Mechanisms of Signal Transduction:

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Interleukin-18 Suppresses Adiponectin Expression in 3T3-L1 Adipocytes via a Novel Signal Transduction Pathway Involving ERK1/2-dependent NFATc4 Phosphorylation*

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An inverse correlation between the pro-inflammatory cytokine interleukin-18 and the anti-atherogenic adipokine adiponectin has been reported in the chronic pathological conditions obesity, insulin resistance, coronary artery disease, and metabolic syndrome. We investigated whether this relationship is coincidental or has a causal basis. Here we show that interleukin-18 (IL-18) suppresses adiponectin transcription, mRNA expression, and secretion by 3T3-L1 adipocytes. IL-18 suppresses adiponectin promoter-reporter activity, an effect reversed by deletion or mutation of the NFATc4 core DNA-binding site. IL-18 induces NFATc4 phosphorylation (Ser^{676}), nuclear translocation, and *in vivo* DNA binding. IL-18 induces ERK1/2 phosphorylation and enzyme activity, and pretreatment with the MEK inhibitor U0126, ERK1/2 inhibitor PD98059, or small interference RNA targeted to ERK1/2 attenuates ERK1/2 activation and NFATc4 phosphorylation. Finally, inhibition of ERK1/2 or NFATc4 knockdown reverses IL-18-mediated adiponectin suppression. In contrast to its inhibitory effects on adiponectin expression, IL-18 potently stimulates PAI-1 secretion. These data demonstrate for the first time that IL-18 selectively suppresses adiponectin expression via ERK1/2-dependent NFATc4 activation and suggest that the inverse relationship observed between IL-18 and adiponectin in various chronic pathological conditions is causally related. Thus, targeting IL-18 expression may enhance adiponectin expression and mitigate disease progression.

Adiponectin is an anti-inflammatory cytokine secreted primarily by adipocytes (1, 2). Paradoxically, the increased body fat in obesity is associated with decreased circulating adiponectin levels (3, 4). Secretion of the adipokines leptin and nerve growth factor, on the other hand, are not affected, and in fact, their systemic levels are increased by several folds by obesity (5, 6), suggesting that obesity is specifically associated with reduced adiponectin levels.

Adiponectin signals mainly via adiponectin receptors 1 and 2 (7). A wide variety of cells express adiponectin receptors, including vascular endothelial and smooth muscle cells, and tissue macrophages. Thus, the cells that are known to play key roles in the pathobiology of vascular disease are all targets of adiponectin. Adiponectin inhibits endothelial cell activation by suppressing cytokine, chemokine, and adhesion molecule expression, and can inhibit smooth muscle cell proliferation (8, 9). Adiponectin inhibits foam cell formation by suppressing macrophage uptake of low density lipoprotein (10). Together, these findings suggest that adiponectin acts as an anti-inflammatory and anti-atherogenic cytokine.

In contrast to adiponectin, plasma levels of the pro-inflammatory cytokine interleukin (IL)[2]-18 are increased during obesity (10–12). Plasma IL-18 levels are also increased in other chronic inflammatory conditions such as type II diabetes, coronary artery disease, heart failure, and metabolic syndrome (10–15). Importantly, plasma levels of IL-18 show a positive correlation with intimal-medial thickening and predict future cardiovascular events (16, 17). Thus, plasma IL-18 levels can serve as a marker of chronic inflammation. Expression of IL-18 and its receptor has been demonstrated in human atherosclerotic lesions localized predominantly to smooth muscle cells (18). However, IL-18 affects all three major cell types involved in the development and progression of atherosclerotic vascular disease. IL-18 stimulates smooth muscle cell migration and proliferation (19) and stimulates cytokine, chemokine, and adhesion molecule expression in endothelial cells (20). IL-18 also modulates foam cell formation by stimulating scavenger receptor and chemokine expression (21). In an animal model of atherosclerosis, the IL-$18^{-/-}$ apo$E^{-/-}$ double-knockout mice show fewer lesions and delayed lesion progression compared

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[2] The abbreviations used are: IL, interleukin; ChIP, chromatin immunoprecipitation; IBMX, 3-isobutyl-l-methylxanthine; IL-18R, interleukin-18 receptor; NFAT, nuclear factor of activated T cells; PAI-1, type-1 plasminogen-activator inhibitor; siRNA, small interfering RNA; nt, nucleotide(s); ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; qPCR, quantitative PCR; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; MEK, MAPK/ERK kinase; C/EBP, CAAT/enhancer-binding protein; ANOVA, analysis of variance; GKLF, gut-enriched Kruppel-like factor; JNK, c-Jun N-terminal kinase.

with the apoE$^{-/-}$ single knockout mice (22). Together, these reports indicate that IL-18 exerts both pro-inflammatory and pro-atherogenic effects.

