Differential expressions of NOD-like receptors and their associations with inflammatory responses in rheumatoid arthritis

H.W. Kim[1], Y.-J. Kwon[1], B.W. Park[2], J.J. Song[1], Y.-B. Park[1], M.-C. Park[1]

[1]Division of Rheumatology, Department of Internal Medicine, Yonsei University College of Medicine, Seoul, South Korea; [2]Chadwick International School, Songdo, Incheon, South Korea.

Abstract

Objective

To determine the differential expressions of nucleotide oligomerisation domain (NOD)-like receptors (NLRs) and to investigate their association with inflammatory responses in rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS).

Methods

Gene expression and protein levels of various NLRs, including NOD1, NOD2, NLRP1, NLRP3, NLRP12, NLRX1, and NLRC3, were determined in FLS and synovial tissues from patients with RA and patients with osteoarthritis (OA) using quantitative real-time PCR and immunohistochemistry. After transfection of NOD2 RNAi plasmids or a pcDNA3.1-NLRX1 vector, gene expression levels of pro-inflammatory cytokines in RA FLS and the protein levels of these cytokines in culture supernatants were determined using quantitative real-time PCR and enzyme-linked immunosorbent assays. The effects of NLR gene regulation on NF-κB and caspase-1 were evaluated using Western blot analysis.

Results

Gene expression levels of NOD1, NLRP1, NLRP3, NLRP12, and NLRC3 were not different between RA and OA samples. NOD2 gene expression and protein levels were significantly increased in RA samples, whereas the levels of NLRX1 were significantly decreased. Downregulation of NOD2 gene expression by transfection with NOD2 RNAi plasmid significantly reduced pro-inflammatory cytokine levels in RA FLS, while transfection with adenoviral vectors encoding NLRX1 had no effect on pro-inflammatory cytokine levels. Downregulation of NOD2 gene expression significantly decreased NF-κB, TRAF6, and IKK levels, but not caspase-1 levels, in RA FLS.

Conclusion

NOD2 is upregulated in RA FLS; moreover, downregulation of NOD2 gene expression reduces pro-inflammatory cytokine and NF-κB levels in RA FLS. These findings provide evidence that NOD2 exerts pro-inflammatory effects in RA.

Key words

rheumatoid arthritis, NOD-like receptors, NOD2, cytokine, NF-κB

Hye Won Kim, MD
Yong-Jin Kwon, MD
Byung Wook Park, MD
Jason Jungsik Song, MD
Yong-Beom Park, MD, PhD
Min-Chan Park, MD, PhD

Please address correspondence and reprint requests to:
Dr Min-Chan Park,
Gangnam Severance Hospital,
Yonsei University College of Medicine,
211 Eonjuro, Gangnam-gu,
06273 Seoul, South Korea.
E-mail: mcpark@yuhs.ac

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Introduction

Pattern recognition receptors are integral to the innate immune system as a first line of defense against pathogenic microorganisms or endogenous damaged tissue. These pattern recognition receptors include the well-recognised toll-like receptors (TLRs) and the more recently discovered nucleotide oligomerisation domain (NOD)-like receptors (NLRs). NLRs act as intracellular sensors for pathogens and endogenous products of tissue injury. In humans, the NLR family is comprised of 22 proteins that share structural similarities in their NOD and leucine-rich repeat domains (1-4). A new annotation system subdivides the NLR family into four subfamilies (A, B, C and P), based on variation in the N-terminal domain structures for each NLR (5). NOD1 and NOD2, also known as NLRC1 and NLRC2, respectively, are the prototypical NLRs that propagate inflammatory signals. These NLRs initiate host defense mechanisms by activating nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) after the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular pattern molecules (DAMPs) (6). NLRP1 and NLRP3 activate inflammasomes and interact with caspase-1 to drive interleukin (IL)-1β-dependent inflammation. Moreover, NLRP1 and NLRP3 can induce programmed cell death, resulting in cellular lysis and the release of inflammatory contents (7, 8). An additional subfamily, NLRX1, has no homology to the N-terminal domains of proteins in any of the other four subsets and have been shown to facilitate mitochondria-targeted signalling and reactive oxygen species production (9). Some other NLR members have been reported to have a regulatory role in inflammatory response. For example, NLRP12 has been suggested to downregulate NF-κB and extracellular signal-regulated kinase activation in macrophages (10), and NLRC3 attenuates T cell activation by reducing IL-2 and CD25 protein levels (11).

