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Induction of IL-12 production by the activation of discoidin domain receptor 2 via NF-κB and JNK pathway

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ABSTRACT

We investigated the mechanism involving discoidin domain receptor 2 (DDR2) mediated production of interleukin 12 (IL-12). When compared to control, collagen I upregulated the IL-12 luciferase activity on DDR2 expressing cells. Collagen I induced the phosphorylation of DDR2 and enhanced the phosphorylation of mitogen activated protein kinase (MAPK) kinases. In addition, NF-κB binding activity was enhanced when the cells expressing NF-κB reporter were exposed to collagen I. Moreover, when IL-12 reporter transfected cells were treated with biochemical inhibitors of c-Jun N-terminal kinase (JNK) and NF-κB, collagen-induced IL-12 promoter activity was significantly downregulated in comparison to non-treated cells. Similarly, confirmatory experiments on murine dendritic cells revealed that IL-12 promoter activity is dose dependently downregulated upon NF-κB and JNK inhibitor treatment on collagen I stimulation. In summary, DDR2 is involved in the collagen I-induced IL-12 production via NF-κB and JNK pathway.

1. Introduction

Interleukin-12. (IL-12) is a heterodimeric cytokine composed of two subunits, p35 and p40 [1]. It is produced primarily by antigen-presenting cells (APCs) and induces IFN-γ production from T and natural killer (NK) cells, enhances the proliferation and cytotoxic activity of T and NK cells, and promotes the differentiation of naïve T cells into Th1 effectors [2,3]. The IFN-g production in turn promotes IL-12 production and activation of macrophages, which provides the basis of an auto-regulatory positive feedback loop resulting in a strong immune/inflammatory response directed against the antigen [4].

T helper lymphocytes can acquire distinct highly polarized cytokine profiles [2,5,6]. Early in the immune response, IL-12 also plays a critical role in directing the development of Th1 vs. Th2 cell differentiation characterized by an increased production of IFN and IL-2 (Th1 cytokines) and suppression of IL-4 (Th2 cytokine) formation. IL-12 production and a Th1 cytokine response are indispensable in the defense against intracellular pathogens [7,8]. But excessive production of IL-12 and Th1 cytokines can lead to autoimmune diseases such as multiple sclerosis, inflammatory bowel disease, diabetes, and rheumatoid arthritis as well as contribute to other inflammatory states such as septic shock, and the Schwarzman reaction [9–14].

In *in vivo*, the development of APCs takes place in contact to the extracellular matrix (ECM) proteins. In murine systems, it has been demonstrated that collagen can affect the function of dendritic cells in terms of their allostimulatory capacity, co-stimulatory molecules expression and secretion of cytokines [15,16]. In our previous studies, we have shown that IL-12 production is enhanced by collagen I and is mediated through a collagen receptor tyrosine kinase, discoidin domain receptor 2 (DDR2) [17,18]. This study investigates the role of DDR2 on IL-12 production and demonstrates that collagen I-induced IL-12 production is mediated through DDR2 and is associated with alteration of the activation of nuclear factor kappa B (NF-κB) and c-Jun terminal kinase (JNK).

2. Materials and methods

2.1. Animals

Female 8–10 week-old C57BL/6 mice were obtained from Samtaco (Seoul, Korea). All animal studies were performed in accordance with the protocol approved by the Institutional Animal Care and Use of Committee of Chonbuk National University Medical School.

2.2. Materials

Anti-phosphotyrosine mAb (p-Tyr-100) was from Cell Signaling (Beverly, MA). Anti-DDR2 (C-19), and anti-β-actin were from Santa

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Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated or nonphosphorylated p38, JNK and ERK were from Cell Signaling Technology (Beverly, MA). Fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG was obtained from BD Pharmingen (San Diego, CA). SB203580, PD98059, SP600125, and staurosporine were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). Pyrrolidinedithiocarbamate ammonium (PDTC) was purchased from Sigma (St. Louis, MO, USA). All reagents were from Sigma Co. (St. Louis, MO) unless otherwise stated.

pGL3-Luc (Luciferase) vector harboring the murine IL-12p40 promoter was a gift from Dr. Tae Sung Kim (Korea University, Seoul, Korea).

2.3. Cell culture

HEK293 cells were obtained from ATCC Global Bioresource Center (Manassas, VA) and cultured in Dulbecco's modified Eagle's Medium (DMEM, HyClone) containing 10% FBS (FBS, HyClone) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), under a 95% air, 5% CO_2 atmosphere at 37 °C. Generation of bone marrow-derived dendritic cells (mDCs), culture, and confirmation by flow cytometry has already been reported in our earlier study [18].

