Induction of pro-inflammatory cytokines by 29-kDa FN-f via cGAS/STING pathway

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Short title: Activation of cGAS/STING by 29-kDa FN-f

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Materials

Etoposide, 29-kDa FN-f from human plasma and an antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against γH2AX, cGAS, IKK, TBK1/phospho-TBK1, IRF3/phospho-IRF3, and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα)/phospho-IκBα (Ser32) were obtained from Cell Signaling Technology (Danvers, MA, USA). An antibody against STING was purchased from LifeSpan BioScience (Seattle, WA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). Small interfering RNAs (siRNAs) against cGAS, STING, TLR-2, and NOD2 were purchased from Bioneer (Daejeon, South Korea). Primers for cytokines were obtained from Cosmo Genetech Co. (Seoul, South Korea).

Western blot analysis

Cells were harvested and were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer. After determination of protein concentration, equal amount of proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) nonfat milk in TBST (Tris buffered saline and 0.1% Tween 20), sequentially incubated with primary (1: 1000 dilution) and secondary antibodies (1: 5000 dilution), and then developed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology).

Transfection with siRNA and overexpression vectors

Human chondrocytes were transfected with siRNA at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s

instructions. The siRNA sequences are shown as follows: control (sense, 5′-CCU-ACG-CCA-CCA-AUU-UCG-U-3′; antisense, 5′-ACG-AAA-UUG-GUG-GCG-UAG-G-3′), NOD2 (sense, 5′-UAU-UGU-UAU-CGC-GCA-AAU-ACA-GAG-C-3′; antisense, 5′-GCU-CUG-UAU-UUG-CGC-GAU-AAC-AAU-A-3′), TLR-2 (sense, 5′-GGC-UUC-UCU-GUC-UUG-UGA-C-3′; antisense, 5′-GUC-ACA-AGA-CAG-AGA-AGC-C-3′), cGAS (sense, 5′- CCU-UGU-ACC-CAA-GCA-UGC-A-3′; antisense, 5′-UGC-AUG-CUU-GGG-UAC-AAG-G-3′), and STING (sense, 5′-UCU-UGC-GUA-AUC-AUG-ACU-A-3′; antisense, 5′- UAG-UCA-UGA-UUA-CGC-AAG-A -3′).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Normal cartilage from the femoral heads of patients (n=6, 72 ± 22.1) with femoral neck fractures and OA cartilage from the knee joints of OA patients (n=8, 74.6 ± 5.2) were frozen at -80°C and were grinded to a fine powder in liquid nitrogen. Total RNA was isolated from cultured chondrocytes or from powdered cartilage tissue using TRIzol reagent and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR were performed in master mix containing SYBR Green PCR master mix, forward and reverse primer, and an equal amount of cDNA. Ct values were normalized to GAPDH and expressed as fold change to the untreated control. The primer sequences are shown as follows; GM-CSF forward, 5’-GGG-CAG-CCT-CAC-CAA-GCT-3’; GM-CSG reverse, 5’-CTT-GTA-GTG-GCT-GGC-CAT-CA-3’; G-CSF forward, 5’-AGA-GCC-CCA-TGA-AGC-TGA-TG-3’; G-CSF reverse, 5’-GCT-TCC-TGC-ACT-GTC-CAG-AGT-3’; IFN-α forward, 5’- AGG-CTG-TGG-GTT-TGA-GGC-AGA-TCA-3’; IFN-α reverse, 5’-TGT-GGG-TTT-GAG-GCA-GAT-CA-3’; c-GAS forward, 5’-CGG-GCG-GTT-TTG-GAG-AA-3’; c-GAS reverse, 5’-GCC-GCC-GTG-GAG-ATA-TCA-3’; STING forward, 5’-GCT-TTA-GCC-GGG-AGG-ATA-GG-3’; STING reverse, 5’-CCT-CAA-GTG-TCC-GGC-AGA-A -3’.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Mann–Whitney U test or two-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.