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Optineurin in Synovial Fibroblasts Plays a Protective Role against Joint Destructions in Rheumatoid Arthritis

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Running head: Optineurin and Joint Destructions in RASFs

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Abstract

Objectives: Optineurin (OPTN) is an autophagy adaptor/receptor which acts as an intrinsic negative regulator of osteoclast differentiation. Receptor activator of nuclear factor-κB ligand (RANKL) expressed by rheumatoid arthritis synovial fibroblasts (RASFs) is primarily responsible for bone erosions in RA. We aim to explore the role of OPTN in the pathogenesis of joint destructions in RA.

Methods: Synovial fibroblasts (SFs) from RA patients were incubated with/without TNF-α/IFN-γ and the expression of OPTN was analyzed using RT-qPCR and western blot. RANKL and osteoprotegerin (OPG) expression were evaluated in OPTN siRNA transfected RASFs with/without TNF-α/IFN-γ. OPTN-reduced RASFs were cocultured with monocytes and stained with Tartrate-Resistant Acid Phosphatase (TRAP). Matrix metallopeptidase 3 (MMP3), IL-6, GATA-3, carbohydrate sulfotransferase 15 (CHST15), and hyaluronan synthase 1 (HAS1) mRNA levels were analyzed by RT-qPCR. Protein levels of IκBα, NF-κB1 and RelA were measured to evaluate NF-κB signaling.

Results: OPTN levels were upregulated after TNF-α/IFN-γ stimulation. RANKL was increased following treatment with TNF-α/IFN-γ and the effect was further pronounced in OPTN-reduced RASFs OPG mRNA levels remained unchanged. Monocytes cocultured with OPTN-reduced RASFs differentiated more into $TRAP^+$ multinucleated-cells compared to those cocultured with control RASFs. MMP3 was upregulated while GATA-3, CHST15 and HAS1 were downregulated in OPTN-reduced RASFs. IκBα degradation and nuclear NF-κB1, RelA expression following TNF-α treatment was prolonged in OPTN-reduced RASFs.

Conclusion: OPTN plays a protective role in RA with its upregulation when induction by pro-inflammatory cytokines. Absence of OPTN might worsen RA by generating joint destructive state including increased RANKL expression on RASFs and subsequent osteoclast differentiation.

Keywords: rheumatoid arthritis, OPTN, RANKL, joint destruction, osteoclast

Words: 3938

Introduction

Rheumatoid arthritis (RA) is characterized by chronic joint inflammation leading to progressive cartilage and bone destruction. Synovial fibroblasts (SFs) are the main stromal cells which produce extracellular matrix components of synovial fluid in normal joint. However, in RA, SFs proliferate excessively and produce a huge amount of inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs), destroying adjacent cartilage (1).

Receptor activator of nuclear factor kappa B ligand (RANKL) plays an essential role in osteoclast formation by binding to RANK expressed on osteoclast precursors (2). RANKL exists in a soluble form and in a cellular, membrane-bound form (3). A recent study has reported that, among different forms, RANKL expressed on SF membrane plays a major role in bone erosions during joint inflammation (4).

Optineurin (OPTN) is an autophagy adaptor/receptor with multiple properties and its expression is enhanced by tumor necrosis factor-α (TNF-α) and interferons (IFNs) (5). OPTN has ubiquitin-binding domain and LC3 interacting region and therefore promotes the degradation of polyubiquitinated substrates which is termed selective autophagy. Among selective autophagy, OPTN particularly targets aggregated proteins, mitochondria, and bacteria which are termed aggrephagy, mitophagy, and xenophagy, respectively (6). OPTN has been reported to act as a negative regulator of osteoclast differentiation *in vitro* where loss of OPTN increased osteoclast activity and bone turnover (7). The insufficient function of OPTN has been reported to be related to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (8). Further investigation on the role of OPTN in other diseases including autoimmune disease has recently been explored (9). A number of studies have clarified the multifunction of OPTN in cell homeostasis and disease

pathogenesis (5). Among these functions, OPTN regulates nuclear factor kappa B (NF-κB) signaling, one of the most important inflammatory pathways in RA (10)

Given the possible involvement of OPTN in RA, we herein investigated the role of OPTN in SFs in the pathogenesis of joint destructions in RA, particularly its role in the expression of RANKL on SFs and subsequent osteoclast differentiation.

Materials and Methods

Patient synovial tissues and cell preparation

The protocol for collecting synovial samples and the use of human whole blood were approved by the institutional review boards (IRBs) of Hokkaido University Hospital (Approval number: 008-0103). Synovial tissues were obtained during synovectomy or total joint replacement surgery from patients with RA with written informed consent. All patients with RA fulfilled the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 criteria for the classification of RA (11). Demographic characteristics of patients are summarized in Supplementary Table 1. A portion of the obtained synovial tissue in each instance was evaluated immunohistochemically (Supplementary File, Method). RASFs were isolated by enzymatic digestion of the synovial tissues as described elsewhere (12). RASFs with passage 4-8 were used in this study. The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin with 100 μg/ml streptomycin (Sigma Aldrich). Human monocytes were isolated from whole blood of healthy individuals, using RosetteSep^{TM} human monocyte enrichment cocktail (Stemcell) according to manufacturer's instructions.

Proinflammatory cytokine stimulation in RASFs

TNF-α and IFN-γ were selected for treatment of RASFs because of their major involvement in the pathogenesis of RA (13). 10ng/ml or 100ng/ml of cytokines were selected for treatment after referring to previous studies (14-23). RASFs were seeded at a density of 1×10^5 cells/well in 6 well plate overnight. RASFs were treated with 10 ng/ml or 100 ng/ml of TNF-α (Sigma Aldrich) or IFN-γ (Biolegend) for 4 or 24 hours and the expression of OPTN was

analyzed by real-time PCR. Similar procedures were performed after 24 hours cytokine stimulation to analyze the OPTN protein production by Western blotting. For control, RASFs were treated with matched amount of phosphate-buffered saline (PBS).

siRNA transfection in RASFs

RASFs were seeded at a density of 1×10^5 cells/well in 6 well plate overnight and transfected by OPTN siRNA (s19720, ThermoFisher) or negative control siRNA for 24 hours using lipofectamine RNAiMAX (ThermoFisher). Knockdown efficiency was verified by real-time PCR 24 hours after transfection or and Western blotting 48 hours after transfection.

Real-time quantitative RT-PCR

Total RNA was obtained from RASFs using TRIzol RNA Reagent (Life Technologies, USA), according to the manufacturer's instructions. The RNA samples (2μg) were reverse-transcribed using SuperScript VILO (Invitrogen, USA) according to the manufacturer's instructions. The cDNA samples were diluted 100 times before running PCR. A standard qPCR approach was then used to monitor the levels of transcripts in RASFs. In the experiments, the level of transcripts was normalized to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript using an Applied Biosystems 7500 Real-Time PCR System and TaqMan MGB primers specific for the transcripts that encode *RANKL* (Hs00243522_m1), *OPG (Hs00900358_m1)*, *IL-6* (Hs00985639_m1), *MMP-3* (Hs00968305_m1) and *GAPDH* (Hs99999905_m1). Relative quantification was performed using the comparable cycle threshold (CT) method in which ΔCT is the level of the transcripts in the RNA sample relative to that of the GAPDH transcript. The difference in the expression of the transcripts among each sample was defined as fold changes in mRNA levels by $2^{-\Delta\Delta CT}$. For other mRNA expression

analysis, Fast SYBR Green MasterMix (ThermoFisher) was used according to manufacturer's protocols. Primers for human genes were shown in Supplementary Table 2.