Because circulating levels of adiponectin show an inverse relationship with IL-18 in several pathological conditions (11–15, 23, 24), we investigated whether this relationship is coincidental or has a causal basis. Here we demonstrate for the first time that IL-18 suppresses adiponectin expression by adipocytes via a pathway featuring ERK1/2-dependent NFATc4 phosphorylation and activation. These data suggest that the relationship between IL-18 and adiponectin plasma levels has a direct causal component, and that targeting IL-18 is a potentially effective strategy for enhancing adiponectin expression and attenuating disease progression.

EXPERIMENTAL PROCEDURES

Materials—Recombinant mouse IL-18 (B002–5), IL-18-neutralizing antibodies (αIL-18Ab; D048–3), anti-mouse IL-18Rα antibody (AF856), anti-mouse IL-18Rβ antibody (AF199), normal goat IgG1 (AB-108-C), recombinant mouse IL-18BPd/Fc chimera (#122-BP), and Fc were purchased from R&D Systems (Minneapolis, MN). Donkey anti-goat fluorescein isothiocyanate-conjugated antibodies (Cat. no. 705-095-147) were from Jackson ImmunoResearch Laboratories (West Grove, PA). The NFAT luciferase reporter plasmid (pNFAT-Luc) was obtained from Panomics (Freemont, CA). Antibodies to NFATc2 (sc-7296) and NFATc4 (sc-13036) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to β-actin (A300–491A) and nucleolin (A300–711A) were purchased from Bethyl Laboratories, Inc. (Montgomery, TX). Antibodies to phospho-Ser^{676} NFATc4, generated using synthetic phospho-peptide (Lys-Arg-Ser(P)-Pro-Thr-Gln-Ser-Phe-Arg-Phe-Leu-Pro-Val-Ile-Cys) encoding human NFATc4 and conjugated to ovalbumin have been described previously (generously provided by Chi-Wing Chow, Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY) (25). Antibodies to total (#9102) and phospho ERK1/2 (#9101S) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). MEK Inhibitor U0126 (Promega), ERK1/2 inhibitor (PD98059; 10 μM in Me_2SO for 1 h) and Me_2SO were from EMD Biosciences (San Diego, CA). Insulin, IBMX, dexamethasone, and all other chemicals were purchased from Sigma-Aldrich.

Cell Culture—3T3-L1 preadipocytes (#CL-173) were purchased from ATCC (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium containing high glucose and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 100 mM minimal essential medium sodium pyruvate, and 10% fetal calf serum. All tissue culture reagents were purchased from Invitrogen. When confluent (day 0), cells were differentiated into adipocytes by supplementing the medium with 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). After 48 h, the medium was replaced with Dulbecco's modified Eagle's medium plus 10% fetal calf serum supplemented with 5 μg/ml insulin. Forty-eight hours later, medium was replaced with fresh Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Five days later, the fully differentiated adipocytes were used in the experiments. In addition to pharmacological inhibitors, ERK1/2 expression was targeted by ERK1 and ERK2 siRNA duplexes. Treatment with pharmacological inhibitors, transfections, knockdown, or adenoviral infection did not affect cell viability (trypan blue dye exclusion).

IL-18 Receptor Expression-RT-PCR and Flow Cytometry—IL-18 receptor expression was analyzed by RT-PCR using DNA-free total RNA isolated from adipocytes and receptor-specific primers: IL-18Rα (GenBank^{TM} accession NM_008365, 195 nt): sense, 5′-GTG CAC AGG AAT GAA ACA GC-3′ (bases 519–537) and antisense, 5′-ATT TAA GGT CCA ATT GCG ACG A-3′ (bases 692–713); IL-18Rβ (GenBank^{TM} NM_010553, 241 nt): sense, 5′-GGA GTG GGA AAT GTC AGT AT-3′ (bases 1321–1340), and antisense, 5′-CCG TGC CGA GAA GGA TGT AT-3′ (bases 1561–1535). Surface expression of IL-18Rα and -β were measured by flow cytometry. In brief, fully differentiated 3T3-L1 cells were gently trypsinized, washed twice in phosphate-buffered saline, fixed in 2% paraformaldehyde for 10 min, and resuspended in phosphate-buffered saline containing antibodies to IL-18Rα, IL-18Rβ, or normal goat IgG at 1:100 dilution for 1 h at 37 °C. Cells were washed in phosphate-buffered saline, and incubated for an additional 1 h at 37 °C with donkey anti-goat IgG conjugated to fluorescein isothiocyanate (1:100). The cell suspension was then washed twice in phosphate-buffered saline and analyzed by fluorescence-activated cell sorting.