2.4. Flow cytometry

HEK293 cells were transfected with pcDNA3.1/mDDR2 using Lipofectime method (Invitrogen), according to the manufacturer's recommendations. After 48 h, cells were washed in phosphate buffer saline (PBS) containing 0.1% sodium azide and incubated on ice for 20 min with anti-DDR2, or control IgG antibodies. FITC-conjugated anti-goat antibody was added after washing once in PBS containing 0.1% sodium azide. Unlabelled cells were also included during the experiments. After 15 min incubation, the cells were analyzed using the FACS Calibur (Becton–Dickinson, San Diego, CA). Data were presented as histograms and mean fluorescence intensity (MFI).

2.5. Western blotting

Cells were lysed in ice-cold lysis buffer (iNtRON Biotech, Korea) for 20 min and centrifuged (15,000*g*) for 20 min. Protein concentrations were measured using a bicinchoninic acid technique. 20 μg lysates were run on an 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech). Then, blocking was done with 5% skimmed milk for 1 h at room temperature, and the membranes were probed with primary antibodies as indicated at 4 °C overnight, washed with tris-buffered saline containing 0.1% Tween 20 (TBST) for 4 times, and subsequently incubated with horseradish peroxidase-conjugated secondary antibody for 45 min. Again washed with TBST for 3 times and proteins were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

2.6. ELISA

Cytokine concentrations for IL-12 p40 were determined with commercially available reagents and ELISA kits purchased from eBioscience (eBioscience, California, USA). Cytokine concentrations were determined using a standard curve obtained from the known concentration of cytokine standards included in each assay plate according to manufacturer's instructions.

2.7. Electrophoresis mobility shift assay (EMSA)

EMSA was performed as described previously in detail [19,20].

2.8. Luciferase activity assay

Cells were transiently transfected with an IL-12 luciferase reporter gene construct or a control construct using Lipofectime method (Invitrogen), as previously described [20]. Cells were allowed to recover for 24 h, and stimulated as indicated. Then, cell lysates were prepared. Luciferase activity was evaluated using the Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

2.9. Statistical analysis

All values are presented as means ± SEM. Statistical significance was determined using the Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. Collagen I induces enhanced IL-12 production via DDR2

Our previous studies have shown that DDR2 enhances IL-12 production by dendritic cells upon stimulation with collagen I [17,18]. However, the pathway regulating the collagen-induced IL-12 production through DDR2 remained to be elucidated. To explore the associated pathways, we overexpressed DDR2 in HEK293 cells, analyzed the efficiency of transfection using flow cytometry (Fig. 1A and B), and subsequently applied the cells in the experiments. Fig. 2A shows that when DDR2 positive cells were cultured on none or collagen coated dishes, the relative luciferase activity of IL-12 was enhanced on collagen coated dishes only when compared to that of none coated or DDR2 negative cells on both the dishes. These results indicated that DDR2 is involved in the IL-12 production by collagen I. Next, we sought to examine the phosphorylation of DDR2 upon treatment with collagen I. Results indicated that DDR2 is phosphorylated upon collagen I stimulation

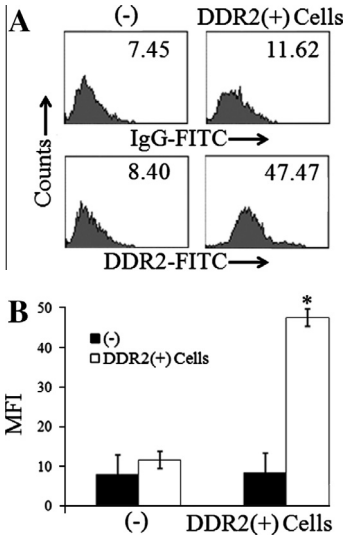

Fig. 1. Screening of transfection efficiency of discoidin domain receptor 2 (DDR2) in HEK293 cells. (A) The cells were transfected with pcDNA3.1/mDDR2 and after 24 h, the cells were analyzed by flow cytometry. (B) Bar diagram represents the mean ± SEM of three independent experiments' mean fluorescence intensity (MFI) (*$p < 0.05$).

