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Interleukin-33 acts as a transcriptional repressor and extracellular cytokine in fibroblast-like synoviocytes in patients with rheumatoid arthritis

Eun-Ju Lee [a,c,1], Min Wook So [b,1], Seokchan Hong [c], Yong-Gil Kim [c], Bin Yoo [c], Chang-Keun Lee [c,*]

[a] Asan Institute for Life Science, Asan Medical Center, Seoul 138-736, Republic of Korea
[b] Division of Rheumatology, Department of Internal Medicine, Pusan National Yangsan Hospital, Yangsan 626-770, Republic of Korea
[c] Division of Rheumatology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 138-736, Republic of Korea

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ABSTRACT

The present study aimed to assess the functions of interleukin (IL)-33 in fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA). Enzyme-linked immunosorbent assays (ELISAs) were used to quantify interleukin (IL)-33 in plasma obtained from patients with RA and osteoarthritis (OA). To evaluate functions of intracellular IL-33, levels of inflammatory mediators and matric metalloproteinases (MMPs) were measured in RA FLS transfected with IL-33 small- interfering RNA (siRNA) or plasmids, and changes in the expression and regulation of nuclear factor kappaB (NF-κB) were determined using western blotting and reporter gene assays. In addition, to examine the extracellular effects of IL-33, *IP*10 and receptor activator of NF-κB ligand (*RANKL*) mRNA levels were measured after treatment with IL-33 and blocking antibodies to ST2, the IL-33 receptor. To evaluate whether extracellular IL-33 regulated osteoclastogenesis, human $CD14^+$ monocytes cocultured with IL-33-stimulated FLS were stained with tartrate-resistant acid phosphatase (TRAP). IL-33 levels were higher in plasma obtained from patients with RA than in those obtained from patients with OA. The expression levels of IL-33 were elevated in RA FLS that had been stimulated with poly I:C, IL-1β, and tumor necrosis factor (TNF)-α. Silencing of IL-33 increased the levels of pro-inflammatory molecules and MMPs, promoted inhibitor of kappaB (IκBα) degradation, and increased NF-κB activity; these effects were reversed in IL-33 plasmid-transfected FLS. Stimulation with exogenous IL-33 increased *RANKL* and *IP-10* mRNA expression. These increases were blocked by anti-ST2 treatment. Furthermore, we confirmed that extracellular IL-33 stimulated the formation of $TRAP^+$ multinucleated osteoclasts through RA FLS. These results suggested that intracellular IL-33 acted as a transcriptional repressor of NF-κB, which may provide negative feedback against inflammatory responses, whereas, extracellular IL-33 functioned as an activator of osteoclastogenesis. Therefore, increased plasma IL-33 levels in patients with RA could be a possible biomarker to reflect the potential risks of bone erosion.

1. Introduction

The pathology of rheumatoid arthritis (RA) is defined by immune-mediated joint inflammation and the destruction of cartilage and bone [1,2]. In particular, RA fibroblast-like synoviocytes (FLS) in the synovial intimal lining produce cytokines that perpetuate inflammation and pro-destructive proteases, inducing a unique and aggressive phenotype known as pannus. Pannus eventually leads to invasion into the extracellular matrix of the adjacent cartilage and bone, further exacerbating joint damage [3].

Interleukin (IL)-33, the most recently discovered cytokine in the IL-1 family, was originally described as a nuclear protein in canine cerebral arteries [4,5]. Since then, nuclear IL-33 expression has been identified both *in situ* and *in vitro* in several cell types, including endothelial cells, epithelial cells, and fibroblasts [6–9]. Furthermore, overexpression of the IL-33 protein has been detected in the nuclei of RA FLS [10]. An association between IL-33 and heterochromatin in endothelial cells has been functionally linked to transcription [11,12], and overexpression of intracellular IL-33 reduces

* Corresponding author at: Division of Rheumatology, Department of Internal Medicine, University of Ulsan, College of Medicine, Asan Medical Center, Seoul 138-736, Republic of Korea.
E-mail address: cklee@amc.seoul.kr (C.-K. Lee).
[1] Eun-Ju Lee and Min Wook So contributed equally to this work.

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NF-κB-mediated gene expression, thereby suppressing proinflammatory signaling [13].

ST2, a receptor of IL-33, is expressed on endothelial cells, mast cells, macrophages, and fibroblasts. Once IL-33 is released into extracellular areas, the IL-33/ST2 system can also be involved in the progression of RA. Recent studies performed in murine models of collagen-induced arthritis (CIA) suggest that the IL-33/ST2 pathway is involved in the pathogenesis of RA [14–16]. Indeed, the administration of a soluble ST2 fusion protein or an antibody specific for ST2 receptors to mice reduced disease severity in comparison with untreated animals. IL-33 treatment also markedly exacerbated synovial hyperplasia and mononuclear and polymorphonuclear cell infiltration into the joints, which was accompanied by marked cartilage and bone erosion. IL-33 and ST2, both of which are expressed in the human RA synovium, are elevated in the sera and synovial fluids of RA patients and demonstrate a positive correlation with disease progression [17]. Moreover, the mRNA expression levels of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) were reduced in the joints of anti-ST2-treated mice [10], suggesting that IL-33/ST2 signaling affects RANKL-mediated bone erosion.