Rheumatoid arthritis (RA) is an inflammatory disease characterised by synovial hyperplasia and inflammatory cell infiltration into the synovium, which causes joint inflammation and destruction. Although the pathogenesis of RA remains unclear, activation of the innate immune system by endogenous or exogenous stimuli has been suggested to play a pivotal role (12, 13). NOD1 and NOD2 expression has been demonstrated in fibroblast-like synoviocytes (FLS) and synovium samples from patients with RA (14-16); moreover, an accumulating body of evidence indicates that NLRs may play a role in the pathogenesis of RA (14-18). However, the expression levels of various NLRs in RA have not yet been comprehensively evaluated and their dominant signalling pathways in RA are unclear.

Considering the large number of NLRs and their complex and intersecting functions, most of which have yet to be determined, a thorough comparison of NLR gene expression levels and their associations with inflammatory responses will be needed to identify the precise roles of NLRs in RA. In this study, we determined the expression levels of various NLRs in RA FLS and evaluated their associations with pro-inflammatory cytokine levels. We also identified the signalling pathway that is responsible for the actions of NLRs in RA FLS.

Materials and methods

Isolation and culture of FLS

Synovial tissue samples were obtained from five patients with RA and three patients with OA who underwent total knee replacement surgery. The samples were washed with phosphate-buffered saline, cut into small pieces, and treated with 1 mg/ml type I collagenase (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) for 2 h at 37°C. Isolated FLS were suspended in DMEM containing 10% fetal bovine serum (Gibco BRL), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells obtained from the fourth to sixth passages were used in all experiments. Human monocytic THP-1 cells (ATCC, Rockville, Maryland, USA) were maintained in DMEM containing 10% fetal calf serum (Gibco BRL) and differentiated for 72 h with 167 nmol/L of phorbol-12-myristate-13-acetate.

This study was approved by our Ethics Committee and all study subjects provided written informed consent prior to study enrolment.

RNA extraction and quantitative real-time PCR

RA FLS were treated with or without 100 ng/mL of lipopolysaccharide (LPS) for 24 h where indicated, after which total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Genomic DNA was removed by DNase I digestion (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. RNA was homogenised using Qiashredder columns (Qiagen), and the obtained RNA pellet was dissolved in 30 μl of RNase-free water and stored at -80°C. The RNA was then reverse transcribed into complimentary DNA (cDNA) using a SuperScript III synthesis kit (Invitrogen), after which gene expression was analysed using TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7300 Real Time PCR System. The conditions for amplification were as follows: 50°C for 2 min and 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 1 min. The PCR reactions had a total volume of 50 μl, and each PCR sample underwent 30 cycles of amplification to ensure that the reactions had not reached the plateau phase of amplification. All reactions were performed in triplicate. The results were evaluated using the $2^{-\Delta\Delta Ct}$ method as previously described (19), and the calculated number of copies was normalised to the number of GAPDH mRNA copies in the samples.

Immunohistochemistry

Formalin-fixed synovial tissue samples were embedded in paraffin and sectioned to a thickness of 5 μm. Deparaffinisation of the paraffin-embedded slides was performed by xylene immersion followed by dehydration in ethanol. Slides were incubated overnight at 4°C with primary antibodies reactive to NOD2 and NLRX1 (Abcam, Cambridge, MA, USA) followed by the appropriate secondary antibodies (ISU Abxis, Seoul, Korea). All tissue samples were counterstained with haematoxylin. After immunohistochemical staining, the expression levels of different markers in the synovial tissue samples were scored semiquantitatively on a four-point scale. Scoring was performed independently and blindly by two investigators, after which averages were calculated. A score of 0 represented minimal expression, 1 represented mild expression, 2 represented moderate expression, and 3 represented abundant marker expression (20).

RNA interference, construction of expression plasmids and transient transfection

After treatment with 100 ng/mL of LPS for 24 h, RA FLS were seeded at 5×10^4 cells per well. Six hours after plating, the RA FLS were transfected with NOD2 RNAi plasmids or negative control siRNA (25 pmol) using Lipofectamine 2000 (Bioneer, Alameda, CA, USA) according to the manufacturer's protocol. Cells were incubated for 72 h at 37°C in a 5% CO_2 incubator. An adenoviral vector containing cDNA of wild-type NLRX1, pcDNA3.1 NLRX1 (Bioneer), or the empty vector was transiently transfected into RA FLS to enhance the expression of NLRX1. The full-length pcDNA3.1 NLRX1 vector was made by cloning the full-length PCR product of NLRX1 with PFU DNA polymerase (Bioneer). The plasmid sequence was confirmed by DNA sequencing. For the transient transfection experiments, RA FLS were plated on a 24-well plate at a density of 2×10^5 cells per well for 24 h prior to transfection. Lipofectamine 2000 (Bioneer) was used to perform transfection with a 2.0-mg pcDNA3.1 NLRX1 vector or 2.0-mg pcDNA3.1 empty vector.

