Online Support Materials

Materials and Methods

Cell culture and adenovirus transduction: The murine monocyte/macrophage cell line RAW264.7 was purchased from American Type Culture Collection (ATCC, Virginia). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% FBS, 100 IU/ml penicillin in an incubator with 5% CO_2 and 95% air at 37°C. RAW264.7 cells were transduced with adenoviral vectors at a defined multiplicity of infection (MOI). The adenoviral vectors used were as follows; Adv-CMV-FoxO1 expressing wild type FoxO1 ($1.0x10^{11}$ pfu/ml), the null adenovirus Adv-null ($1.25x10^{11}$ pfu/ml), Adv-FoxO1-RNAi vector encoding a 19-bp DNA (5'-CGCCCCAGGTGGTGGAGAC-3') that is complementary to FoxO1 mRNA sequence (10-29 nt) under the control of mouse U6 promoter, and a control adenovirus containing the scrambled RNAi (5'-GGACTCGGGCCACCGGGTA-3') under the control of mouse U6 promoter, as described (1). All adenoviral vectors were produced in HEK293 cells and purified as described (2). For LPS treatment, cells were cultured in DMEM in the presence of 100 ng/ml LPS (LPS from Escherichia coli 0127:B8, Sigma-Aldrich, St Louis, MO). Likewise, cells were treated with 200-nM bovine serum albumin (BSA)-bound palmitate, as described (3).

BSA-bound palmitate solution was prepared as described (3). Palmitate (20 mg) was dissolved in pre-boiled 0.1 N NaOH, followed by 10-fold dilution in DMEM containing 12% w/v BSA (A6003, Sigma-Aldrich, St. Louis, MO). This yields a molar ratio of 1.5:1.0 between palmitate and BSA, which is equivalent to the corresponding ratio in human serum (3; 4). The solution was adjusted to pH 7.4 and used in culture medium. Palmitate-free BSA (12% w/v) solution was used as control.

Cytokine quantification: Medium concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, GM-CSF and IFN-γ were determined using the Beadlyte Mouse Multi-Cytokine Detection System 2 (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's instructions. Plasma IL-1β levels were determined using the mouse-specific IL-1β ELISA kit (R&D, Minneapolis, MN).

Transfection: RAW264.7 cells were grown to 80% confluence in DMEM medium supplemented with 10% FBS. About 5 x 10^5 cells were collected and washed once with DMEM medium, and resuspended with 100-µl electroporation buffer (Amaxa, Gaithersburg, MD) containing 2-µg plasmid DNA. After electroporation, cells were plated in 6-well dishes and cultured in DMEM medium. For transfection of cells with siRNA, 1.2-µg of siRNA against NFκB P50 (sc-29408, Santa Cruz Biotechnology) or control siRNA (sc-36869, Santa Cruz Biotechnology) were included in the electroporation.

RNA isolation and real time RT-PCR: RNA isolation from RAW264.7 cells was performed using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Real-time quantitative RT-PCR was used for quantifying mRNA concentrations using the Roche LightCycler-RNA amplification kit (Roche Diagnostics, Indianapolis, IN), as described (5). The primers used are IL-1β forward 5'-GAAGATGGAAAAACGGTTTG-3' (corresponding to the mouse *Il1b* cDNA nucleotide 630-649) and IL-1β reverse 5'-GGAAGACACGGATTCCATGG-3' (mouse *Il1b* cDNA nucleotide 788-807). Primers for FoxO1 mRNA and 18S rRNA have been described (5; 6). All primers were obtained commercially from Integrated DNA Technologies (Coralville, IA).

Western blot analysis: RAW 264.7 cells (~1x10^6 cells) were lysed in 200-μl M-PER (Pierce, Rockford, IL, USA) containing 2-μl Halt Protease Inhibitor Cocktail (Pierce). Protein extracts were obtained by centrifugation at 15,000 g for 10 min. The proteins were resolved on 4–20% SDS-polyacrylamide gels and subjected to immunoblot analysis. Proteins were blotted onto Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA), and probed with mouse anti-NFκB P50 antibody (1:200 dilution, Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-phosph FoxO1 (Ser256, 1:1000 dilution, Cell signaling Technology, Danvers, MA), or anti-FoxO1 antibody as described (5). Monoclonal anti-actin antibody (1:1000 dilution, Sigma-Aldrich, Saint Louis, MO) was used as loading controls. Proteins bands were detected by autoradiography and their relative intensities were quantified by densitometry using NIH (National Institutes of Health, Bethesda, MD) image software as described (5).

Immunoprecipitation: Co-immunoprecipitation was used for assaying potential interactions between FoxO1 and NFκB P50 subunit. Raw264.7 cells (1.5 x 10^6 per 60-mm dish) were plated in dishes. After 24-h incubation, cells were treated with LPS (100 ng/ml) or mock-treated with PBS, and alternatively with palmitate (0.2 μM) or BSA as control for 24 h. Cells were harvested and lysed in 200-μl M-PER lysis buffer containing 4-μl Halt Protease Inhibitor Cocktail (Pierce). Aliquots of lysates (100-μg protein) were incubated with 10-μl anti-FoxO1 (5), anti-P50 antibody (Santa Cruz Biotech, Santa Cruz, CA) or anti-IRS2 (Upstatet Biotechnology, Lake Pacid, NY) for 16 h at 4°C. The reaction mixtures were incubated with 50-μl protein-A Sepharose for 1 h at 4°C. After washing the Sepharose beads three times with ice-cold M-PER lysis buffer, proteins were

extracted using Laemmli buffer and subjected to immunoblot analysis using anti-FoxO1, anti-P50 or anti-phosphotyrosine (Clone 4G10, Upstate Biotechnology) antibodies, respectively.

