack of NF-kappaB activation in NEMO/IKKgamma-deficient mice. Genes & development 2000;14:854-62.

44. Bonnard M. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. The EMBO Journal 2000;19:4976-85.

45. Hemmi H, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, et al. The Roles of Two IκB Kinase-related Kinases in Lipopolysaccharide and Double Stranded RNA Signaling and Viral Infection. Journal of Experimental Medicine 2004;199:1641-50.

Figure legends:

Figure 1. IKKε expression in human cartilage and chondrocytes.

A, Left, representative immunohistochemical staining for IKKε in human normal and OA cartilage (bars = 100 μm). Right, quantification of IKKε-positive cells in human normal and OA cartilage. Values are means ± SD (n=5). B, Relative mRNA levels of *IKKε* in human normal and OA chondrocytes. Values are means ± SD (n=5). C, Relative mRNA levels of *IKKε* in human normal (left) and OA (right) chondrocytes treated with 10 μg/ml LPS, 1 ng/ml IL1β, and 10 ng/ml TNFα for 6 hours as determined by qRT-PCR. Values are means ± SD (normal; n=6, OA; n=5). *P < 0.05; ***P < 0.001.

Figure 2. Effects of IKKε or TBK1 knockdown by siRNA or BAY-985 on mRNA levels of catabolic factors.

A, Relative mRNA levels of catabolic factors in siIKKε-transfected human OA chondrocytes with or without LPS. Values are means ± SD (n=6). B, Relative mRNA levels of catabolic factors in siTBK1- or siTBK1/IKKε-transfected human OA chondrocytes with LPS. Values are means ± SD (n=7). C and D, Changes in LPS-induced gene expression in human normal (C) and OA (D) chondrocytes treated with BAY-985 (1, 5, or 10 μM) for 48 hours. Values are means ± SD (n=7). *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 3. Effects of IKKε overexpression and BAY-985 on mRNA levels of catabolic factors.

A, Western blot (left) and quantification (right) of IKKε expression with IKKε (Ad-IKKε) or GFP (Ad-GFP) overexpression in human normal chondrocytes treated with 10 μg/ml LPS. GAPDH was used as a loading control. Values are means ± SD (n=4). B, Changes in LPS-induced gene expression in human normal chondrocytes with or without IKKε overexpression and with or without BAY-985 treatment. Values are means ± SD (n=6). **P < 0.01; ***P < 0.001.

Figure 4. IKKε overexpression activates NF-κB signaling and BAY-985 reverses the effect.

A, Western blot (left) and quantification (right and below) of expression of p-IκBα, IκBα, p-p65, p65, p-C/EBPβ, and C/EBPβ with IKKε overexpression and BAY-985 treatment in human normal chondrocytes with or without LPS (20 minutes) stimulation. GAPDH was used as a loading control. Values are means ± SD (n=6). B, Changes in LPS-induced gene expression in human normal chondrocytes with IKKε overexpression and sip65 transfection. Chondrocytes were stimulated by LPS for 6 hours. Values are means ± SD (n=6). *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 5. In vivo effects of intra-articular administration of BAY-985 on surgically induced OA mice 4 weeks after surgery.

A, Representative immunofluorescence images of Ikkε (red) and quantification of Ikkε-positive cells 4 weeks (Left) or 8 weeks (Right) after DMM+MCL transection surgery or sham surgery in mice (bars = 100 μm). Values are means ± SD (n=5). B, Left, representative images of Safranin O–fast green staining of the knee joints of mice subjected to sham surgery or DMM+MCL transection 4 weeks after surgery, and treated

with saline or BAY-985 (bars = 100 μm). Right, quantification of cartilage degradation, as measured by OARSI score, cartilage thickness, synovitis, osteophyte maturity and size 4 weeks after surgery in the indicated groups. Values are means ± SD (n=9 per group). C and D, Left, representative immunofluorescence images of phospho-IκBα (C), Il6 (D), and Mmp13 (D) 4 weeks after sham or DMM + MCL transection surgery in mice treated with saline or BAY-985 (bars = 100 μm). Right, Quantification of p-IκBα– (C), Il6- (D), and Mmp13 (D)-positive cells. Values are means ± SD (n=9). **P < 0.01; ***P < 0.001.

Figure 6. In vivo effects of intra-articular administration of BAY-985 on surgically induced OA mice 8 weeks after surgery.

