Interleukin-12B is upregulated by decoy receptor 3 in rheumatoid synovial fibroblasts

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Abstract. Decoy receptor 3 (DcR3) competitively binds to three ligands, Fas ligand, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells and tumor necrosis factor-like ligand 1A (TL1A), to prevent their effects. Recent studies have suggested that DcR3 directly affects cells as a ligand. Using a microarray assay, our group newly identified interleukin (IL)-12B, which encodes the p40 subunit common to IL-12 and IL-23, as one of the genes for which expression in fibroblast-like synoviocytes from patients with rheumatoid arthritis (RA-FLS) is induced by DcR3. The present study demonstrated that IL-12B mRNA expression was upregulated by DcR3-Fc in RA-FLS in a dose-dependent manner, but not in OA-FLS. IL-12B p40 protein in RA-FLS was increased when stimulated with DcR3-Fc. Pre-treatment with anti-TL1A antibody suppressed the upregulation of IL-12B mRNA in RA-FLS stimulated with DcR3-Fc. DcR3 mRNA expression in RA-FLS was induced by IL-23, but not by IL-12. These results indicated that DcR3 may increase IL-12 or IL-23 by inducing IL-12B p40 expression via membrane-bound TL1A on RA-FLS and that IL-23 reciprocally induces DcR3 expression in RA-FLS. DcR3 and IL-23 may interact in a feedback loop that aggravates local inflammation in patients with RA.

Introduction

Rheumatoid arthritis (RA) is an inflammatory joint disease that features hyperplasia of the synovial tissue and formation of pannus, and their invasive growth into the cartilage,

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which results in the destruction of cartilage and bone (1). Inflammatory cytokines, including interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF) α , are expressed and functionally active in synovial tissues. Within a complex regulatory network, cytokines are implicated in specific immunological processes that promote chronic inflammation, autoimmunity and tissue destruction (2).

Decoy receptor 3 (DcR3)/TR6/M68/TNFRSF6b is a member of the TNF receptor (TNFR) superfamily, but is a secreted protein, as it lacks the transmembrane domain of conventional TNFRs (3). The three ligands of DcR3 are the TNF superfamily members Fas ligand, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT) and TNF-like ligand 1A (TL1A) (4). Death receptor 3 (DR3) is the receptor for TL1A that induces apoptosis and the activation of nuclear factor κ-light-chain-enhancer in activated B cells. DcR3 antagonizes TL1A/DR3 signaling event (5). DcR3 is expressed in certain types of normal tissues, including the colon, stomach, spleen, lymph nodes, spinal cord, pancreas and lungs (3,6), but not in NIH3T3 human fibroblast cells (7); furthermore, DcR3 is frequently overexpressed in various tumor cell types (3,6,8). In tumors, overexpression of DcR3 may facilitate the evasion of the cytotoxic and regulatory effects of Fas ligand (3,9), LIGHT (10) and TL1A (5). A previous study by our group reported that DcR3 is expressed in fibroblast-like synoviocytes from patients with rheumatoid arthritis (RA-FLS) and that DcR3 expression induced in RA-FLS by TNFa protected the cells from Fas-induced apoptosis (11). These results led to the hypothesis that DcR3 is a key regulatory molecule for the proliferation of RA-FLS.

Studies have suggested that DcR3 directly induces monocytes to form osteoclasts (12) and that reverse signaling of DcR3 triggers enhanced adhesion of monocytes (13). A previous study by our group also reported that DcR3 induces very late antigen-4 expression in THP-1 macrophages to inhibit cycloheximide-induced apoptosis (14). Another study by our group found that DcR3 binds to TL1A expressed on RA-FLS, resulting in negative regulation of inflammatory cytokine-induced cell proliferation (15). Furthermore, a comprehensive genetic analysis using microarrays by our group demonstrated that DcR3 regulates gene expression in RA-FLS (16).

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From these gene expression profiles, IL-12B was identified by our group as a gene which is induced by DcR3 in RA-FLS (16). IL-12B encodes the IL-12B p40 subunit, which is common to IL-12 and IL-23 (17). IL-12 consists of IL-12A p35 and IL-12B p40 and induces T-helper cell (Th)1 immune responses, which are linked to autoimmune diseases, including inflammatory bowel disease and psoriasis (18). IL-23 is comprised of IL-23A p19 and IL-12B p40 and is linked to autoimmune diseases, including multiple sclerosis and inflammatory bowel disease, via Th17 immune responses (19). IL-12 (20) and IL-23 (21,22) have also been reported to be associated with the pathogenesis of RA.