IL-1β promoter-directed luciferase reporter system and luciferase assay: A 2-kb DNA fragment containing the mouse IL-1β promoter was amplified from mouse genomic DNA (BioChain Institute Inc, Hayward, CA) by PCR using primers for forward (5'-CATCTCTTACACACGATTAAATG-3') and reverse reactions (5'-CTCGAACCACTGCAGGGTTTG-3'). After verifying its nucleotide sequence by DNA sequencing, the IL-1β promoter was cloned into the luciferase reporter pGL3-Basic vector (Promega, Madison, WI), resulting in pHD386. For luciferase assay, RAW264.7 cells in 6-well microplates were transfected with 1-μg pHD386 DNA by electroporation. In each transfection, 1-μg plasmid pCMV-LacZ DNA was included and the amount of □-gal activity was used as an internal control for normalizing transfection efficiency. After 24-h incubation, cells were collected and resuspended in 400-μl lysis buffer (Promega) for preparation of cell lysates. After centrifugation at 15,000 g for 3 minutes, aliquots (20 μl) of the supernatant were used to determine luciferase and β-gal activities, as described (2). When NFκB P50 or P65 was co-expressed with FoxO1 in RAW264.7 cells, plasmids encoding the human P50 or P65 subunit of NFκB under the control of CMV promoter were included in electroporation. In cases where a dual luciferase reporter assay system was employed, the plasmid pGL4.75 encoding Renilla luciferase was used as internal control. The relative promoter activity was defined as the ratio of Firefly and Renilla luciferase activities according to the manufacturer's instructions (Promega).

Chromatin immunoprecipitation (ChIP) assay: Chromatin immunoprecipitation (ChIP) was used to study the interaction between FoxO1 protein and the *Il1b* promoter DNA. RAW264.7 cells ($2x10^5$ cells) were transfected with pHD386 in the presence of FoxO1 vector at an MOI of 200 pfu/cell in triplicate. Plasmid pHD386 was derived from pGL3-basic vector, in which the luciferase gene was under the control of a 2-kb mouse *Il1b* promoter (-2,063/+4 nt). After 24-h incubation, cells were cross-linked with 1% formaldehyde, followed by sonication in a Microson 100 Watt Ultrasonicator (Structure Probe, West Chester, PA) at 30% of maximum power for 10 consecutives cycles of 10-second pulses. After centrifugation at 15,000 g for 10 min, the supernatant was subjected to control IgG or anti-FoxO1 immunoprecipitation using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) as described (1). The immunoprecipitates were analyzed by PCR assay to detect co-immunoprecipitated DNA using the *Il1b* promoter-specific primers (forward 5′-TCTATTTCCCTTCAGTGCTG-3′, and reverse 5′-TTCATGAGCACAGTCCATCT-3′) that flank the consensus FoxO1 binding site (-1,359/-903 nt) in the mouse IL-1β promoter.

Site-directed mutagenesis: Site-directed mutagenesis was used to alter the insulin response element (IRE) in the IL-1β promoter in pHD386, using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two primers used in the reaction were 5′-CTTATAGAAACAAGAATTTTCCgAgACAATTTTTTAG-3′ (with mutant bases in lower cases) and 5′-GGAAAATTCTTGTTTCTATAAGATCTGGGGA-3′. After confirmation by DNA sequencing, the mutant IL-1β promoter containing an altered IRE was subcloned into the luciferase reporter plasmid pGL3-basic. The resulting plasmid pHD386Mut was used for promoter activity assay.

***Statistical analysis*:** Data were presented as mean ± SEM. Statistical analysis was performed using two-tailed unpaired Student's *t*-test or by analysis of variance (ANOVA) using StatView software (Abacus Concepts). Pair-wise comparisons were performed to study the significance between different conditions. *P*-values <0.05 were considered statistically significant.

References:

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Supplementary Table 1. Effect of FoxO1 on cytokine production profiles in unstimulated RAW264.7 cells.

	PBS	Control	FoxO1	*P* value
IL-1β (pg/ml)	94±21	84±27	70±12	NS
IL-2 (pg/ml)	77±8.6	75±5.9	71±13	NS
IL-4 (pg/ml)	78±5.8	70±8.7	74±9.4	NS
IL-5 (pg/ml)	69±7.5	59±5.8	55±3.1	NS
IL-6 (pg/ml)	90±9	117±30	139±11	NS
IL-10 (pg/ml)	122±8.7	114±26	128±23	NS
IL-12 (pg/ml)	113±7.1	107±6.2	106±8.2	NS
IFN-γ (pg/ml)	79±18	83±11	73±12	NS
TNF-α (pg/ml)	15603±376	14301±899	21459±1139*	$P<0.05$
GM-CSF (pg/ml)	61±2.4	66±8.7	53±5.1	NS

RAW264.7 cells were mock-treated with PBS, or transduced with Adv-FoxO1 or control Adv-null vector at a fixed dose (MOI, 200 pfu/cell). After 24-h incubation in the absence of LPS, conditioned medium was harvested for profiling the production of cytokine IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, INF-γ, TNF-α, GM-CSF. Data were obtained from three independent experiments. NS, not significant. *$P<0.05$ vs. control by ANOVA.