Immunoblotting

RASFs were lysed using cell lysis buffer (Wako, Japan) including protease and phosphatase inhibitors (Sigma-Aldrich). For the analysis of nuclear NF-κB1 and RelA, RASFs were lysed using EpiQuik Nuclear Extraction Kit (Epigentek) according to the manufacturer's protocol. Supernatant of the cell lysates were loaded onto a gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to a polyvinylidene difluoride membrane. The membrane was blocked by 0.02% of bovine serum albumin (Sigma) for 1 hour at room temperature. After blocking, the membranes were probed with antibodies against OPTN (1:200 dilution, Abcam, ab23666), IκBα (1:1000 dilution, Cell Signaling, 4814), NF-κB1 p105/p50 (1:1000 dilution, Cell Signaling, 13586), RelA/p65 (1:1000 dilution, Cell Signaling, 8242) or β-actin (Sigma-Aldrich, A3854) and histone-3 (1:1000 dilution, Cell Signaling, 4499) as endogenous control and incubated overnight at 4 °C in Solution 1 (Toyobo, Japan). All primary antibodies are rabbit-derived antibodies except IκBα which is mouse-derived antibody. HRP-conjugated secondary antibodies were added after washing. As secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit (Sigma-Aldrich, A4914) and goat anti-mouse antibodies (Sigma-Aldrich, A8642) were used. Amerisham® ECL® Western Blotting Detection Reagents (GE Healthcare, UK) were used for detection. Signals were detected using enhanced chemiluminescence Western blot detection reagents (GE healthcare) and LAS-4000 imaging (Fujifilm). For quantification of protein expression, densitometric analysis was performed using ImageJ software. To avoid inaccuracy, the bands were only compared within the same blot.

Flow cytometry

Single-cell suspensions of RASFs were detached by accutase digestion. Cell were counted and suspended at 1×10^6 /ml in FACS buffer and double-stained with PE-conjugated anti-human RANKL monoclonal antibody (Biolegend, 347504) or PE-conjugated anti-mouse IgG2b, κ (Biolegend, 400314) and propidium iodide (PI) (Sigma-Aldrich, P4170) for 30mins on ice in the dark with direct staining method. Stained cells were applied to BD FACSCalibur™ (BD Biosciences) and analyzed using FlowJo software (Tree Star). PI^- RASFs were gated as live cells to further evaluate $RANKL^+$RASFs.

Proliferation assay

RASFs were cultured in 96-well plates at a density of 1×10^4 cells/well for 72 hours after OPTN or control siRNA transfection. to evaluate the cell proliferation rate using cell proliferation ELISA, BrdU kit (Roche, 1647229). RASFs were then incubated for 24 hours after adding bromodeoxyuridine (BrdU). Pyrimidine analogue BrdU is incorporated in place of thymidine into the DNA of proliferative cells during this labeling period. The incorporated BrdU was detected using anti-BrdU antibody conjugated with peroxidase and quantified by substrate reaction. The results were measured using a Multiskan Ascent Plate reader (Thermo Electron Corporation, USA) with wavelength at 370nm (reference wavelength: 492nm) and were expressed as final optical density.

Coculture of monocytes and RASFs

RASFs were seeded onto 24-well plate (1×10^3 cells/well) and transfected with OPTN siRNA or control siRNA for 24 hours. As described above, isolated human monocytes (3×10^4 cells/well) were added to the OPTN-reduced or control RASFs with fresh media. The cells were cocultured for 9 days in α-Minimum Essential Medium and 10% FBS in the presence of

25ng/ml macrophage colony-stimulating factor (R&D, 216), without antibiotics. The medium was changed every 3 days. The addition of 50 ng/ml recombinant RANKL protein (R&D, 390) was used as a positive control (24) and it was added on day 0, day 3 and day 6 when the fresh medium was replaced. Tartrate-resistant acid phosphatase (TRAP)-positive cells were identified using a leukocyte acid phosphatase kit according to the manufacturer's recommended protocol (Sigma-Aldrich, 387A). TRAP-positive cells with 3 or more nuclei were considered as osteoclasts. $TRAP^+$ multinucleated cells (MNC) were counted in each whole well under light microscope with ×100 magnification.

Statistical analysis

Statistical analysis was performed using Graphpad Prism® software. Student's *t* test or one-way analysis of variance (ANOVA) was used for ~~analyzation~~ analysis. The data are expressed as the mean ± SD. P values less than 0.05 were considered significant. Parametric analysis is used for normal distribution and ~~which~~ non-parametric analysis is used for non-normal distribution.

Results

OPTN is upregulated in the presence of TNF-α or IFN-γ

To mimic the inflammatory conditions developing in RA joints, we first treated RASFs with TNF-α or IFN-γ. The expression of OPTN was evaluated by real-time PCR or Western blotting following the cytokine stimulation. OPTN mRNA expression was upregulated 4 hours after stimulation with TNF-α or IFN-γ. The upregulation of OPTN mRNA in RASFs was sustained and further pronounced 24 hours after the stimulation, about 2-fold higher with TNF-α or 4-fold higher with IFN-γ (Figure 1A, 1B). The upregulation of OPTN was confirmed at protein levels by Western blot analysis of cells following 24 hours treatment with TNF-α or IFN-γ (Figure 1C, 1D).

OPTN is successfully knockdown by siRNA

We successfully downregulated OPTN at both mRNA (96.663%±2.025%) and protein (80.67%±6.064%) levels (Figure 2A, 2B) by siRNA and lipofectamine RNAiMAX transfection system.

Downregulation of OPTN increases the expression of RANKL on RASFs

Given the role of OPTN as a negative regulator of osteoclast differentiation (7), we next downregulated OPTN using siRNA and evaluated RANKL expression on RASF membrane. The expression of RANKL on RASFs was slightly increased following treatment with TNF-α or IFN-γ, and significantly increased with both cytokines, in both control RASFs and OPTN-downregulated RASFs. Further, on OPTN-downregulated RASFs, compared to RASFs transfected with control siRNA, the increase of RANKL was more pronounced with statistical significance (Figure 2C, 2D). TNF-α significantly upregulated RANKL mRNA

expression in control RASFs but there was no significant difference in OPTN-downregulated RASFs. This might due to the wide range of standard deviation in patients. However, the increase of RANKL was also observed at mRNA levels in OPTN-downregulated RASFs (Figure 2E). Although the RANKL mRNA levels in OPTN-downregulated RASFs had higher fold change (range from 1.500 to 12.865) compared to the TNF-α-and IFN-γ response control RASFs (range from 1.285 to 7.753) (Figure 2C, right panel), the two groups had no significant difference. Different from mRNA expressions, the cell surface RANKL in cytokine mixtures control RASFs was significantly higher than OPTN-downregulated RASFs (Figure 2D, right panel). One of the possible reasons is the duration of cytokine stimulation in evaluating cell surface RANKL was much longer (6 hours stimulation in mRNA evaluation and 72 hours stimulation in cell surface RANKL evaluation). Single cytokine (TNF-α but not IFN-γ) could slightly increase cell surface RANKL expression without significant difference on top of OPTN siRNA. Cytokines mixtures treated OPTN downregulated-RASFs had strong significant difference in cell surface level ($P<0.0001$) suggesting that without OPTN, RASFs might produce an enormous amount of RANKL when they are in active state.