Overall, these findings suggest that IL-33 acts as a dual-function cytokine that exerts unique biological effects on the cell nucleus independent of its binding to cell surface receptors. Nevertheless, to date, studies investigating this cytokine have primarily focused on extracellular IL-33, rather than the function of intracellular IL-33 as a repressor of transcription. Accordingly, in the present study, we investigated the dual functions of IL-33 in RA FLS.

2. Methods

2.1. Plasma samples from RA patients and osteoarthritis (OA) patients

Plasma samples were obtained from patients who were referred to Asan Medical Center in 2010 and 2011. We recruited 30 patients with early RA (less than a 12-month history of symptoms) who fulfilled the American College of Rheumatology criteria for RA [18]; we excluded patients who had been previously treated with disease-modifying antirheumatic drugs. Plasma samples were obtained from OA patients. All patients provided written informed consent, and the ethics committee of our institution approved the study protocol.

2.2. Primary culturing of FLS obtained from RA and OA patients

FLS were isolated from synovial tissues obtained from RA patients during synovectomy. The tissues were washed with phosphate-buffered saline (PBS; Welgene, Korea), minced, and incubated with collagenase (Invitrogen Life Technologies, CA, USA) for 5 h at 37 °C with gentle agitation. Isolated cells were collected by centrifugation and washed with serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies). The cell suspensions were then cultured at 37 °C in a humidified incubator with 5% CO_2. After overnight incubation, nonadherent cells were removed to obtain only adherent synoviocytes. FLS obtained after 4–6 passages were used in the experiments.

2.3. Silencing IL-33 in RA FLS

IL-33 was silenced using small interfering RNA (siRNA). The siRNA duplex used in this study was designed to target the human IL-33 sequence (HSS132061). Cells that achieved ⩾80% confluence were transfected with IL-33 siRNA or scrambled RNA (Invitrogen Life Technologies) using the RNA MAXi transfection reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. After transfection for 24 h, the cells were stimulated with 10 ng/mL TNF-α (R&D Systems, MN, USA), 10 ng/mL IL-1β (R&D Systems), and 10 μg/mL poly I:C (Sigma, Anbour, USA) for 24 h. The cells and supernatants were collected and proinflammatory cytokines and matrix metalloproteinases (MMPs) were analyzed as described in subsequent sections.

2.4. Overexpression of IL-33 in RA FLS

The coding sequence of IL-33 was cloned into pLenti6/V5 vectors. To produce the lentivirus, 293FT cells were transfected with a ViraPower packaging mix (Invitrogen Life Technologies), and a pLenti IL-33 plasmid or an empty pLenti expression plasmid (as a control) using Lipofectamine 2000 and Opti-MEM® I medium. Virus-containing supernatants were collected. For IL-33 overexpression, RA FLS were transduced with the virus-containing supernatant in the presence of Polybrene. The cells and supernatants were collected and the proinflammatory cytokines and MMPs were analyzed.

2.5. RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA extraction was carried out using the TRIsure reagent (Bioline, London) according to the manufacturer's instructions. Reverse transcription for cDNA synthesis was accomplished using 1 μg of total RNA and oligo dT primers (Promega, WI, USA). Quantitative RT-PCR was performed in 96-well plates in a total volume of 20 μL using the LightCycler 480 SYBR Green I Master system (Roche Diagnostics, IN, USA). After incubation at 95 °C for 10 min using the LightCycler 480 system and 45 rounds of amplification for 10 s at 95 °C, 20 s at 55–60 °C, and 30 s at 72 °C, relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$. GAPDH was used as the internal control. The primers used in this study are listed in Supplementary Data 1.

2.6. Protein extraction and immunoblot analysis

Whole cell lysates were prepared in lysis buffer (iNtRon, Daejon, Korea). Protein samples were separated using 10% SDS–PAGE and transferred to PVDF membranes (Amersham Pharmacia Biotech Uppsala, Sweden). The membranes were then blocked, and incubated for 2 h at room temperature in primary antibody against the inhibitor of κB (1:1000 IκB; Cell Signaling, MA, USA). Following the washes, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling) for another 1 h at room temperature. Bands were detected using ECL Prime detection solution and hyperfilm (Amersham Pharmacia Biotech).

2.7. Enzyme-linked immunosorbent assay (ELISA)

IL-6, IL-8, MCP-1, MMP-1, MMP3, and MMP-13 levels in the cultured supernatant of FLS were determined using ELISA according to the manufacturer's instructions (R&D Systems). The levels of intracellular IL-33 were determined by resuspending FLS in cell lysis buffer and incubating the samples with gently agitation for 30 min at room temperature. After centrifugation at 15,700*g* for 5 min, IL-33 and total protein quantities in cell lysates were determined using ELISA kit (cat.D3300; R&D Systems) and a protein assay reagent (Bio-Rad Laboratories, Inc., CA, USA), respectively. Intracellular IL-33 levels were adjusted according to the amount of total protein. That is, intracellular IL-33 levels (pg/mL) were divided by the quantity of total protein (mg/mL) in the whole cell lysate. IL-33 levels in the plasma samples were measured using ELISA Duoset (R&D Systems).