Plasminogen Activator Inhibitor-1 Production—Adipocytes were treated with IL-18 (10 ng/ml) for 48 h. PAI-1 levels in culture supernatants were quantified by ELISA (Mouse PAI-1 ELISA kit, Molecular Innovations, Inc., Southfield, MI). Specificity of IL-18 was verified by treating cells with neutralizing IL-18 antibodies or IL-18BP/Fc chimera (10 μg/ml) for 1 h prior to IL-18 treatment. Normal rat IgG_1 and Fc served as controls.

Adiponectin Expression—Adiponectin mRNA expression was quantified by real-time PCR. DNA-free total RNA was prepared using the RNAqueous®-4PCR kit (Ambion). RNA quality was assessed by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples used for quantitative PCR had RNA integrity numbers of >9.1 (scale = 1–10), as assigned by default parameters of the Expert 2100 Bioanalyzer software package (v2.02). A total of 2 μl of cDNA was used, and amplification was performed in duplicate in an ABI PRISM 7900 Sequence Detector (PE Applied Biosystems, Foster City, CA) using the following primer sets for adiponectin: sense primer, 5′-AAG GAC AAG GCC GTT CTC T-3′; antisense primer, 5′-CGC ACG ATT TCC CTC TCA GCT G-3′. The results were quantified by the ΔΔCt method using β-actin (sense, 5′-CA GGG TGT GAG TTG GGG AAT G-3′; antisense, 5′-CGC ACG ATT TCC CTC TCA GCT G-3′) as internal standard. Effects of IL-18 on adiponectin gene transcription were determined using nuclear run-on assays (26), and on adiponectin mRNA stability by actinomycin D pulse (26), followed by RT-qPCR.

Adiponectin secretion was quantified by an ELISA (Mouse adiponectin ELISA kit, Cat. no. EZMADP-60K, Millipore, Billerica, MA) that detects all forms of adiponectin except the globular form (Technical data sheet, Millipore).

Adiponectin Promoter-Reporter Assays—The region of the mouse adiponectin gene promoter spanning −1138 to +30 bp

in the pGL3-Basic vector (p1138-Luc) has been described previously (27). The deletion construct p312-Luc was generated using the following primers: sense, 5′-*ggtacc*GGTACCGAGGATAATTTTCATTGCAC-3′; antisense, 5′-*ctcgag*TCGTCAGATCCACTGACAATCGT-3′. Mutation of the NFAT binding site (from AAACTTATGGGAAAGGGAG to AAACTTATCTTAAAGGGAG; sense, 5′-AGG ACA AAC TTA TCT TAA AGG GAG GTC TCC TGA C-3′) in p1138-Luc construct was generated by site-directed mutagenesis (28). Adipocytes were transfected with 3 μg of the promoter-reporter vectors and 100 ng of the control *Renilla* luciferase vector pRL-TK (Promega) using Lipofectamine® (19, 26). After incubation for the indicated time periods, the cells were harvested for the dual-luciferase assay. Data were normalized by dividing firefly luciferase activity with that of the corresponding *Renilla* luciferase. All plasmids were purified using EndoFree Plasmid Maxi kit (Qiagen), and transfections did not result in cell death.

Western Blotting, Enzyme Activity, and Immunocomplex Kinase Assays—NFAT levels were determined in whole cell homogenates by immunoblotting using isotype-specific antibodies (19, 26). Similarly, phospho-NFATc4 levels in cytoplasmic and nuclear extracts were determined using phospho-specific (Ser^{676}) antibodies. β-Actin and nucleolin served as markers of purity of cytoplasmic and nuclear protein extracts, respectively, and to verify equal loading of proteins. ERK1/2 activation was determined by immunoblotting using activation-specific antibodies and immune-complex kinase assays (ERK1/2, p44/42 MAPK Assay Kit, Cell Signaling Technology, Inc.) (29).