OPTN does not affect osteoprotegerin levels

Osteoprotegerin (OPG) is an endogenous decoy receptor for RANKL and thereby counteracts the upregulation of RANKL. We therefore evaluated its expression in OPTN-downregulated RASFs. The expression of OPG was not different in OPTN-downregulated RASFs and control RASFs (Figure 3A). In contrast to RANKL, the expression of OPG was increased by treatment with TNF-α but not by that with IFN-γ. OPTN downregulation did not affect the increase of OPG induced by TNF-α (Figure 3B).

Downregulation of OPTN in RASFs promotes osteoclast formation

To confirm the functional regulation of RANKL by OPTN in RASFs, we next cocultured monocytes and OPTN-downregulated RASFs, evaluating osteoclast differentiation. Since the coculture experiment takes 9 days, we verified the sustained downregulation of OPTN in RASFs from three patients by performing real-time PCR and Western blotting on cells with 10 days interval from siRNA transfection independently before starting coculture (Figure 4A, 4B). Compared to monocytes cultured with not transfected or control siRNA-transfected RASFs, monocytes cultured with OPTN-reduced RASFs showed increased differentiation into TRAP-positive multinucleated cells which were considered as osteoclasts (Figure 4C). Quantitative analysis revealed that osteoclast formation was about three times higher from monocytes cultured with OPTN-downregulated RASFs than those cultured with control RASFs. These results indicate that OPTN functionally downregulates RANKL in RASFs.

Downregulation of OPTN activates NF-κB signaling pathway in RASFs

Chronic inflammation is the key mediator for local and systemic bone loss in RA patients (25). OPTN has also been reported to be involved in regulating the NF-κB signaling pathway (5). Hence, we investigated the activity of the NF-κB signaling pathway in OPTN-downregulated RASFs. There was no significant difference in the IκBα expression between control RASFs and OPTN-downregulated RASFs (Figure 5A, 5B); however, the nuclear p50 and p65 were lower in OPTN-downregulated RASFs (Figure 5A, 5D, 5E). According to previous reports, nuclear localization of NF-κB takes about thirty minutes whereas a cycle of the NF-κB will take about 100 minutes to process (26). Therefore, we stimulated RASFs with 10ng/ml TNF-α for 0, 1 and 2 hours. IκBα, which anchors NF-κB in the cytoplasm and thereby inhibits its activity, was temporarily decreased 1 hour after treatment with TNF-α but rapidly restored to the baseline level 2 hours after the treatment in control RASFs. In contrast, in OPTN-downregulated RASFs, the IκBα decrease was sustained until 2 hours after

treatment with TNF-α, indicating the prolonged activation of NF-κB by OPTN downregulation (Figure 5A, 5B). Consistent with this, NF-κB1/p50 and RelA/p65 were increased in the nuclei of OPTN-downregulated RASFs compared to control RASFs (Figure 5A, 5D, 5E). The OPTN expression was suppressed by OPTN siRNA and TNF-α slightly increased OPTN expression in control RASFs after 2 hours stimulation (Figure 5B).

OPTN regulates major molecules involved in homeostasis of joint environment

RASFs produce a number of cytokines, chemokines and MMPs which cause chronic joint inflammation and progressive joint destruction (27). Among them, we evaluated IL-6 and MMP-3 which are known to be particularly pathogenic molecules in RA (28, 29). MMP-3 was significantly increased with approximately 5-fold change following OPTN downregulation (Figure 6A). As IL-6 is a key player in the pathogenesis of RA, anti-IL-6 therapy has become available for clinical use (30). Therefore, we analyzed the expression of IL-6 after downregulation of OPTN. Although IL-6 was obviously increased after downregulating OPTN, there was no significant difference compared to control RASFs due to the large variation in the response to OPTN si between patients (Figure 6B). We next investigated several molecules involved in joint or bone protection, including GATA3, CHST15 and HAS1. Whereas GATA3 is classically an important transcription factor in T cells development (31), a recent report has revealed the function of GATA3 in regulating the bone healing process after bone fractures (32). We observed the decreased expression of GATA3 following OPTN downregulation (Figure 6C). CHST15 is one of the sulfotransferases responsible for biosynthesis of sulfated chondroitin sulfate which has been reported to be related to bone shortening and reduced chondrocyte proliferation (33). CHST15 was significantly decreased in OPTN-downregulated RASFs (Figure 6D). HAS1 is an enzyme responsible for hyaluronate/hyaluronan biosynthesis and its deficiency has been

reported to result in chronic joint inflammation *in vitro* (34). Downregulation of OPTN resulted in significantly decreased levels of HAS1 (Figure 6E). A recent study reported the inflammatory role of GATA1 in RASFs (35), hence we evaluated the expression of GATA1 in OPTN-downregulated RASFs. Overall, it had obviously higher expression after downregulating OPTN but no significant difference compared to RASFs due to the wide range of standard deviation. This result is compatible with the study as GATA1 has been reported to be IL-6 dependent and our data showed the similar pattern in IL6 expression in OPTN-downregulated RASFs.

Discussion

This study provides the first demonstration of autophagy adaptor/receptor OPTN as a negative regulator of RANKL and its protective role against joint destruction in RA. Since both OPTN and RANKL are upregulated by TNF-α and IFN-γ (Figure 1, 2, S20), OPTN may counteract, to some extent, RANKL and inhibit excessive joint destruction in inflammatory conditions developing in RA.

A previous study indicated the degradation of polyubiquitinated proteins by proteasome and autophagy as one of the key mechanisms to maintain homeostasis of RASFs (36). The degradation of polyubiquitinated proteins by autophagy, called selective autophagy, requires autophagy adaptors/receptors which have ubiquitin-binding domain and LC3, a component of autophagosome, -interacting region. The impairment of some autophagy adaptors/receptors have been reported to be related to the development of neurodegenerative diseases (37). We have evaluated the expression of six major autophagy adaptors/receptors in RASFs, including p62, OPTN, Neighbour of BRCA1 gene 1 (NBR1), Nuclear dot protein 52kDa (NDP52), Tax1 binding protein 1 (TAX1BP1) and Toll-interacting protein (TOLLIP), following treatment with inflammatory cytokines, including TNF-α, IFN-γ, IL-1β, TGF-β, IL-17, IL-22,

platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Supplementary File, Figure S1-S11). Among them, p62 and OPTN were significantly upregulated by inflammatory cytokines, particularly by TNF-α and IFN-γ (Supplementary File, Figure S1, S4, S6, S7). These results suggest a role for p62 and OPTN in RASFs exposed to inflammatory conditions. The role of p62 to regulate stress-induced cell death in RASFs has been previously reported (36).

OPTN has been implicated in many signaling pathways and cellular processes, but its role in osteoclast differentiation may be particularly important because of the implications for human diseases. Obaid, et al. (7) have demonstrated that susceptibility of Paget's disease of bone is mediated by a common variant at the OPTN locus, which is associated with reduced expression of OPTN. They further showed that OPTN in osteoclast precursor cells inhibited their differentiation into osteoclasts by blunting NF-κB activity and enhancing IFN-β signaling and that mice with a loss-of-function mutation in OPTN had increased bone turnover. Mice with global deletion of OPTN (*Optn*$^{-/-}$) have been reported to have increased osteoclastogenesis and have become an essential mouse model of Paget's disease (38).