A, Histologic assessment by Safranin O–fast green staining of the knee joints of mice subjected to sham surgery or DMM+MCL transection 8 weeks after surgery, and treated with saline or BAY-985 (bars = 100 μm). Above, representative images of cartilage, synovium, and osteophyte. Below, quantification of cartilage degradation, as measured by OARSI score, cartilage thickness, synovitis, osteophyte maturity and size 8 weeks after surgery in the indicated groups. Values are means ± SD (n=9 per group). B, Knee hyperalgesia assessed using the PAM device before surgery and 2, 4, 6, and 8 weeks after surgery in mice treated with saline or BAY-985. Values are means ± SD (n=9). **P < 0.01;

***P < 0.001.

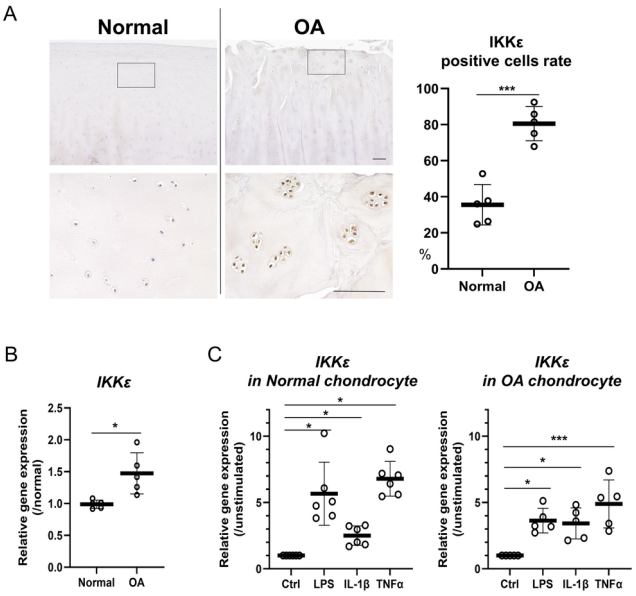
Table S1. Primer sequences used for real-time PCR

Figure S1. Effects of IKKε knockdown by siRNA on gene expression of catabolic factors in response to IL1β.

Changes in gene expression in response to IL1β in human OA chondrocytes transfected siIKKε. Values are means ± SD (n=6). *P < 0.05; **P < 0.01; ***P < 0.001.

Figure S2. Representative images of synovium and osteophyte in 4 weeks after surgery.

A and B, Representative images of synovium (A) and osteophyte (B) 4 weeks after sham or DMM+MCL transection surgery, with saline or BAY-985.



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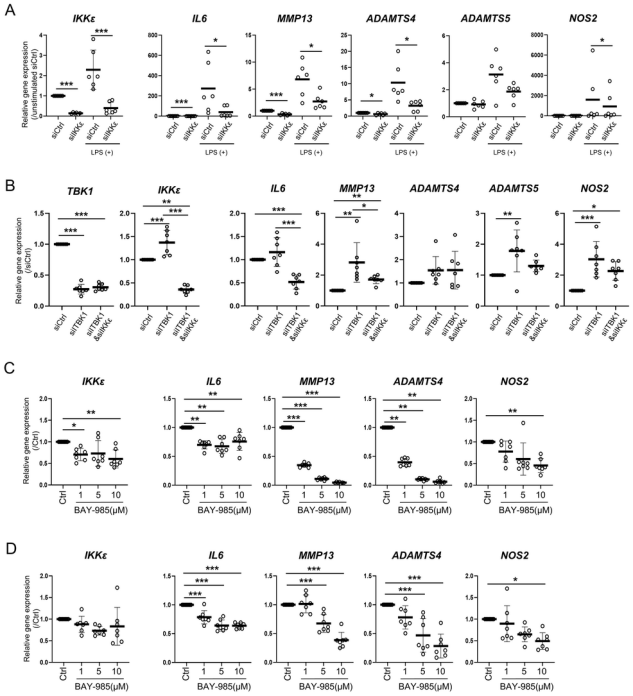

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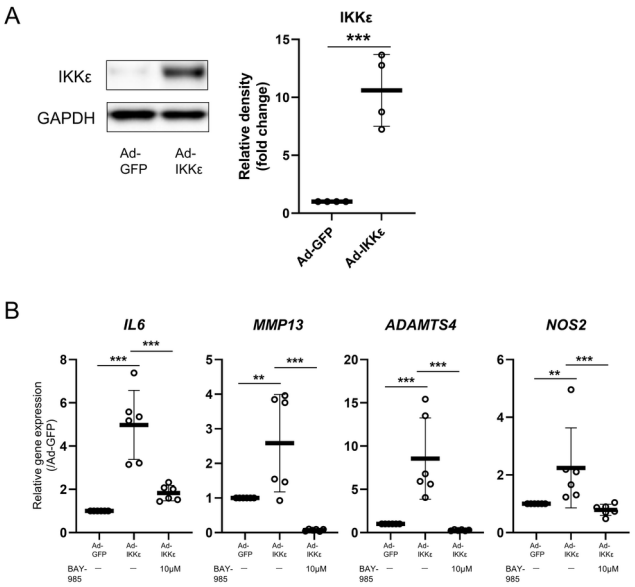