Reporter and Chromatin Immunoprecipitation Assays—NFAT activation was analyzed by reporter assays using adenoviral transduction of NFAT reporter vector (Ad-NFAT-Luc, 50 multiplicity of infection, catalog no. 1665, Vector Biolabs, Philadelphia, PA). Ad-MCS-Luc (50 multiplicity of infection; catalog no. 1680, Vector Biolabs) served as a control. Ad-β-gal (50 multiplicity of infection; catalog no. 1080, Vector Biolabs) served as an internal control. Twenty-four hours after infection, cells were treated with IL-18 (10 ng/ml) for an additional 12 h. Firefly luciferase activity in cell extracts was determined as previously described (19, 26, 29). β-Galactosidase activity in cell extracts was determined using a Luminescence β-Galactosidase Detection Kit II (BD Biosciences), and the results are expressed in relative light units as a ratio of firefly luciferase to β-galactosidase activity.

In vivo DNA binding of NFATc4 to the adiponectin promoter was confirmed by ChIP assays (29). Adipocytes were treated with IL-18 (10 ng/ml) for 1 h. The ChIP assay was carried out as previously described (29) using the Chromatin Immunoprecipitation Assay kit (catalog no. 17-295, Upstate Biotechnology Inc., Lake Placid, NY). Immunocomplexes were prepared using anti-p-NFATc4(Ser^{676}) antibody. The supernatant of an immunoprecipitation reaction carried out in the absence of antibody was used as the total input DNA control. PCR was carried out on 1 μl of a 1:100 dilution of each sample using adiponectin promoter-specific primers: sense (−432 to −413), 5′-GCT TCA CAT TTA GTT ACA AA-3′ and antisense (−292 to −273), 5′-ATA ATT CAG CAT GTT TCT GA-3′, that would amplify a 160-bp fragment, followed by analysis on 2% agarose gels. Primers predicted to amplify a 200-bp fragment of exon 3 of the adiponectin gene (sense, 5′-GTC TCC ACG ACT CTT ACA TG-3′ and antisense, 5′-ACA TTC ATA CAC TCA GCC TG-3′) were used as the PCR control.

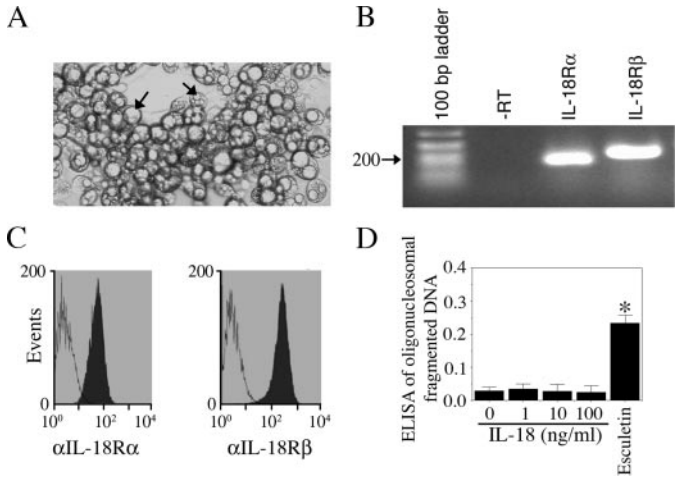

FIGURE 1. **IL-18 does not affect adipocyte viability.** *A*, fully differentiated 3T3-L1 adipocytes. 3T3-L1 preadipocytes were treated with a combination of insulin, dexamethasone, and IBMX as described under "Experimental Procedures." *Arrows* indicate lipid droplets in differentiated adipocytes. *B*, RT-PCR showing the expression of the ligand-binding α and the signal-transducing β subunits of the IL-18 receptor by adipocytes under basal condition. *C*, flow cytometry showing the membrane expression of α and β subunits of the IL-18 receptor by adipocytes under basal conditions. *D*, IL-18 does not induce adipocyte death. Adipocytes were treated with IL-18 for 72 h. Esculetin (400 μM for 48 h) served as a positive control. *, $p < 0.001$ *versus* untreated by ANOVA.

siRNA—GKLF (sc-35479), C/EBPβ (sc-29862), NFATc2 (sc-36056), NFATc4 (sc-38116), ERK1 (sc-29308), and ERK2 (sc-35336) siRNA were purchased from Santa Cruz Biotechnology, Inc. Non-targeting siRNA (5′-UUC UCC GAA CGU GUC ACG Utt-3′; no. 1022076, Qiagen, Valencia, CA) served as a negative control. Cells were transfected with 100 nM (GKLF, C/EBPβ, NFATc2, or NFATc4) or 25 nM (ERK1 and ERK2) siRNA using Oligofectamine^{TM} reagent (Invitrogen). 48 h later, knockdown of corresponding proteins was confirmed by Western blotting using whole cell homogenates.