Western blot analysis

FLS were washed and harvested by centrifugation at 13,000×g for 20 sec. Total protein extracts were generated using PRO-PREP protein extraction solution (Intron Biotechnology, Gyeongi-do, South Korea) according to the manufacturer's instructions. Protein standards were prepared with diluted albumin (BSA), and the protein concentrations were estimated using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo, Rockford, IL, USA). After quantitation, proteins were electrophoresed and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 2–3 h at room temperature and subsequently incubated with primary antibodies against NOD2, NLRX1, NF-κB, TNF receptor associated factor 6 (TRAF6), I kappa B kinase (IKK), and caspase-1 (Abcam) overnight at 4°C. The membranes were then incubated with secondary goat anti-rabbit IgG-horseradish peroxidase antibodies for 1 h at room temperature. Immunoreactive protein bands were detected by enhanced chemiluminescence (ECL, Pierce) and visualised using Agfa film. Bands were quantified by densitometric analysis using ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of various pro-inflammatory mediators, including IL-1β, TNF-α, and IL-6, in the culture supernatants of RA FLS were determined using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. Intra- and inter-assay coefficients of variation were lower than 8% for all tests.

Statistical analysis

All statistical analyses were conducted using SPSS v. 20; results are expressed as the mean ± standard error of the mean. Each result represents the mean of at least three experiments performed on different days. Differences were assessed by one-way and two-way repeated-measures analysis of variance (ANOVA), followed by the Bonferroni post hoc test for comparison of multiple groups. Independent *t*-tests or Mann-Whitney U-tests were also used as appropriate. *P*-values less than 0.05 were considered statistically significant.

Results

Expression and production of NLRs in RA and OA FLS and synovium

Quantitative real-time PCR showed that NOD2 mRNA expression levels in

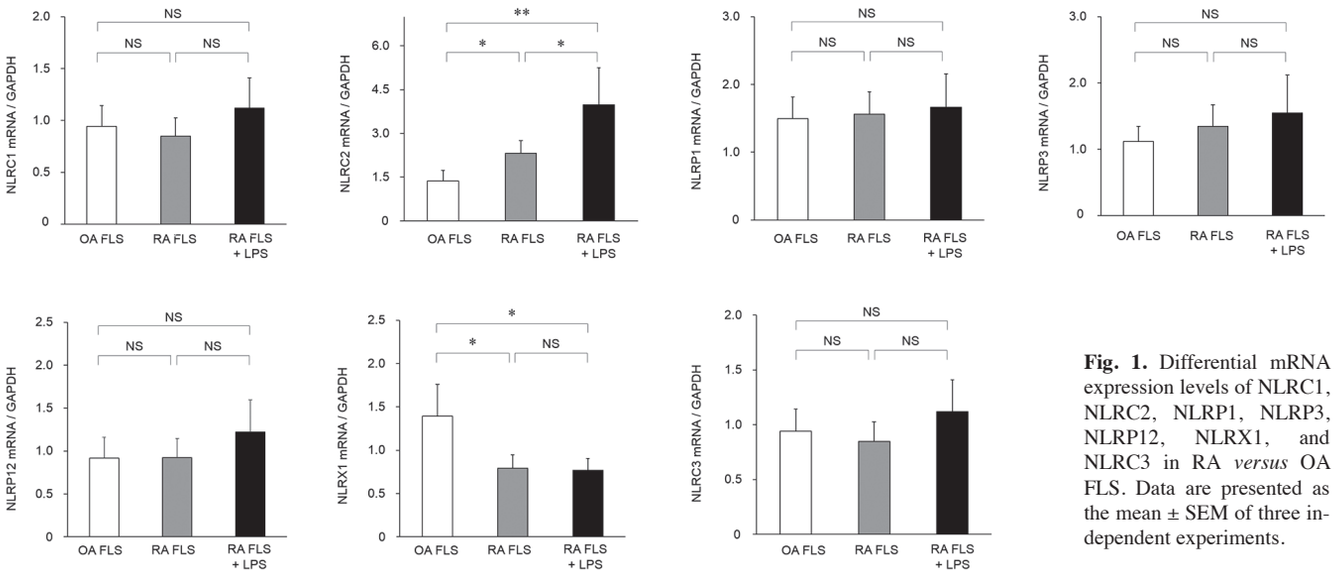

Fig. 1. Differential mRNA expression levels of NLRC1, NLRC2, NLRP1, NLRP3, NLRP12, NLRX1, and NLRC3 in RA *versus* OA FLS. Data are presented as the mean ± SEM of three independent experiments.