The data obtained in our study would further support the role of OPTN as a negative regulator of osteoclast differentiation. We newly identified the role of OPTN in RANKL-expressing RASFs, whereas the study by Obaid, et al. focused on its role in RANKL-responsive cells. RANKL is mainly produced by cells of the osteoblast lineage, T cells and SFs (39). Among the RANKL-expressing cells, SFs are primarily responsible for the local formation of osteoclasts in the joints in RA models (4). Taken together, OPTN in RASFs may act as a negative regulator of bone erosions in RA.

Previous reports have shown that TNF-α and IFN-γ synergistically activates NF-κB signaling by inducing NF-κB -like factor binding elements or enhancing the degradation of IκBα and IκBβ, the inhibitor proteins of NF-κB (40, 41). Our data showed an upregulated nuclear p50

and p65 expression but no significant difference in IκBα expression between control RASFs and OPTN-downregulated RASFs (Figure 5). A previous study reported that NF-κB is a transcription factor in activating OPTN promoter in response to TNF-α and OPTN itself functions as a negative regulator of NF-κB (42). The author knockdown OPTN by shRNA and there was no significant difference in the IκBα expression but IκBα decrease in TNF-α stimulated OPTN-knockdown HeLa cells. This data is consistent to our results. Further, the author also reported that OPTN over-expressed HeLa cells inhibit NF-κB activity, therefore, we believe the presence of OPTN could inhibit NF-κB-induced RANKL expression and would be expected to attenuate TNF-α+IFN-γ induction of RANKL in a negative feedback loop.

Further, we have recently reported that NF-κB1 is a transcription factor for the RANKL gene (43). Hence, we treated RASFs with TNF-α or IFN-γ or both to evaluate the expression of RANKL. With the combination of cytokines, RANKL expression was significantly increased in control RASFs and was even more pronounced in OPTN-downregulated RASFs.

To exclude the possibility that the increased osteoclast formation in monocytes cultured with OPTN-downregulated RASFs is due to the increased proliferation of OPTN-downregulated RASFs, we analyzed the proliferation rate of OPTN-downregulated RASFs and that of control RASFs by BrdU assay. There was no difference in the proliferation rate between OPTN-downregulated and control RASFs (Figure S12). These results support the previous findings that the increased osteoclast formation was mediated by the increased RANKL expression at the single cell level.

A previous report has mentioned that downregulated OPTN increased osteoclast differentiation in bone-marrow-derived macrophages (BMDMs) isolated from mice and mutated OPTN knock-in mice ($Optn^{D477N/D477N}$) had high bone turnover ability compared to wildtype mice (7). Hence we consider that it is difficult to identify the role of OPTN in

fibroblasts via in vivo experiments as it has an intrinsic role in osteoclast precursors. Therefore, we decided to use coculture of human monocytes and OPTN-reduced RASFs to evaluate its function instead of an animal model. Further, in this study, we used osteoarthritis SFs (OASFs) as control SFs due to the difficulty of getting healthy SFs (Figure S13-S17). Overall, there was no difference between RASFs and OASFs. The immunohistochemistry findings in human synovial tissue confirmed that OPTN was expressed in OA in the lining of synovium whereas it was expressed in both lining and sub-lining layers of synovium tissues in RA (Figure S19).

Our further investigation of OPTN downregulation in RASFs confirmed the protective role of OPTN against joint destruction as the pathogenic molecules MMP-3 and IL-6 were increased while the protective molecules GATA3, CHST15 and HAS1 were decreased. Among them, the expression of MMP-3 was most obviously changed in OPTN-downregulated RASFs with approximately 5-fold higher mRNA levels compared to control RASFs. MMP-3 is a proteolytic enzyme produced mainly by SFs and degrades many types of collagen and proteoglycans (44). MMP-3 also activates other MMPs such as MMP-2 and MMP-9 (28). Serum levels of MMP-3 are correlated with disease activity and progressive joint damage in patients with RA (45). These findings suggest the direct role of OPTN to protect the joint by inhibiting expression and activity of MMPs. Among the six genes we have evaluated, there were several studies suggest their relationships with NF-κB. For example, NF-κB activation was found necessary for the induction of MMP-3 and IL-6 (46, 47). CHST15 is necessary for the synthesis of chondroitin sulfate, a matrix component which is able to diminish NF-κB activation in synovial membrane (48). IL-1β (an NF-κB activator) has been reported to upregulate GATA-1 (49).

Previous studies have already shown the regulation of NF-κB signaling by OPTN in nerve cells and indicated its involvement in neurodegenerative diseases (8). Our current study has

confirmed the corresponding regulation of OPTN in RASFs. The confirmation is of significance since NF-κB signaling is one of the most important inflammatory pathways in RA (10) and other rheumatic diseases (50).

In conclusion, OPTN plays a protective role against joint destruction in RA with its upregulation when induction by pro-inflammatory cytokines in RASFs. Absence of OPTN might worsen RA by generating a joint destructive state including increased RANKL expression on RASFs, subsequent osteoclast differentiation, and the dysregulation of molecules involved in joint homeostasis. These direct and indirect roles of OPTN may contribute to our understanding of the mechanisms of joint destruction in RA which are still rarely completely prevented anti-inflammatory treatments.

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Author contributions

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. Wen Shi Lee had full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis

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Figure Legends

Figure 1. mRNA and protein expression of OPTN increased following treatment with TNF-α or IFN-γ in RASFs. mRNA expression of OPTN 4 hours (left panel) and 24 hours (right panel) after treatment with TNF-α **(A)** or IFN-γ **(B)**. Protein expression of OPTN with densitometric analysis 24 hours after treatment with TNF-α **(C)** or IFN-γ **(D)**. The mRNA levels of OPTN were evaluated by three independent experiments using RASFs from three different patients. The average Ct value of each group was normalized to unstimulated RASFs. Blots are representative of four independent experiments using RASFs from four different patients. OPTN/βactin ratio was normalized to unstimulated RASFs. Values are presented as mean ± S.D. *P<0.05, **P<0.01 versus control, by one-way ANOVA. RQ, relative quantification.

Figure 2. RANKL expression in OPTN-downregulated RASFs. OPTN mRNA **(A)** and protein levels **(B)** after 24 and 48 hours siRNA transfection, respectively. **(C)** RASFs RANKL cell surface expression after 24 hours siRNA transfection followed by relevant cytokines stimulation for 72 hours. Histograms are representative of five independent experiments using RASFs from five different patients. **(D)** Quantification of **C**, presented as mean fluorescent intensity (MFI) ratio. **(E)** RASFs RANKL mRNA levels were evaluated by ten independent experiments from ten different patients. RASFs were transfected with siRNA for 24 hours then treated with cytokines for 6 hours. Values are presented as mean ± S.D. *P<0.05, **P<0.01, ****P<0.0001, each group versus control siRNA (without cytokine stimulation). #P<0.05, ##P<0.01, ###P<0.001, each group of OPTN downregulated-RASFs versus its corresponding group in control siRNA transfected RASFs. Data in A, B, C (left panel) and D (left panel) were analyzed by Student's t-test and data in D (right panel) and E

(right panel) were analyzed by one-way ANOVA. Left panels in D and E compared control RASFs and OPTN-downregulated RASFs whereas right panels compared each group with or without cytokines stimulation. Ctrl si, control siRNA; OPTN si, optineurin siRNA; RQ, relative quantification.