Cell Death Detection ELISA—Adipocytes were treated with IL-18 at the indicated concentration for up to 3 days. Cells were harvested and analyzed for mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by ELISA (Cell Death Detection ELISA^{PLUS} kit, Roche Applied Science, Indianapolis, IN) (30). Esculetin (6,7-dihydroxy-2*H*-1-benzopyran-2-one, 400 μM for 48 h, Sigma-Aldrich), a coumarin derivative and a known inducer of adipocyte death (31), was used as a positive control.

Statistical Analysis—Results are expressed as means ± S.E. For statistical analysis we used ANOVA followed by an appropriate *post hoc* multiple comparison test (Tukey method). Each experiment was performed at least three times, and data were considered statistically significant at $p < 0.05$.

RESULTS

IL-18 Does Not Induce Cell Death in Adipocytes—Treatment with insulin, IBMX, and dexamethasone induces the differentiation of 3T3-L1 preadipocytes into fully differentiated adipocytes as characterized by the intracellular accumulation of lipid droplets (Fig. 1*A*, *arrows*). In all subsequent experiments, fully

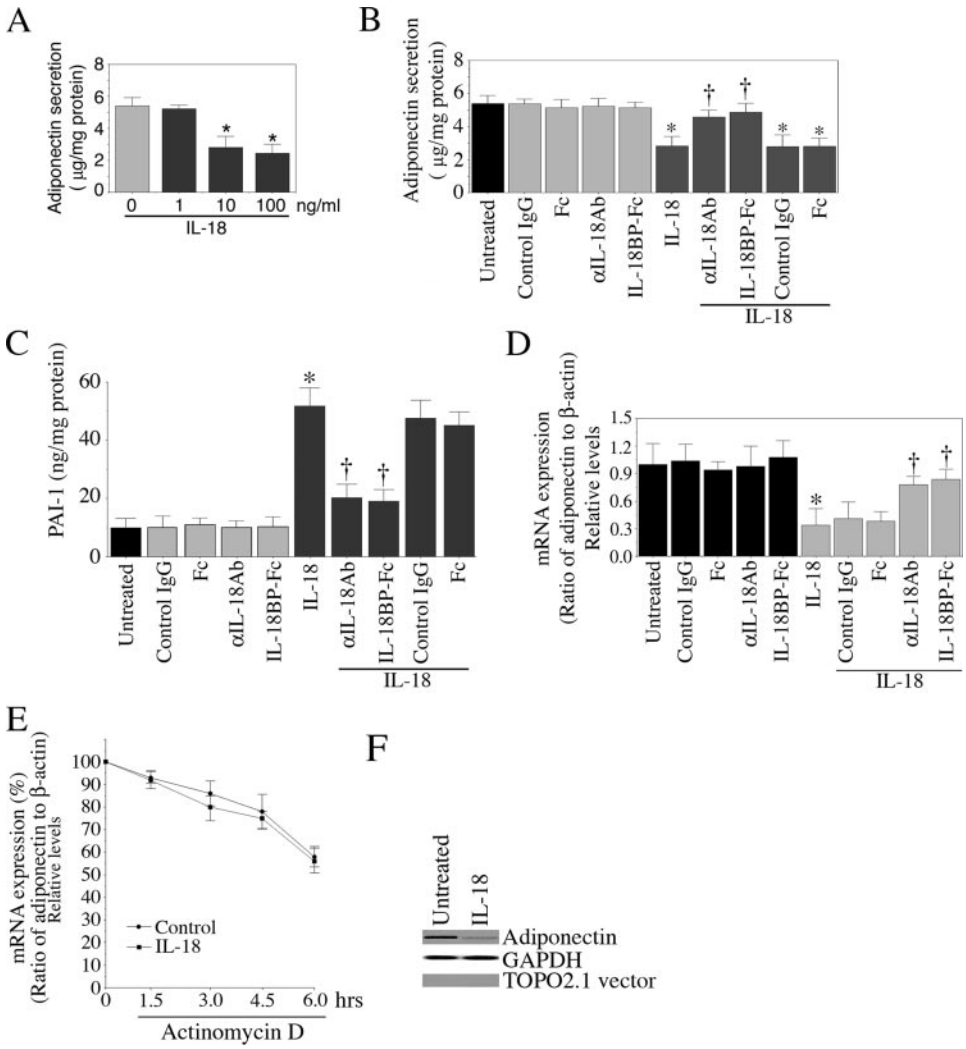

FIGURE 2. **IL-18 suppresses adiponectin expression.** *A*, IL-18 suppresses adiponectin secretion. Adipocytes were treated with IL-18 for 24 h. Adiponectin levels in culture supernatants were quantified by ELISA. *, $p < 0.01$ *versus* untreated. *B*, IL-18 neutralizing antibodies and IL-18BP:Fc inhibit IL-18-mediated suppression in adiponectin secretion. Adipocytes were treated with IL-18 antibodies or IL-18BP:Fc (10 μg/ml for 1 h) prior to IL-18 addition (10 ng/ml for 24 h). Adiponectin levels were quantified as in *A*. *, $p < 0.01$ *versus* untreated, †, $p < 0.05$ *versus* IL-18. *C*, IL-18 stimulates PAI-1 secretion. Culture supernatants of adipocytes treated as in *B* were analyzed for PAI-1 levels by ELISA. $p < 0.001$ *versus* untreated, †, $p < 0.01$ *versus* IL-18. *D*, IL-18 suppresses adiponectin mRNA expression. Adipocytes were treated with IL-18 neutralizing antibodies or IL-18BP:Fc prior to IL-18 (10 ng/ml for 24 h). Adiponectin mRNA expression was quantified by RT-qPCR. *, $p < 0.01$ *versus* untreated, †, $p < 0.05$ *versus* IL-18 by ANOVA. *E*, IL-18 does not alter adiponectin mRNA half-life. Adiponectin mRNA half-life in IL-18-treated and control adipocytes was evaluated by actinomycin D pulse followed by RT-qPCR at the time points indicated ($n = 6$). *F*, IL-18 suppresses adiponectin gene transcription. Adiponectin gene transcription following IL-18 exposure was evaluated by nuclear run-on assays ($n = 3$). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control and TOPO 2.1 served as a vector control.