The present study demonstrated that DcR3 induces IL-12B p40 expression in RA-FLS by binding to membrane-bound TL1A. In turn, IL-23 upregulates DcR3 expression in RA-FLS. These results suggested that DcR3 and IL-23 may interact in a feedback loop that aggravates local inflammation in patients with RA.

Materials and methods

Isolation and culture of synovial fibroblasts. Synovial samples were obtained from patients with RA who fulfilled the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (23) and who had never been treated with biologics during total hip or knee replacement. Patients included 3 males and 27 females aged 69.0±10.3 years old. Written informed consent to participate in this study was obtained from all patients in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The Medicine Ethics Committee of Kobe University Graduate School of Health Sciences (Kobe, Japan) approved the protocol including consent procedures. Synovial samples from patients with osteoarthritis (OA) were obtained during total knee replacement in a similar manner (8 females; aged 71.1±10.6 years old). To isolate FLS, synovial tissue specimens were minced and digested in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 0.2% collagenase (Sigma-Aldrich) for 2 h at 37°C. Dissociated cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 units/ml penicillin/streptomycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Following incubation overnight and removal of non-adherent cells, adherent cells were further incubated in fresh medium. Cells from passages 3-7 were used in all further experiments (11).

Cell treatments. For quantification of IL-12B mRNA expression in RA-FLS by reverse transcription quantitative polymerase chain reaction (RT-qPCR), cells (1x10⁶/well) were stimulated with 10, 100 or 1,000 ng/ml recombinant human DcR3-Fc chimera protein (DcR3-Fc; R&D Systems, Minneapolis, MN, USA), 1,000 ng/ml immunoglobulin (Ig)G1 (R&D Systems) as a control, or left untreated by incubation in serum-free Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 12 h. Furthermore, RA-FLS (1x10⁶ cells/well) were stimulated with 1,000 ng/ml DcR3-Fc for 0, 6, 12 and 24 h.

In another experiment, RA-FLS (1x10⁶ cells/well) were pre-incubated with 5.0 μ g/ml monoclonal mouse anti-human TL1A antibody (clone 6E6; 322204; Biolegend, San Diego, CA, USA), 5.0 μ g/ml mouse IgG1 (BA343; Acris, San Diego, CA, USA) or serum-free Opti-MEM overnight at 37°C prior to stimulation with 1,000 ng/ml DcR3-Fc for 12 h and analysis of IL-12B mRNA levels by RT-qPCR.

Further batches of RA-FLS (1x10⁶ cells/well) were stimulated with 10 or 100 ng/ml recombinant human IL-12 (R&D Systems), 10 or 100 ng/ml recombinant human IL-23 (R&D Systems) or serum-free Opti-MEM for 12 h for subsequent assessment of DcR3, TL1A and DR3 mRNA by RT-qPCR.

For quantification of IL-12B mRNA expression in OA-FLS stimulated with DcR3-Fc, cells ($1x10^{6}$ /well) were incubated with 1,000 ng/ml DcR3-Fc or IgG₁ in serum-free Opti-MEM for 12 h.

For assessment of the expression of IL-12B p40 protein in RA-FLS by western blot analysis, cells (1x10⁶/well) were stimulated with 1,000 ng/ml DcR3-Fc, 1,000 ng/ml IgG1 or left untreated in serum-free Opti-MEM for 24 h.

RT-qPCR analysis. RA-FLS and OA-FLS were cultured in six-well plates at 1x10⁶ cells/well with various stimulants as described above. RNA was extracted using the QIAshredder and RNeasy mini kits (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Oligo (dT)-primed first-strand complementary DNA (cDNA) was synthesized from 2 μ g total RNA using a High Capacity cDNA Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression levels of mRNA encoding IL-12B p40, DcR3, TL1A and DR3, which also binds to TL1A, were compared using TaqMan[®] real-time PCR on a StepOne[™] real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Pre-designed primers and probes for IL-12B (Hs01011518_m1), DcR3 (Hs00187070_m1), TL1A (Hs00270802_s1), DR3 (Hs00600930_g1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) as the control were obtained from Applied Biosystems (Thermo Fisher Scientific, Inc.). Comparative analyses of each of these genes in individual patients were performed using StepOne[™] 2.1 software (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All amplifications were conducted in duplicate. mRNA expression levels of each gene were calculated using the comparative threshold cycle $(\delta\delta Cq)$ method, as previously described (24).