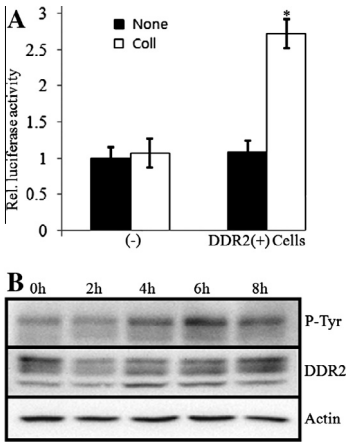

Fig. 2. Collagen I-induced IL-12 luciferase activity and phosphorylation of DDR2. (A) DDR2 was expressed transiently in HEK293 cells. After 48 h, cells were transfected with IL-12 luciferase promoter, followed by treatment with collagen I. Relative luciferase activity was measured. (B) After stimulation of DDR2 over-expressing cells with collagen I for indicated time points, cell lysates were analyzed for DDR2 phosphorylation by western blotting. The experiments were carried out three times with similar results (*$p < 0.05$).

until the indicated time points in DDR2 over expressing cells (Fig. 2B). These results indicated that DDR2 phosphorylation by collagen I results in enhanced IL-12 production.

3.2. Collagen I enhances MAPK phosphorylation and NF-κB binding activity

To identify the molecules associated with DDR2 mediated IL-12 production by collagen I; we examined the role of collagen upon MAPK phosphorylation by western blotting and NF-κB binding activity by EMSA. The data showed that the cells treated with collagen I induced an enhanced phosphorylation of MAPK kinases (Fig. 3A). In addition, EMSA result showed a time dependent increase in nuclear translocation of NF-κB (Fig. 3B). These results indicated that MAPK kinases and NF-κB translocation to the nucleus could be involved in the IL-12 production by collagen I.

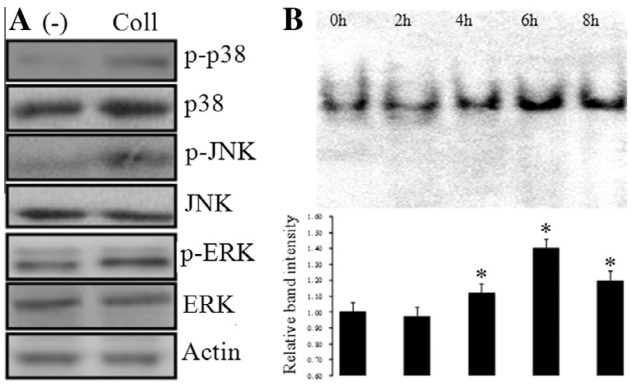

Fig. 3. Collagen I-enhanced MAPK kinases phosphorylation and NF-κB binding activity (A) HEK293 cells were cultured on none or collagen coated dishes for 24 h. Then cell lysates were prepared and western-blotting with specific antibodies to detect the phosphorylated and total forms of ERK, p38 and JNK, was performed. An anti-β-actin antibody was used to normalize the sample loading. (B) At indicated time points, nuclear extracts from the collagen I treated cells were evaluated with regard to NF-κB binding activity by EMSA using biotin labeled sequence containing a NF-κB site. Bar diagram represents relative band intensity of three experiments (*$p < 0.05$).

3.3. Collagen I-induced IL-12 production is regulated by NF-κB and JNK

Fig. 4A shows that the pharmacological inhibition of NF-κB and JNK significantly inhibited IL-12 luciferase activity on collagen coated dishes when compared to control, indicating that collagen I-induced IL-12 production is dependent on activation of NF-κB and JNK.

3.4. IL-12 production in mDCs is via NF-κB and JNK

To confirm the role of NF-κB and JNK on collagen-induced IL-12 production, we examined the IL-12 luciferase activity on mDCs treated or untreated with indicated concentrations of pharmacological inhibitors of NF-κB and JNK. As shown in Fig. 4B, when compared to none treated cells on collagen coated dishes; there was significant downregulation of the IL-12 luciferase activity in the presence of indicated doses of NF-κB inhibitor. Similarly, the IL-12 luciferase activity was significantly downregulated when the indicated doses of JNK inhibitor were treated to the cells (Fig. 4C). These data demonstrate that NF-κB and JNK play a role in DDR2 mediated collagen-induced IL-12 production in mDCs.

4. Discussion

In this study we showed that a novel collagen receptor tyrosine kinase, DDR2, plays an important role in the production of IL-12 with collagen I stimulation through NF-κB and JNK pathway. Our data demonstrated that DDR2 over expressed in HEK293 cells. Our previous studies have shown that DDR2 enhances IL-12 production by dendritic cells upon stimulation with collagen I [17,18]. However, the pathway regulating the collagen-induced IL-12 production through DDR2 remained to be elucidated. IL-12 plays a crucial role in the induction of inflammatory responses [5,6]. Our findings demonstrate that DDR2 inhibition represses collagen I-induced IL-12 production could have crucial value in various inflammatory diseases.