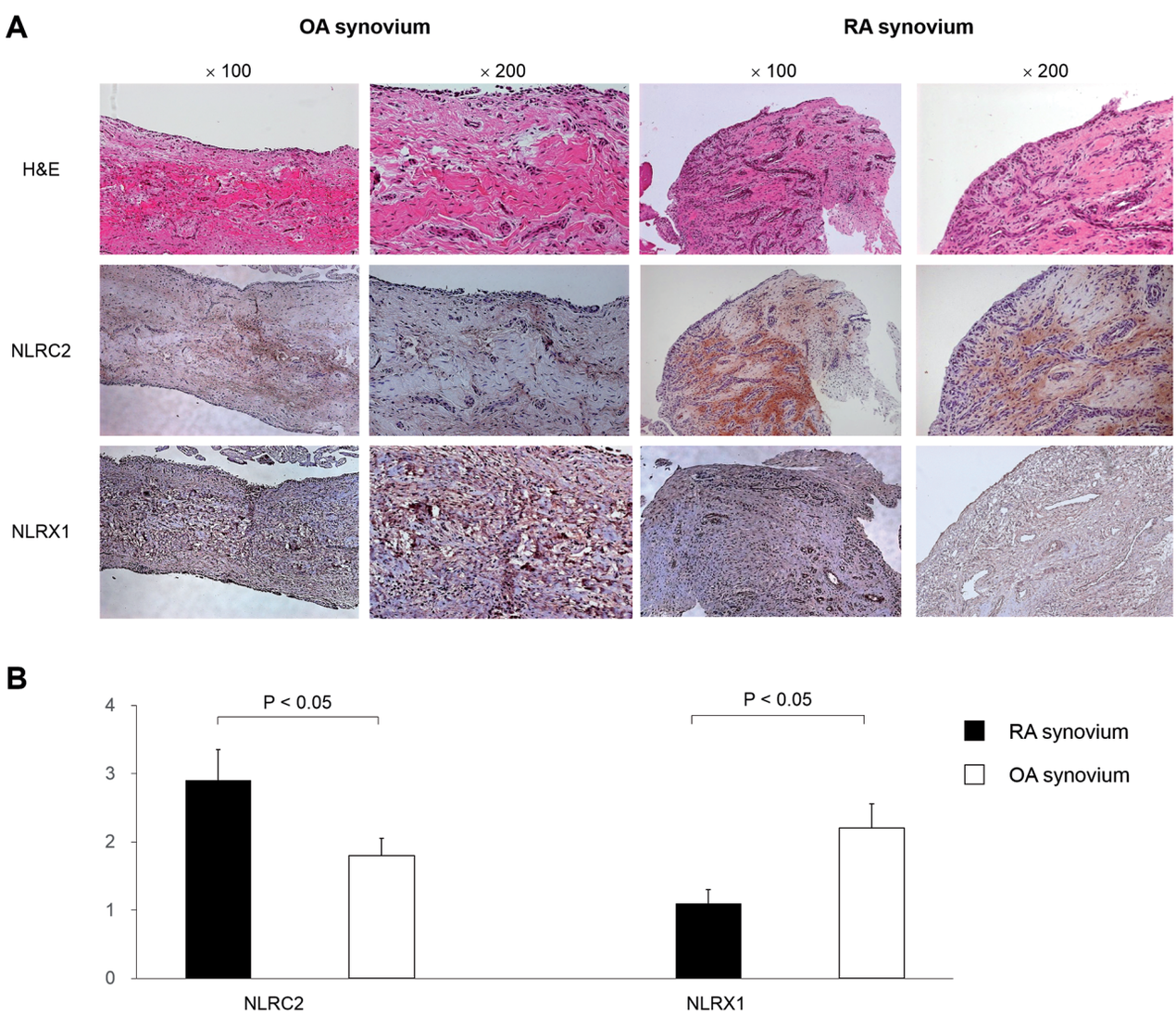

Fig. 2. Immunohistochemical staining of NLRC2 and NLRX1 in RA and OA synovium samples. **A.** RA synovium sample showing increased staining for NLRC2 and decreased staining for NLRX1. **B.** Semiquantitative analysis showing significantly increased NLRC2-positive staining and significantly decreased NLRX1-positive staining in RA synovia compared with OA synovia.