Western blot analysis. Following stimulation, cells were washed on ice and lysed using a solution of protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan), phosphatase inhibitor cocktail 2/3 (Sigma-Aldrich) and hypotonic lysis buffer, which contained 25 mM Tris (Nacalai Tesque Inc.), 150 mM NaCl (Sigma-Aldrich), 1% NP-40 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1.5 mM ethylene glycol tetraacetic acid (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The lysate was incubated for 40 min at 4°C and was subsequently centrifuged at 20,400 x g for 12 min at 4°C in order to isolate the supernatant containing the cytoplasmic proteins. Cytoplasmic proteins were quantified via the Bradford method using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following dilution to an equal concentration with hypotonic lysis buffer



Figure 1. Expression of IL-12B mRNA in RA-FLS. (A) Relative expression levels of IL-12B mRNA in RA-FLS after 12 h of incubation with 10, 100 or 1,000 ng/ml DcR3-Fc or 1,000 ng/ml IgG1, or serum-free medium only. Unstimulated cells were assigned a value of 1. Values are expressed as the mean \pm standard deviation (n=7). (B) Relative expression levels of IL-12B mRNA in RA-FLS after 0, 6, 12 or 24 h of incubation with 1,000 ng/ml DcR3-Fc. Expression at time-point zero was assigned a value of 1. Values are expressed as the mean \pm standard deviation (n=13). *P<0.05; **P<0.01. IL, interleukin; IgG, immunoglobulin G; FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis; DcR3, decoy receptor 3.

(25 mM Tris, 150 mM NaCl, 1% NP-40 and 1.5 mM ethylene glycol tetraacetic acid), each sample was loaded (80 ng/lane) and electrophoresed on a 7.5-15% polyacrylamide gradient gel (Biocraft, Tokyo, Japan) and electrotransferred onto a blotting membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked with 0.05 g/ml skimmed milk (Megmilk Snow Brand Co., Ltd., Tokyo, Japan) diluted with Tris-buffered saline with Tween® 20 [TBST; 20 mM Tris (Nakalai Tesque, Inc.), 150 nM NaCl (Sigma-Aldrich) and 5% Tween® 20 (Bio-Rad Laboratories, Inc.)] for 1 h at room temperature. Following washing three times with TBST, the membrane was incubated with primary antibody diluted with Can Get Signal® Immunoreaction Enhancer Solution 1 (Toyobo Co., Ltd., Osaka, Japan) overnight at 4°C. Following incubation, the membrane was washed three times with TBST and incubated with secondary antibody diluted with Can Get Signal® Immunoreaction Enhancer Solution 2 (Toyobo Co., Ltd.) for 1 h at room temperature.

The expression of IL-12B p40 and α -tubulin was detected using mouse anti-human IL-12B p40 antibody (clone 169516; MAB6091; R&D Systems) and mouse anti-human α -tubulin antibody (clone DM1A; T9026; Sigma-Aldrich) as primary antibodies, respectively. Polyclonal sheep horseradish peroxidase-conjugated anti-mouse IgG antibody (NA931; GE Healthcare) was used as the secondary antibody, and antibodies were visualized using the ECLTM plus reagent (GE Healthcare) according to the manufacturer's protocols using the Chemilumino analyzer LAS-3000 mini (FujiFilm, Tokyo, Japan). Protein expression was evaluated by semi-quantification of digitally captured images using the public domain of the US National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/) with normalization to α -tubulin expression.