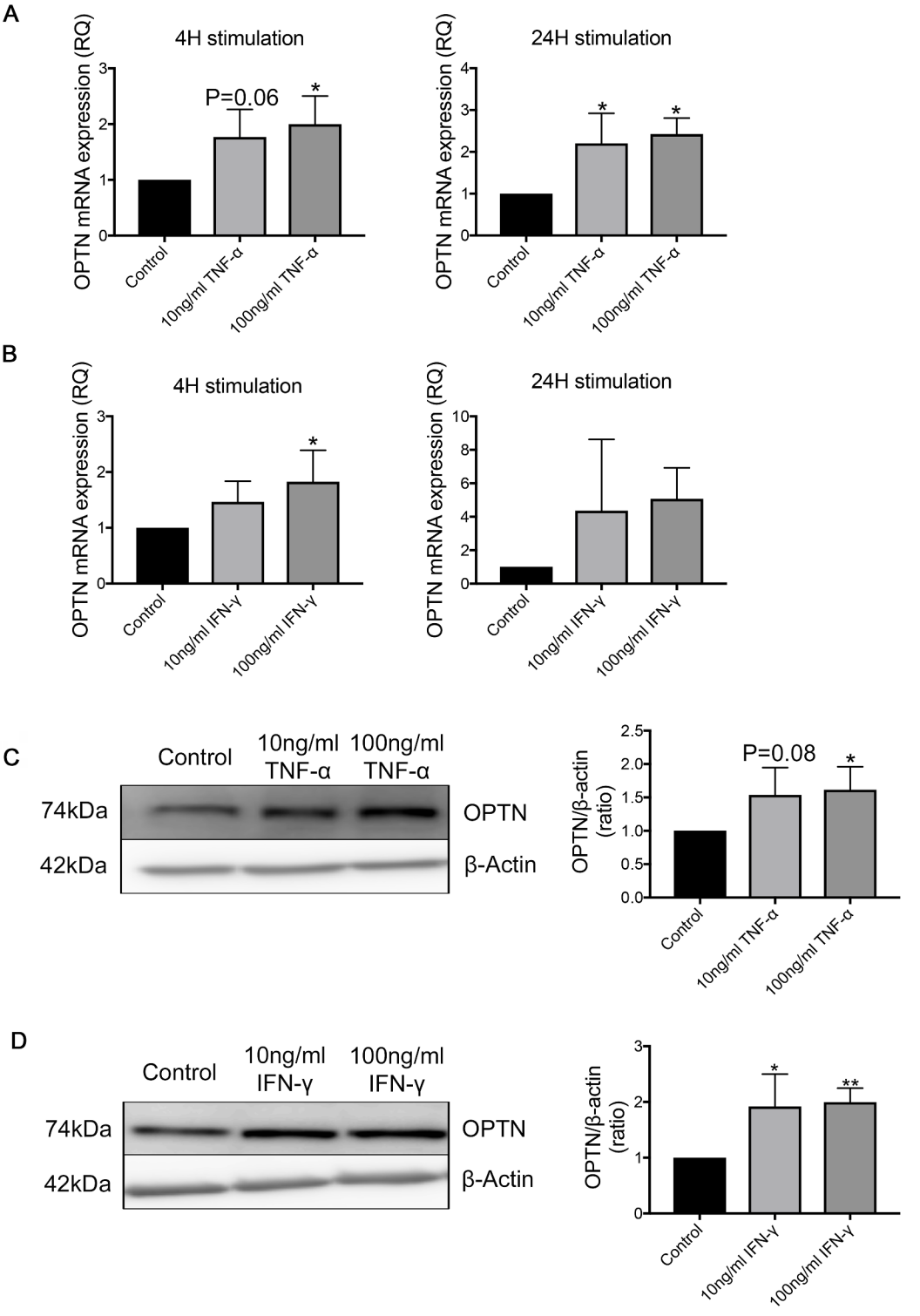
Figure 3. Expression of OPG in OPTN-downregulated RASFs. **(A)** Evaluation of OPG mRNA levels 24 hours after siRNA transfection by five independent experiments using RASFs from five different patients. **(B)** Following siRNA transfection with 24 hour interval, RASFs were treated with TNF-α and/or IFN-γ for 6 hours and OPG mRNA levels were evaluated. Values are presented as mean ± S.D. *P<0.05, **P<0.01, each group versus control siRNA (without cytokine stimulation). There was no significant difference between each group of OPTN siRNA transfected RASFs and its corresponding group in control siRNA transfected RASFs. Data in A was analyzed using Student's *t* test and data in B was analyzed using one-way ANOVA. Ctrl si, control siRNA; OPTN si, optineurin siRNA; RQ, relative quantification; N.S., not significant.

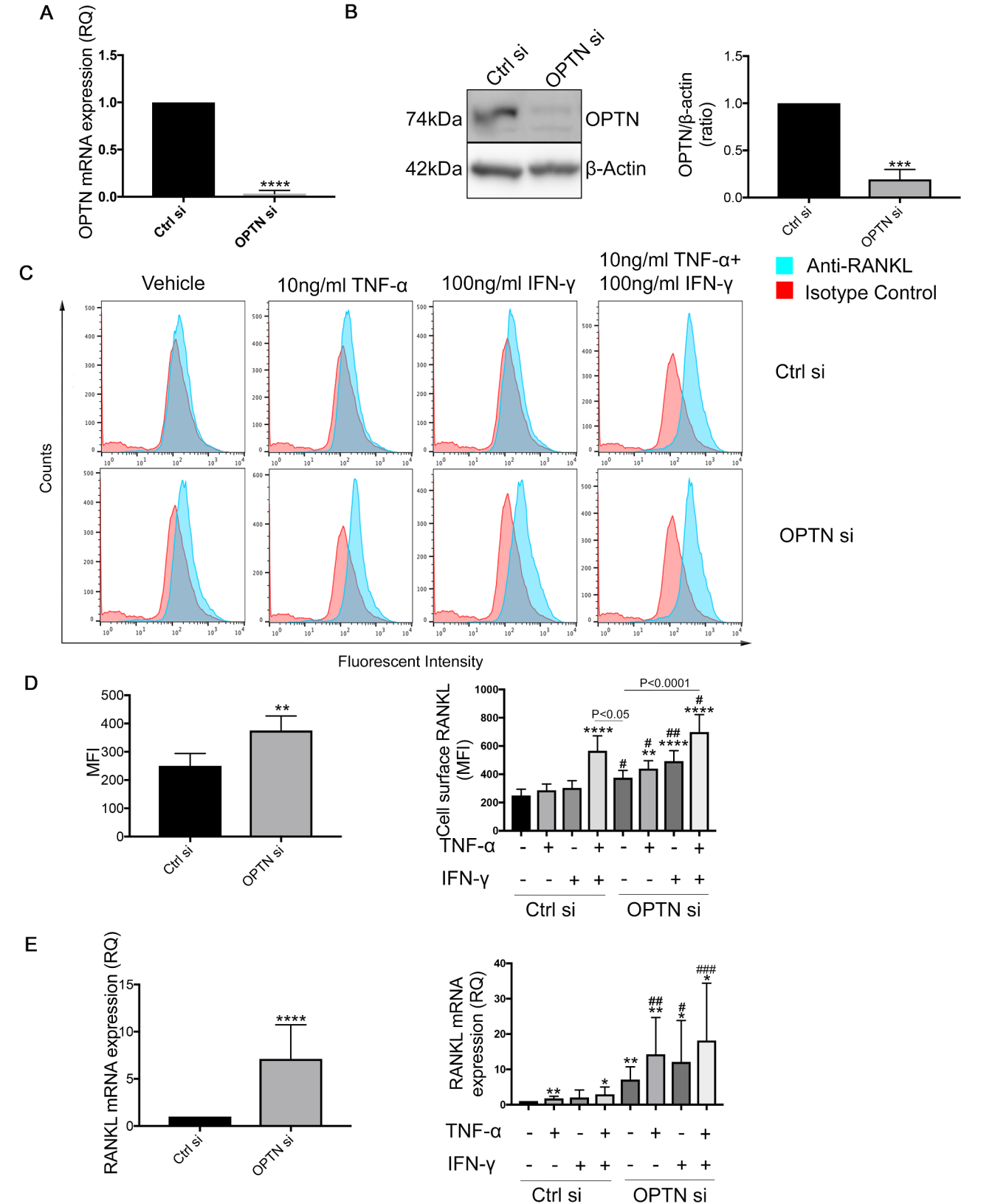
Figure 4. Human monocytes cocultured with OPTN-downregulated RASFs. mRNA levels **(A)** and protein levels **(B)** of OPTN 10 days after siRNA transfection. **(C)** Isolated human CD14+ monocytes were cultured with non-transfected RASFs, control siRNA-transfected RASFs, OPTN siRNA-transfected RASFs, or RANKL in the presence of 25 ng/ml M-CSF and stained with tartrate-resistant acid phosphatase (TRAP). TRAP-positive cells with equal or more than 3 nuclei (MNCs, multinucleated cells) were considered as osteoclasts and were quantified with statistical analysis. Values are presented as mean ± S.D. *P<0.05, ****P<0.0001, data in A and B were analyzed using Student's *t* test and data in C was