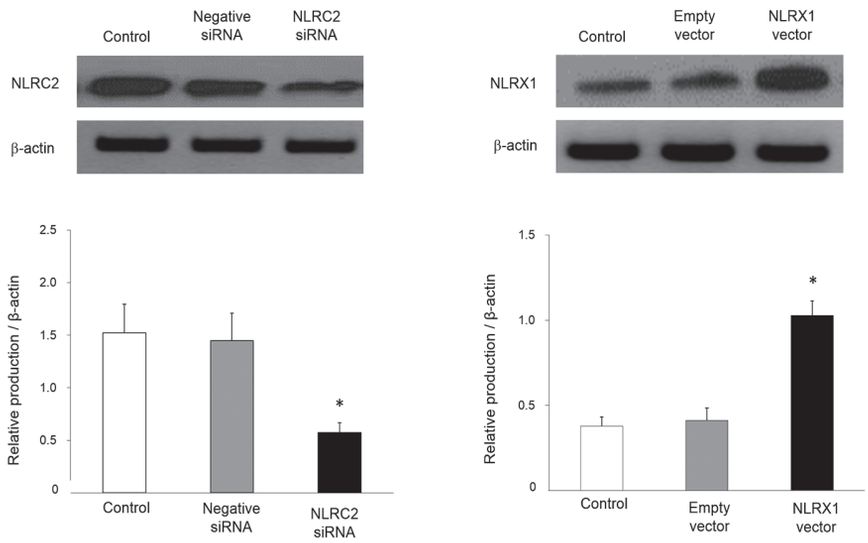

Fig. 3. Effects of NLRC2 and NLRX1 gene regulation on the corresponding protein levels in RA FLS. NLRC2 protein levels were decreased in RA FLS transfected with the NLRC2 RNAi plasmid and NLRX1 protein levels were increased in RA FLS transfected with pcDNA3.1-NLRX1.

RA FLS were higher than those in OA FLS, whereas NLRX1 mRNA expression levels in RA FLS were lower than those in OA FLS. The mRNA expression levels of NOD1, NLRP1, NLRP3, NLRP12, and NLRC3 did not differ between RA and OA FLS (Fig. 1).

Figure 2 shows results from immunohistochemical analyses of NOD2 and NLRX1 expression in RA and OA synovia. Increased NOD2 staining was observed in RA synovia compared to OA synovia, whereas NLRX1 staining was decreased in RA synovia compared to OA synovia (Fig. 2A). Semi-quantitative analysis demonstrated significantly increased NOD2-positive staining and significantly decreased NLRX1-positive staining in RA synovia compared with OA synovia (Fig. 2A).