Statistical analysis. Values are expressed as the mean \pm standard deviation unless otherwise indicated. The Wilcoxon signed-rank test was used to evaluate the differences between



Figure 2. Relative expression of IL-12B mRNA in (A) RA-FLS and (B) OA-FLS after 12 h of incubation with 1,000 ng/ml DcR3-Fc or IgG1 in serum-free medium. Expression was normalized against IgG1, which was assigned a value of 1. Values are expressed as the mean ± standard deviation (n=7 for RA-FLS and n=8 for OA-FLS). **P<0.01. N.S., not significant; IL, interleukin; IgG, immunoglobulin G; RA, rheumatoid arthritis; OA, osteo-arthritis; FLS, fibroblast-like synoviocytes; DcR3, decoy receptor 3.

two groups. The Kruskal-Wallis test was used to evaluate the differences among three or more groups. If the Kruskal-Wallis test indicated statistical significance, a post-hoc analysis was performed for these groups. Statistical analyses conducted using Statcel (version 3; OMS Publishing, Inc., Tokyo, Japan). P<0.05 was considered to indicate a statistically significant difference.

Results

DcR3-Fc increases IL-12B mRNA expression in RA-FLS. RT-qPCR analysis revealed that the expression of IL-12B mRNA in RA-FLS was significantly increased by DcR3-Fc at the highest concentration of 1,000 ng/ml following incubation for 12 h (Fig. 1A). Time-course experiments showed that following 6 and 24 h of stimulation with DcR3-Fc (1,000 ng/ml), the expression of IL-12B mRNA in RA-FLS was

A

B

С



Figure 3. Expression of IL-12B p40 in RA-FLS. Expression levels of IL-12B p40 protein in RA-FLS after 24 h of incubation with 1,000 ng/ml DcR3-Fc, IgG1 or medium only. Values are expressed as the mean ± standard deviation (n=7). *P<0.05; **P<0.01. IL, interleukin; IgG, immunoglobulin G; FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis; DcR3, decoy receptor 3.



Figure 4. Inhibition of IL-12B mRNA expression in RA-FLS by anti-TL1A antibody. RA-FLS were stimulated with 1,000 ng/ml DcR3-Fc for 12 h after pre-incubation overnight with 5.0 μ g/ml anti-TL1A antibody, IgG1 or medium only. Values are expressed as the mean ± standard deviation (n=8). **P<0.01. IL, interleukin; IgG, immunoglobulin G; FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis; DcR3, decoy receptor 3; TL1A, tumor necrosis factor-like ligand 1A.

significantly increased to a similar extent and exhibited a peak at 12 h of stimulation (Fig. 1B).

Upregulation of IL-12B mRNA by DcR3-Fc in FLS is RA-specific. While the expression of IL-12B mRNA in RA-FLS was significantly increased by DcR3-Fc (Fig. 2A), it was not affected in OA-FLS (Fig. 2B). This finding suggested that IL-12B mRNA expression was upregulated by DcR3-Fc in FLS an RA-specific manner.

DcR3-Fc increases IL-12B p40 protein expression in RA-FLS. In accordance with the RT-qPCR results, western blot analysis confirmed that the expression of IL-12B p40 protein in RA-FLS was also significantly increased by DcR3-Fc (Fig. 3).

TL1A antibody suppresses DcR3-induced IL-12B expression in RA-FLS. RT-qPCR analysis revealed that the DcR3-Fc-induced increases in IL-12B mRNA expression in RA-FLS were significantly reduced by pre-treatment with anti-TL1A antibody (Fig. 4). While pre-treatment with IgG as a control slightly but not significantly inhibited DcR3-Fc-induced IL-12B expres-



Figure 5. Expression of (A) DcR3, (B) TL1A and (C) DR3 in RA-FLS stimulated with IL-12 or IL-23. Relative mRNA expression levels in RA-FLS were determined after 12 h of incubation with 0, 10 or 100 ng/ml recombinant human IL-12 or human IL-23. Values are expressed as the mean ± standard deviation (n=8). **P<0.01. N.S., not significant; IL, interleukin; FLS, fibroblast-like synovicytes; RA, rheumatoid arthritis; DcR3, decoy receptor 3; TL1A, tumor necrosis factor-like ligand 1A; DR3, death receptor 3.

sion, and the effect of anti-TL1A on IL-12B expression was significantly higher.

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IL-23, but not IL-12 induces DcR3 mRNA expression in RA-FLS. RT-qPCR revealed that IL-23 induced the expression of DcR3 mRNA, but not that of TL1A or DR3 mRNA, in RA-FLS. However, IL-12 did not induce the expression of DcR3, TL1A or DR3 mRNA (Fig. 5).