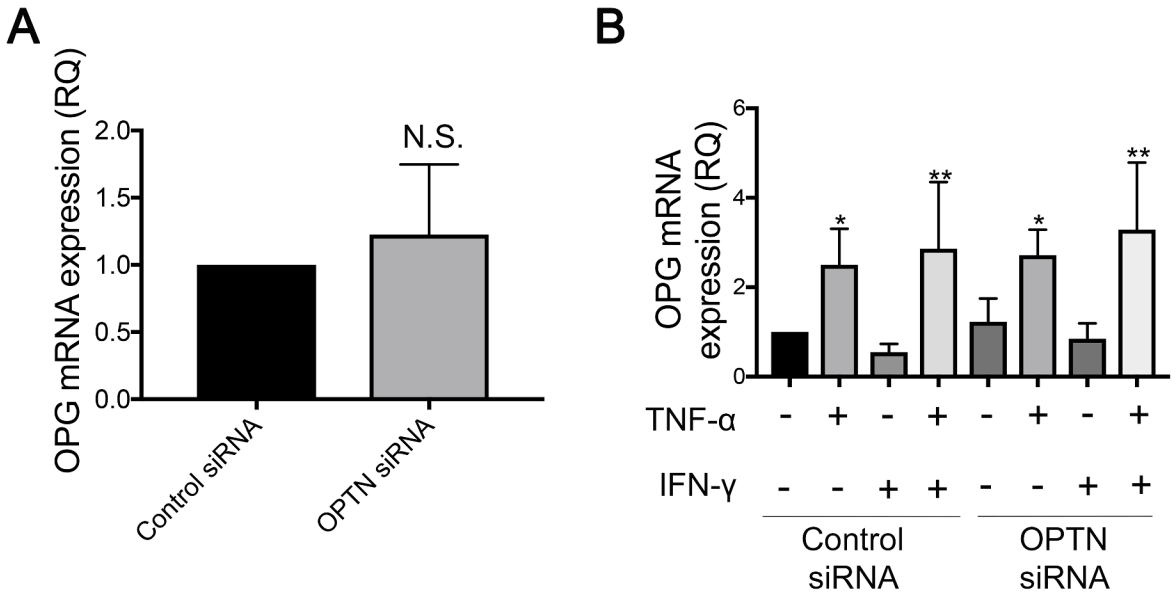
analyzed using one-way ANOVA. Ctrl si, control siRNA; OPTN si, optineurin siRNA; RA, rheumatoid arthritis; RQ, relative quantification.

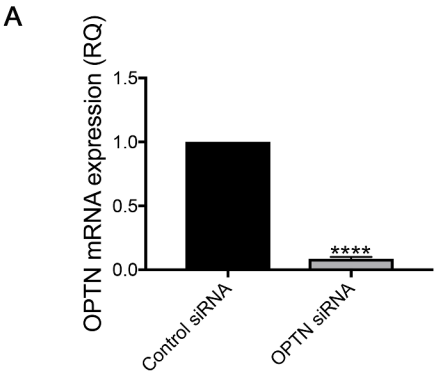
Figure 5. NF-κB signaling in OPTN-downregulated RASFs. **(A)** Control or OPTN-downregulated RASFs were treated with TNF-α for 0, 1, or 2 hours and the expression of IκBα, OPTN and β-actin in the whole cell and that of NF-κB1/p50, RelA/p65 and histone-3 in the nucleus were evaluated. Blots are representative of four independent experiments using RASFs from four different patients. **(B)** Densitometric analysis of IκBα. **(C)** Densitometric analysis of OPTN. **(D)** Densitometric analysis of NF-κB1/p50. **(E)** Densitometric analysis of RelA/p65. Expression of IκBα and OPTN were standardized by that of β-actin while the expression of NF-κB1/p50 and RelA/p65 in nucleus was standardized by that of histone-3. Values are presented as mean ± S.D. *P<0.05, **P<0.01, ***P<0.001, by Student's *t*-test (left panel), and by one-way ANOVA (middle and right panels). Ctrl si, control siRNA; OPTN si, optineurin siRNA.

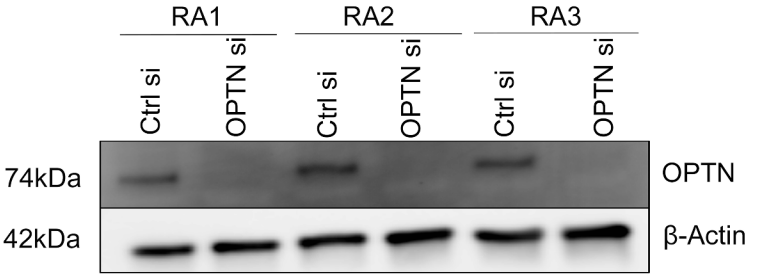
Figure 6. mRNA levels of MMP-3 (A), IL-6 (B), GATA3 (C), CHST15 (D), HAS1 (E) and GATA1 (F) 24 hours after siRNA transfection in RASFs.
Experiments repeated five times for (A) and (B), three times for (C) and (D) and four times for (E) and (F) using RASFs from different patients. Values are presented as mean ± S.D. *P<0.05, ***P<0.001, by ratio paired Student's *t*-test. CHST15, carbohydrate sulfotransferase 15; Ctrl si, control siRNA; HAS1, hyaluronan synthase 1; MMP-3, Matrix metallopeptidase 3; N.S., no significant; OPTN si, optineurin siRNA.