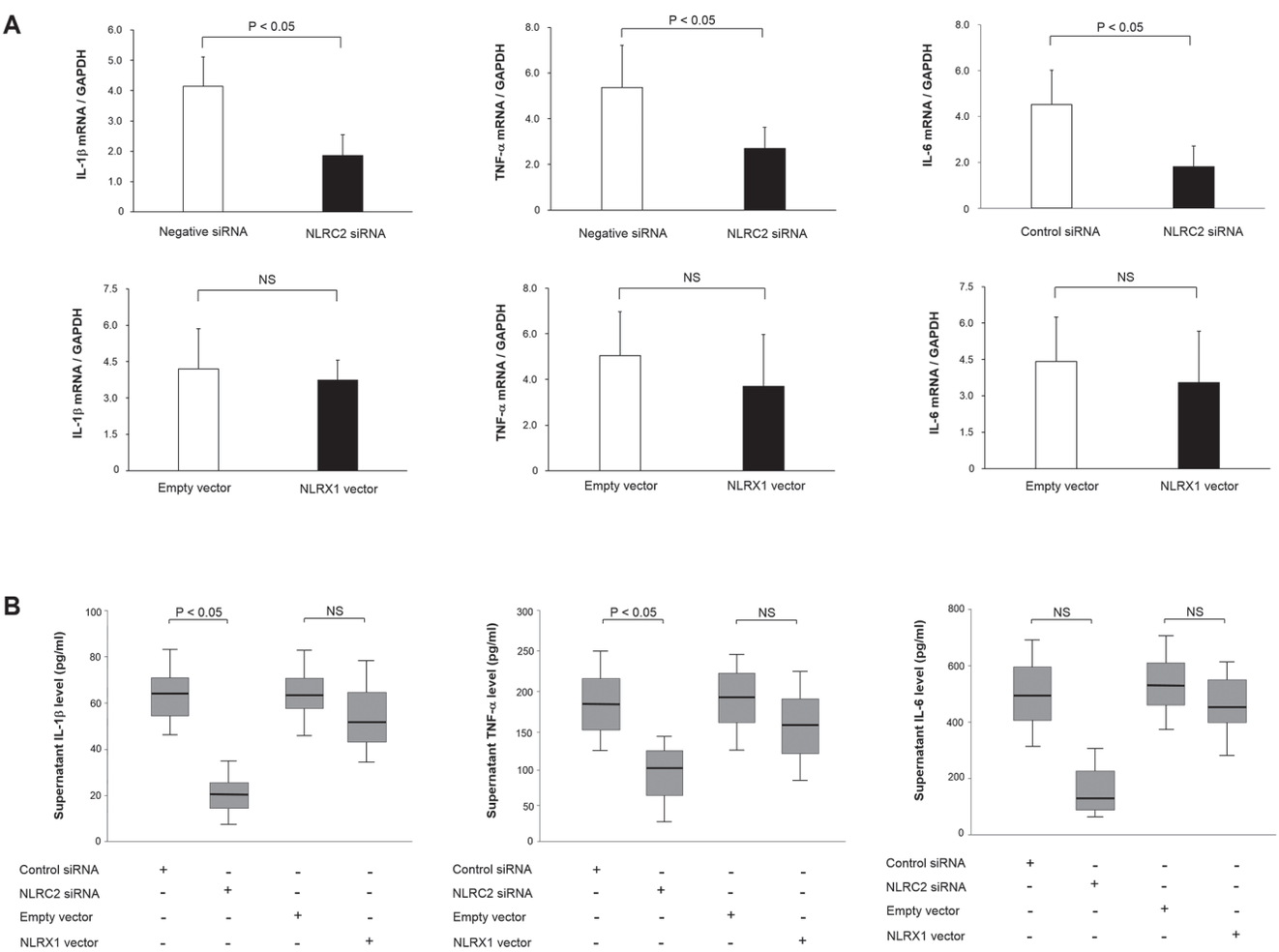

Fig. 4. Effects of NLRC2 and NLRX1 gene regulation on proinflammatory mediator levels in RA FLS. **A.** After treatment with 100 ng/mL lipopolysaccharide (LPS) for 24 h, the mRNA expression levels of IL-1β, TNF-α, and IL-6 in RA FLS transfected with an NLRC2 RNAi plasmid or pcDNA3.1-NLRX1 were determined using quantitative real-time PCR. **B.** After treatment with LPS, the concentrations of IL-1β, TNF-α, and IL-6 in the supernatants of RA FLS transfected with the NLRC2 RNAi plasmid or pcDNA3.1-NLRX1 were measured by ELISA.

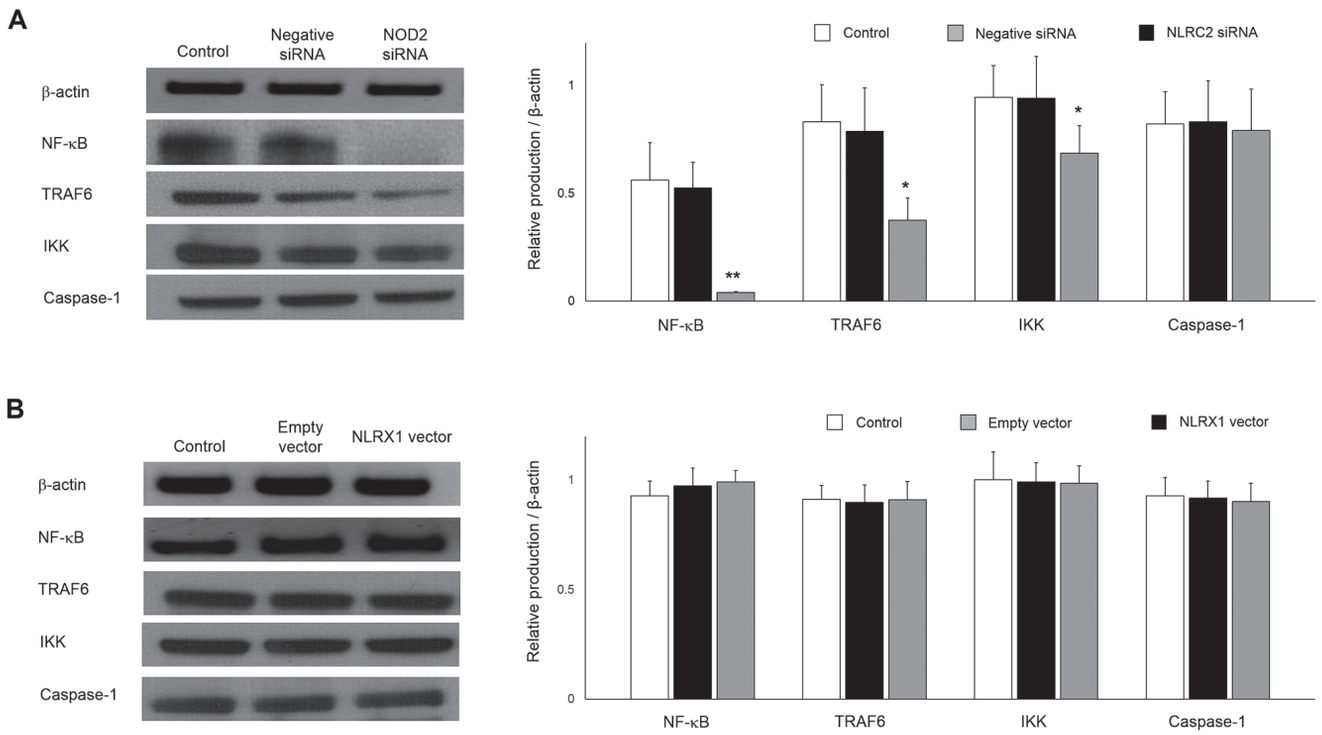

Fig. 5. Effects of NOD2 and NLRX1 gene regulation on NF-κB, TRAF6, IKK, and caspase-1 levels. **A.** Downregulation of NOD2 by transfection of RA FLS with a NOD2 RNAi plasmid resulted in significant reductions of NF-κB, TRAF6, and IKK levels, but not caspase-1. **B**. Augmentation of NLRX1 expression by transfection of RA FLS with pcDNA3.1-NLRX1 did not result in any significant changes in NF-κB, TRAF6, IKK, or caspase-1 levels.