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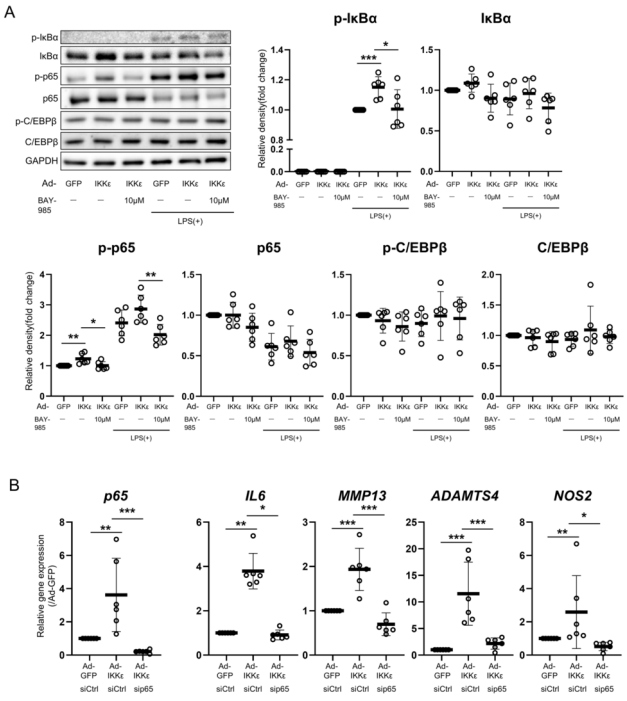

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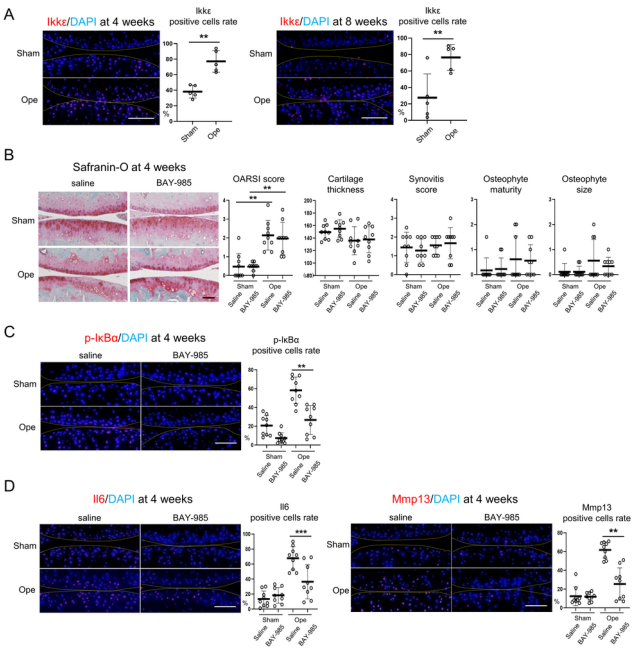

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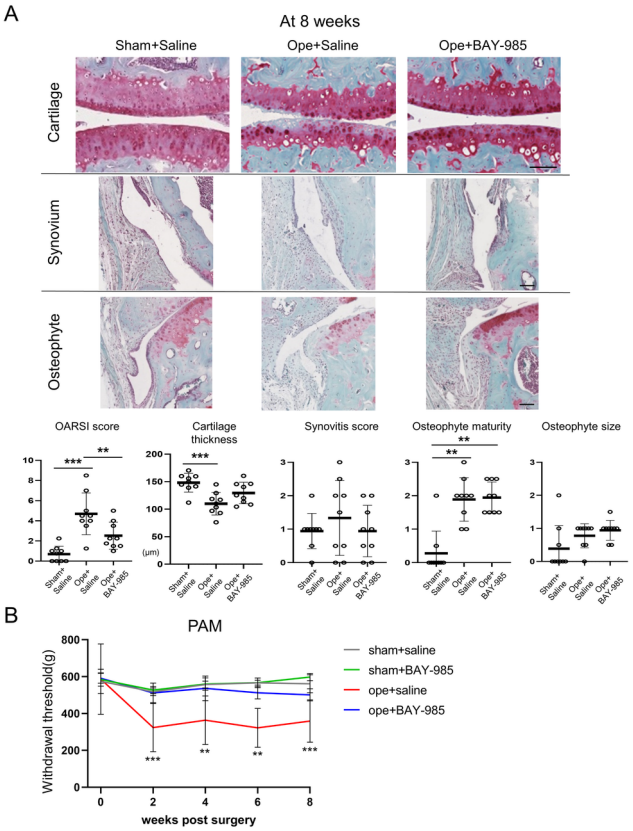

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