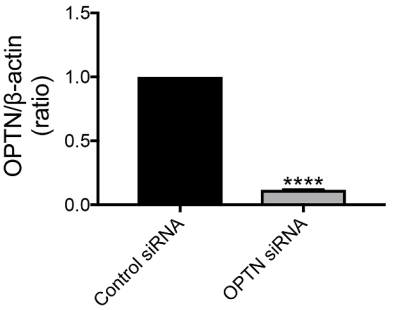

A
4H stimulation
P=0.06
*
OPTN mRNA expression (RQ)
Control
10ng/ml TNF-α
100ng/ml TNF-α
24H stimulation
*
*
B
4H stimulation
*
OPTN mRNA expression (RQ)
Control
10ng/ml IFN-γ
100ng/ml IFN-γ
24H stimulation
C
Control
10ng/ml TNF-α
100ng/ml TNF-α
74kDa
42kDa
OPTN
β-Actin
P=0.08
*
OPTN/β-actin (ratio)
D
Control
10ng/ml IFN-γ
100ng/ml IFN-γ
74kDa
42kDa
OPTN
β-Actin
*
**
OPTN/β-actin (ratio)

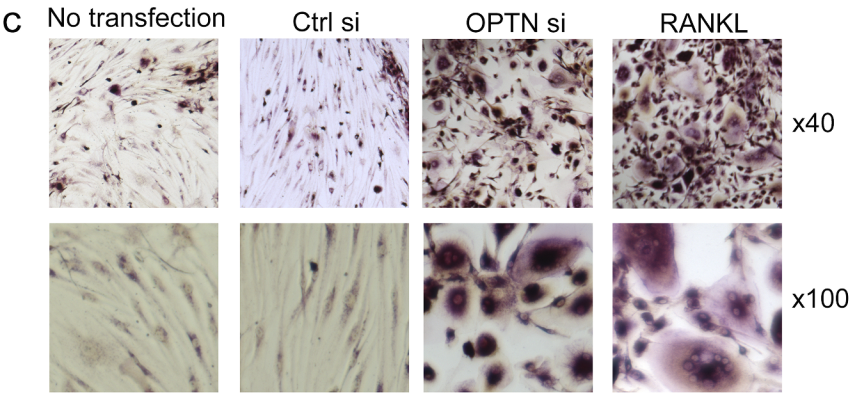

A
OPTN mRNA expression (RQ)
1.5
1.0
0.5
0.0
****
Ctrl si
OPTN si
B
Ctrl si
OPTN si
74kDa
OPTN
42kDa
β-Actin
OPTN/β-actin (ratio)
1.5
1.0
0.5
0.0
***
Ctrl si
OPTN si
C
Vehicle
10ng/ml TNF-α
100ng/ml IFN-γ
10ng/ml TNF-α+ 100ng/ml IFN-γ
Anti-RANKL
Isotype Control
Ctrl si
OPTN si
Counts
Fluorescent Intensity
D
MFI
500
400
300
200
100
0
**
Ctrl si
OPTN si
Cell surface RANKL (MFI)
1000
800
600
400
200
0
P<0.0001
P<0.05
****
**
#
##
TNF-α - + - + - + - +
IFN-γ - - + + - - + +
Ctrl si
OPTN si
E
RANKL mRNA expression (RQ)
15
10
5
0
****
Ctrl si
OPTN si
RANKL mRNA expression (RQ)
40
30
20
10
0
**
*
###
TNF-α - + - + - + - +
IFN-γ - - + + - - + +
Ctrl si
OPTN si

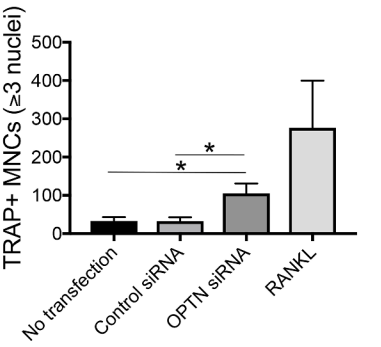

A
OPG mRNA expression (RQ)
2.0
1.5
1.0
0.5
0.0
N.S.
Control siRNA
OPTN siRNA
B
OPG mRNA expression (RQ)
6
4
2
0
*
**
*
**
TNF-α - + - + - + - +
IFN-γ - - + + - - + +
Control siRNA
OPTN siRNA