Effects of NLR regulation on pro-inflammatory cytokine levels in RA FLS

Western blot analysis was next performed to determine the NOD2 levels in RA FLS transfected with NOD2 RNAi plasmids and the NLRX1 levels in RA FLS transfected with pcDNA3.1-NLRX1. As shown in Figure 3, the NOD2 level was decreased upon transfection with NOD2 RNAi plasmids, whereas the NLRX1 level was increased upon transfection with adenoviral vectors expressing NLRX1. Densitometric quantification of Western blot bands from RA FLS showed that these changes were statistically significant.

To investigate the effects of NOD2 and NLRX1 gene regulation on inflammatory responses in RA FLS, the mRNA expression levels of IL-1β, TNF-α, and IL-6 were measured by quantitative real-time PCR in RA FLS transfected with NOD2 RNAi plasmids or pcDNA3.1-NLRX1 after treatment with LPS. The mRNA expression levels of IL-1β, TNF-α, and IL-6 were significantly decreased upon transfection with the NOD2 RNAi plasmids compared with the levels in FLS transfected with the non-silencing RNAi plasmids. Transfection of RA FLS with pcDNA3.1-NLRX1 did not result in any significant changes in the gene expression levels of IL-1β, TNF-α, or IL-6 (Fig. 4A).

We next performed ELISA to determine the effects of NLR gene regulation on pro-inflammatory cytokine levels in cell supernatants. Transfection of RA FLS with NOD2 RNAi plasmids significantly decreased the levels of IL-1β, TNF-α, and IL-6 RA secreted from FLS, whereas transfection with pcDNA3.1-NLRX1 did not result in any significant changes in the secretion of these cytokines (Fig. 4B).

Effect of NLR regulation on NF-κB and caspase-1 levels

To assess the effect of NOD2 and NLRX1 gene regulation on the NF-κB signalling pathway and caspase-1 in RA FLS, the levels of NF-κB, TRAF6, IKK, and caspase-1 were determined by Western blotting in RA FLS transfected with the NOD2 RNAi plasmid or pcDNA3.1-NLRX1. In RA FLS transfected with the NOD2 RNAi plasmid, the NF-κB, TRAF6, and IKK levels were lower than in RA FLS transfected with the non-silencing RNAi plasmid; densitometric quantification of bands from RA FLS showed that this increase was statistically significant. However, the caspase-1 level did not show any significant changes upon transfection of RA FLS with the NOD2 RNAi plasmid (Fig. 5A). Transfection of RA FLS with pcDNA3.1-NLRX1 did not result in any significant changes in NF-κB, TRAF6, IKK, or caspase-1 levels in RA FLS (Fig. 5B).

Discussion

In the present study, we determined the differential gene expression levels of various NLR family members in RA FLS and compared them with their levels in OA FLS. We found that all NLRs tested were constitutively expressed in RA and OA FLS. Moreover, among the various NLRs, NOD2 gene expression was increased and NLRX1 gene expression was decreased in RA FLS compared to OA FLS. Similar results were also found regarding the protein levels of these NLRs. These levels were assessed by semiquantitative scoring of immunohistochemical staining of synovium samples. Our results suggest that

NOD2 and NLRX1 may play a role in inflammatory responses in RA.