We dissected the signaling pathways associated with IL-12 production by using various pharmacological inhibitors. The results suggested that NF-κB and JNK were associated with IL-12 production in mDCs. Consistent with our data, previous reports have described the inhibition of IL-12 through several mechanisms. For instance, vitamin D3 has been shown to inhibit NF-κB binding, subsequently inhibiting IL-12 production [21]. Similarly, glucocorticoids have been shown to inhibit LPS-induced IL-12 production by inhibiting c-Jun [22], which signals through NF-κB [23]. Also, one study showed that a novel cytokine p43, an auxiliary factor of mammalian multiaminoacyl-TRNA synthetases, enhanced IL-12 production via activation of NF-κB in murine macrophages [20].

Previous studies have shown that ERK activation mediates the suppressive effect of *Leishmania phosphoglycans* [24] or *Candida albicans* [25] on IL-12 production. However, we did not observe ERK mediated IL-12 repression upon collagen I stimulation. Consistent with other studies, we demonstrate that IL-12 production was independent of p38 MAPK activation [26], in murine dendritic cells; rather, similar to previous studies, it partly depended on JNK [26,27].

To sum up, IL-12 has crucial role in cellular immunity against intracellular pathogens by directing the Th1 cells development. However, excessive IL-12 production is associated with several inflammatory diseases. Our study identified that DDR2 mediates collagen I-induced IL-12 production through NF-κB and JNK pathway. These results could contribute to fine tuning of immune response and prevention of Th1-mediated immunopathologies through DDR2 inhibition based therapies. Moreover, NF-κB and

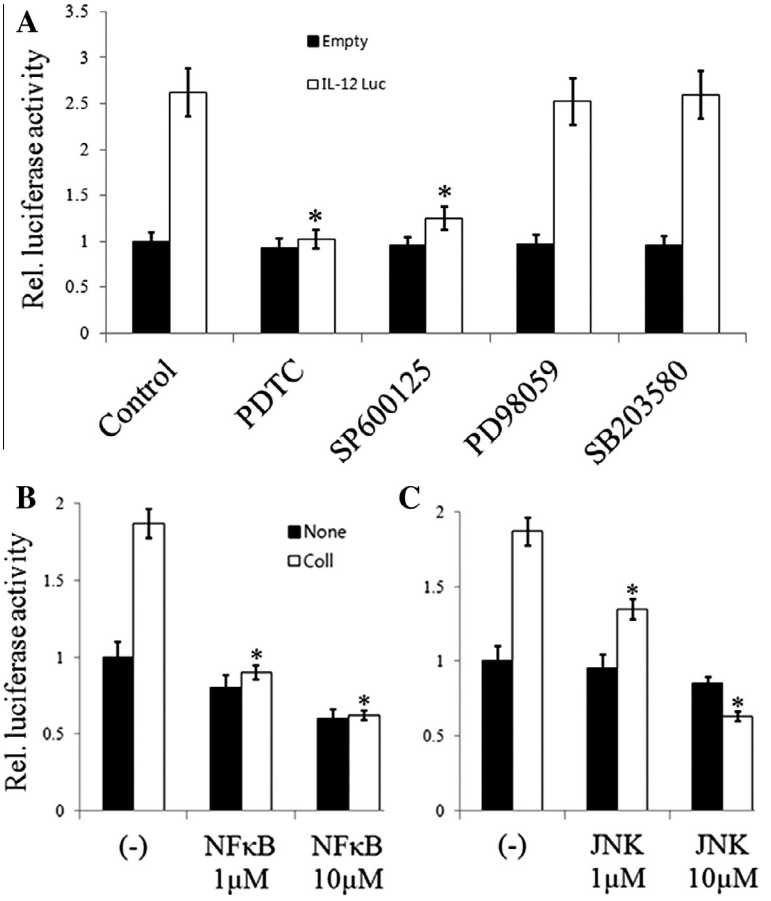

Fig. 4. Collagen I-induced IL-12 promoter activity is inhibited by the biochemical inhibitors of NF-κB and JNK. (A) Transient transfection of the HEK293 cells with IL-12 promoter or empty vectors followed by treatment with indicated biochemical inhibitors in the presence of collagen I. The results are expressed as induction over the values obtained with the untreated HEK293 cells transfected with the empty vector, which was assigned an arbitrary unit 1. (B) Murine dendritic cells were transfected with IL-12 luciferase promoter, followed by incubation on collagen coated or none coated plates in the presence or absence of NF-κB or (C) JNK inhibitors at indicated dosage and relative luciferase activity was measured. The data are representative of three similar experiments ($^*p < 0.05$).

JNK inhibition based therapies could be useful in certain autoimmune and/or inflammatory diseases.

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