Discussion

Previous studies by our group have demonstrated that DcR3 has a substantial role in local inflammation in RA as a decoy receptor (11) and as a ligand for membrane-bound TL1A (15,16). A recent study by our group used a cDNA microarray assay to reveal the expression profiles of genes regulated by DcR3 in RA-FLS (16). The profile revealed upregulation of IL-12B (fold change, 1.65; P=0.008), which was assigned to the major functional clustering categories of cell motility and glycosylation (16). IL-12B mRNA encodes the IL-12B p40 subunit of IL-23 and IL-12. IL-23 regulates Th17 and is involved in the pathogenesis of inflammatory diseases (19,25,26). By contrast, IL-12 shifts the balance of the Th1 vs. the Th2 response towards the Th1 phenotype (18) and induces interferon gamma production via Th1 to be involved in the pathogenesis of inflammatory diseases (27). IL-12 and IL-23 are also key mediators of psoriasis including psoriatic arthritis and are targeted by Ustekinumab, a human anti-p40 monoclonal antibody therapeutic (28). Mannon et al (29) reported that patients with Crohn's disease responded well to treatment with monoclonal antibodies against the p40 subunit of IL-12/IL-23.

The present study confirmed the constitutive expression of IL-12B mRNA and p40 protein in RA-FLS and demonstrated that mRNA and protein expression levels were increased following stimulation with DcR3. Furthermore, while IL-12B mRNA expression was detected in RA-FLS as well as OA-FLS, DcR3 induced overexpression of IL-12B mRNA only in RA-FLS, while not affecting IL-12B expression in OA-FLS. Although the effect of DcR3 on p40 protein expression levels in OA-FLS was not assessed in the present study, it is unlikely to be affected without detectable effects on IL-12B mRNA expression. The results of the present study demonstrated that upregulation of IL-12B mRNA by DcR3-Fc in FLS is disease-specific for RA, as compared with OA which also causes severe destructive arthritis that may necessitate joint replacement but does not accompany autoimmune abnormality. However, other arthritic conditions are less likely to cause joint destruction, and these should be investigated in future studies.

Sakkas *et al* (30) showed that the levels of IL-12B mRNA are higher in the synovial membrane of patients with RA than those in patients with OA, although this difference was not statistically significant. Kitagawa *et al* (31) reported that the level of constitutive IL-12 p40 production by synovial cells (SC) from patients with RA was greater than that by SC from non-RA patients, including those with OA and ankylosing spondylitis, and that IL-12 p40 induction by lipopolysaccharide, a potent inducer of IL-12 production in macrophages and dendritic cells, in SC from patients with RA was significantly higher than that in SC from non-RA patients. Combined with the results of the present study, it is therefore suggested that DcR3/IL-12B mediates functions that comprise a disease-specific pathway in RA. Regarding the mechanisms of the DcR3 - IL-12B interaction, the present study revealed that binding of DcR3 to TL1A, which is expressed on RA-FLS, leads to enhanced expression of IL-12B. In addition, it was demonstrated that IL-23, but not IL-12, induced the expression of DcR3 in RA-FLS. However, the expression of TL1A and DR3 was not affected by either IL-23 or IL-12. These results indicated that DcR3 as a key component of the TL1A/DR3/DcR3 signaling pathway interacts with IL-23 in a feedback loop.

In the present study, the effects of DcR3 on IL-12B mRNA and p40 protein expression levels were investigated. Although the effects of TL1A, Fas ligand and LIGHT on IL-12B and p40 expression levels were were not investigated in the present study, further studies are required in order to assess these effects.

In conclusion, the present study suggested that DcR3 enhances the expression of IL-12B p40 in RA-FLS by binding to membrane-bound TL1A and may increase local IL-23 and IL-12 expression in the rheumatoid synovium. In addition, IL-23, but not IL-12, may induce the expression of DcR3 in RA-FLS. DcR3 and IL-23 may be interact in a feedback loop that aggravates local inflammation in patients with RA. Controlling the expression of local DcR3 or IL-12B may reduce inflammation in the rheumatoid synovium and may represent an approach for developing strategies to treat RA.

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