NOD2 has been shown to be expressed in intestinal cells, oral epithelial cells, gingival fibroblasts, osteoblasts, and fibroblast-like synoviocytes (15, 16, 21-24). These findings indicate that resident tissue cells express NLRs and play an active role in recognition and response to endogenous or exogenous pathogens. Since activation of the innate immune system by endogenous or exogenous stimuli has been suggested to play a role in the pathogenesis of RA (13) and endogenous danger signals and infectious pathogens can be recognised by these pattern-recognition receptors, it is reasonable to hypothesise that sensing endogenous ligands through some NLRs may play a role in the inflammatory process of RA. In the present study, we found that NOD2 gene expression and protein production were enhanced in RA synovia; these results are consistent with previous observations. NOD2 expression has also been shown to be strongly increased in RA synovia, especially at sites of synovial invasion into articular cartilage (14, 15). In the present study, we also found that down-regulation of NOD2 expression in RA FLS via transfection with a NOD2 RNAi plasmid resulted in decreased pro-inflammatory cytokine levels, suggesting that NOD2 plays a pro-inflammatory role in RA. A similar effect of NOD2 has been documented in various inflammatory diseases. For instance, intestinal macrophages isolated from patients with Crohn's disease harbouring disease-associated NOD2 alleles produced elevated levels of pro-inflammatory cytokines (25, 26). Moreover, activation of NOD2 induced IgG4 production by peripheral blood mononuclear cells isolated from patients with autoimmune pancreatitis (27). In murine models of arthritis, NOD2 downregulation was shown to reduce joint inflammation and to reduce pro-inflammatory cytokine and chemokine levels (17, 28). Although pattern recognition receptors, including NLRs, may interact with coexisting infections and stimulate pro-inflammatory mediator production in patients with RA (29), these results, along with our findings, suggest that NOD2 activation can exert a pro-inflammatory influence in RA.

NOD2 induces multiple effector pathways that are involved in inflammatory response. The well-characterised NOD2 effector signalling pathway involves the activation of downstream signalling via receptor-interacting protein kinase 2 (RIP2), a serine/threonine kinase that is essential for NF-κB activation (25, 30, 31). The action of NOD2 is mediated by phosphorylation of NF-κB p65, which enhances p65 transactivation activity. Moreover, activation of the NOD2/RIP2 complex drives the expression of numerous pro-inflammatory cytokines such as IL-1β and TNF-α (34). While NOD2 controls cytokine expression mainly via its ability to activate the NF-κB signalling pathway, several studies have reported that NOD2 has a role in caspase-1 activation and subsequent IL-1β production (35, 36). To identify the dominant signalling pathways through which NOD2 exerts its pro-inflammatory actions in RA FLS, we determined the effect of NOD2 downregulation on NF-κB and caspase-1 levels. We found that downregulation of NOD2 gene expression in RA FLS led to decreased NF-κB, TRAF6, and IKK expression, whereas the caspase-1 level did not significantly change. These observations indicate that the pro-inflammatory action of NOD2 is primarily mediated by NF-κB activation, rather than caspase-1, in RA FLS.

Recent studies have identified a functional subgroup of NLRs, including NLRP12, NLRX1, and NLRC3, that negatively regulates inflammation (37-41). These proteins function as negative regulators of the inflammatory response by modulating canonical and non-canonical NF-κB signalling (11, 41-43). Interestingly, we found significant differences in the gene expression levels of NLRX1, but not NLRP12 and NLRC3, in RA FLS *versus* OA FLS. Specifically, NLRX1 gene expression was reduced in RA FLS and its protein levels were also decreased in RA synovia compared to samples from patients with OA. These findings suggest that the loss of anti-inflammatory NLRX1 function may account for the development and maintenance of rheumatoid synovitis. However, upregulation of NLRX1 gene expression in RA FLS by transfection with pcDNA3.1-NLRX1 had little effect on the gene expression levels and cell supernatant concentrations of multiple pro-inflammatory cytokines. NLRX1 associates with TRAF6 and IKK through an activation signal-dependent mechanism and inhibits subsequent canonical NF-κB activation (43). Since NF-κB signalling is one of the most salient mechanisms in the pathogenesis of RA, the inability of NLRX1 overexpression to affect inflammatory responses in RA FLS was an interesting result. The reason for this negative result remains unclear. We speculate that the stimulation of FLS with LPS prior to exogenous expression of NLRX1 may have been insufficient to activate NLRX1 in the present study. It is also possible that NLRX1 activation requires a specific stimulus other than LPS in FLS, because most studies focusing on the role of NLRX1 have shown that NLRX1 acts as a modulator of PAMP receptors in response to viral exposure (39, 42). Further investigation is needed to clarify this issue.

Our understanding of the role of the innate immune response in RA pathogenesis is growing. Although initial recognition of microbial pathogens is the first step in the activation of the innate immune system, self-peptides may be the primary target in autoimmune diseases such as RA (44). As intracellular sensors, NLRs are attractive target molecules for better understanding the pathogenesis of RA. Our study provides evidence that RA FLS exhibit selective overexpression of NOD2 and decreased expression of NLRX1. These findings indicate that sensing of the endogenous DAMP signal released during the inflammatory RA cascade may perpetuate inflammation by activating specific NLRs. To the best of our knowledge, this study is the first to comprehensively evaluate and compare the expression levels of various NLRs and to determine their association with inflammatory cytokine levels in RA. Our data provide evidence that NLRs, particularly NOD2, play a role in the pathogenesis of RA and suggest that NOD2 may serve as a therapeutic target for the treatment of RA.

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