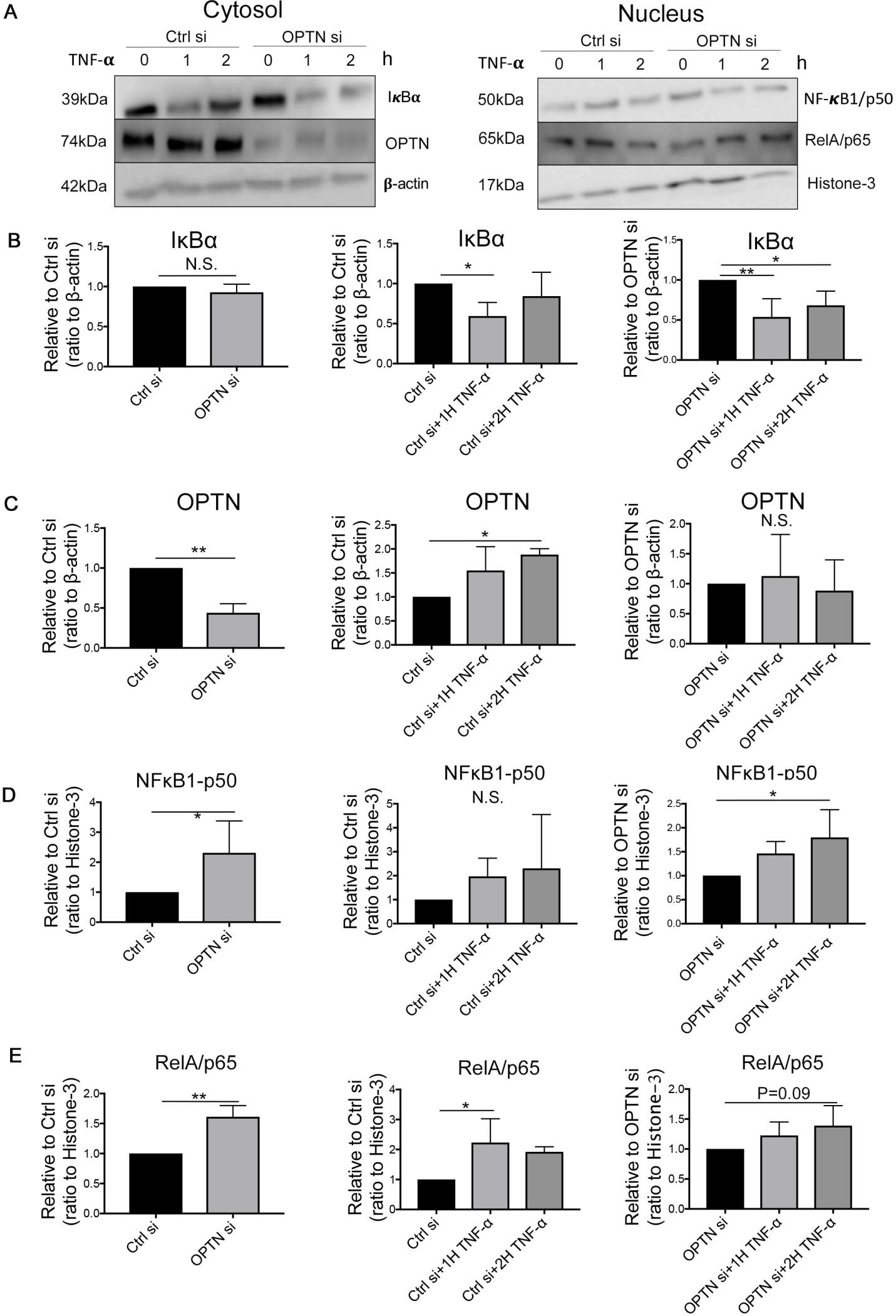

A
OPTN mRNA expression (RQ)
1.5
1.0
0.5
0.0
****
Control siRNA
OPTN siRNA

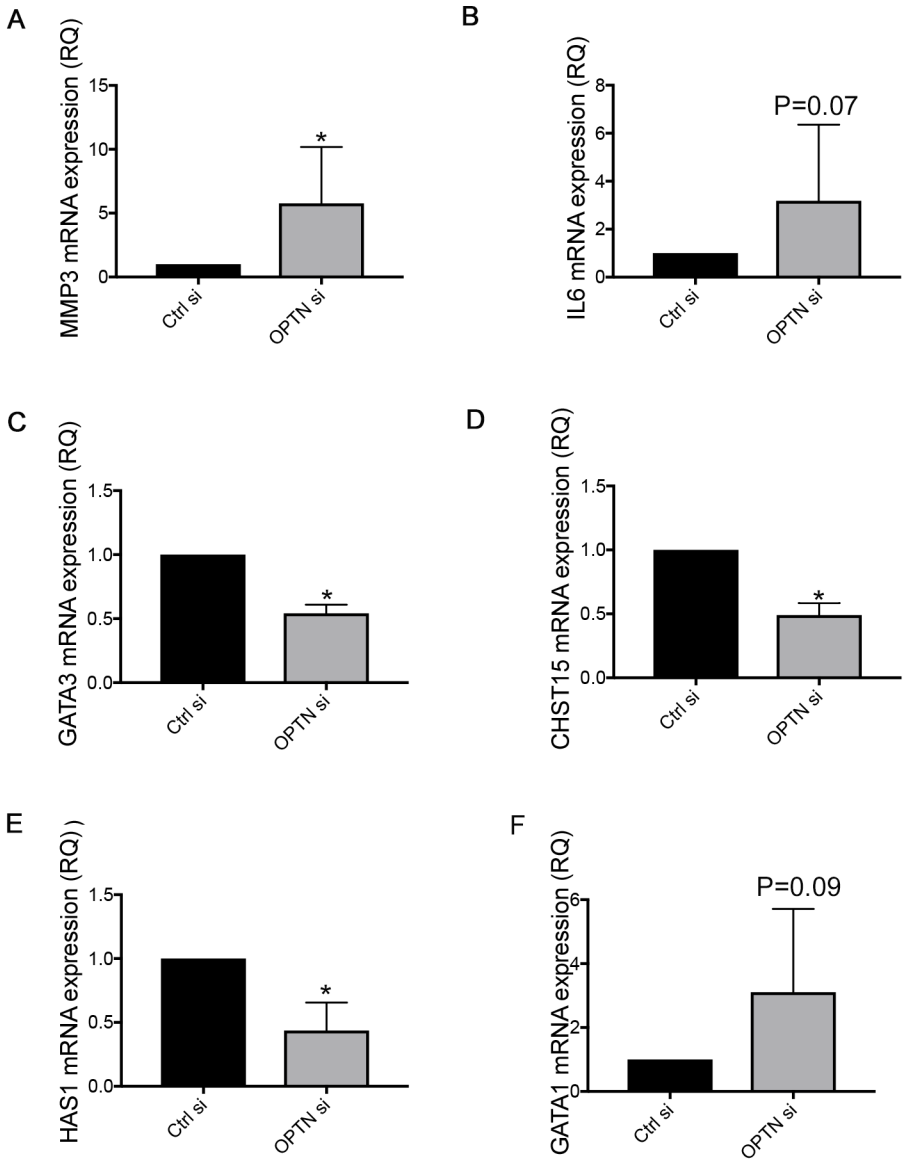

B
RA1
RA2
RA3
Ctrl si
OPTN si
Ctrl si
OPTN si
Ctrl si
OPTN si
74kDa
42kDa
OPTN
β-Actin


OPTN/β-actin (ratio)
1.5
1.0
0.5
0.0
****
Control siRNA
OPTN siRNA


C
No transfection
Ctrl si
OPTN si
RANKL
x40
x100


TRAP+ MNCs (≥3 nuclei)
500
400
300
200
100
0
*
*
No transfection
Control siRNA
OPTN siRNA
RANKL


A
Cytosol
Ctrl si
OPTN si
TNF-α 0 1 2 0 1 2 h
39kDa
IκBα
74kDa
OPTN
42kDa
β-actin
Nucleus
Ctrl si
OPTN si
TNF-α 0 1 2 0 1 2 h
50kDa
NF-κB1/p50
65kDa
RelA/p65
17kDa
Histone-3
B
IκBα
N.S.
Relative to Ctrl si (ratio to β-actin)
Ctrl si
OPTN si
IκBα
*
Relative to Ctrl si (ratio to β-actin)
Ctrl si
Ctrl si+1H TNF-α
Ctrl si+2H TNF-α
IκBα
*
**
Relative to OPTN si (ratio to β-actin)
OPTN si
OPTN si+1H TNF-α
OPTN si+2H TNF-α
C
OPTN
**
Relative to Ctrl si (ratio to β-actin)
Ctrl si
OPTN si
OPTN
*
Relative to Ctrl si (ratio to β-actin)
Ctrl si
Ctrl si+1H TNF-α
Ctrl si+2H TNF-α
OPTN
N.S.
Relative to OPTN si (ratio to β-actin)
OPTN si
OPTN si+1H TNF-α
OPTN si+2H TNF-α
D
NFκB1-p50
*
Relative to Ctrl si (ratio to Histone-3)
Ctrl si
OPTN si
NFκB1-p50
N.S.
Relative to Ctrl si (ratio to Histone-3)
Ctrl si
Ctrl si+1H TNF-α
Ctrl si+2H TNF-α
NFκB1-p50
*
Relative to OPTN si (ratio to Histone-3)
OPTN si
OPTN si+1H TNF-α
OPTN si+2H TNF-α
E
RelA/p65
**
Relative to Ctrl si (ratio to Histone-3)
Ctrl si
OPTN si
RelA/p65
*
Relative to Ctrl si (ratio to Histone-3)
Ctrl si
Ctrl si+1H TNF-α
Ctrl si+2H TNF-α
RelA/p65
P=0.09
Relative to OPTN si (ratio to Histone-3)
OPTN si
OPTN si+1H TNF-α
OPTN si+2H TNF-α


A
MMP3 mRNA expression (RQ)
15
10
5
0
*
Ctrl si
OPTN si
B
IL6 mRNA expression (RQ)
8
6
4
2
0
P=0.07
Ctrl si
OPTN si
C
GATA3 mRNA expression (RQ)
1.5
1.0
0.5
0.0
*
Ctrl si
OPTN si
D
CHST15 mRNA expression (RQ)
1.5
1.0
0.5
0.0
*
Ctrl si
OPTN si
E
HAS1 mRNA expression (RQ))
1.5
1.0
0.5
0.0
*
Ctrl si
OPTN si
F
GATA1 mRNA expression (RQ)
6
4
2
0
P=0.09
Ctrl si
OPTN si