differentiated 3T3-L1 preadipocytes will be termed adipocytes. We investigated whether adipocytes express subunits α and β of the IL-18 receptor. RT-PCR of DNA-free total RNA and receptor-specific primers show that, under basal conditions, adipocytes express both the ligand-binding α and the signal-transducing β subunits of IL-18 receptor (Fig. 1*B*). Flow cytometry confirmed membrane expression of IL-18 receptors α and β (Fig. 1*C*). Because IL-18 can exert pro-apoptotic effects in some cell types, we next investigated whether IL-18 affects adipocyte survival. Adipocytes were treated with IL-18 at the indicated concentrations for 72 h. Cell death was analyzed by quantifying mono- and oligonucleosomal fragmented DNA in the cytoplasmic extracts. Even at the maximum concentration of 100 ng/ml, IL-18 had no detectable effect on adipocyte viability (Fig. 1*D*). However, esculetin, a known inducer of adipocyte apoptotic cell death (31), induced significant cell death ($p < 0.001$). These results indicate that (i) adipocytes express the ligand binding α and the signal transducing β subunits of IL-18 receptor and (ii) IL-18 does not induce adipocyte death under the conditions used in our experiments (Fig. 1).

IL-18 Suppresses Adiponectin, but Not PAI-1, Expression in Adipocytes—Since an inverse relationship between systemic IL-18 and adiponectin levels has been reported for various chronic pathological conditions (11–15, 23, 24), we investigated whether this relationship may have a causal basis. Addition of IL-18 to the adipocytes resulted in a dose-dependent suppression of adiponectin secretion, with significant suppression detected at 10 ng/ml (Fig. 2*A*). No further suppression of adiponectin secretion was observed at IL-18 concentrations of up to100 ng/ml. The suppressive effect of IL-18 was blocked by IL-18 neutralizing antibodies and by an IL-18BP:Fc chimera (Fig. 2*B*), demonstrating specificity for IL-18. To show that the IL-18-induced suppression of adiponectin secretion was the result of a selective effect of IL-18 on the adipocytes, rather than from a global inhibition of all secretory activity, we also quantified the levels of PAI-1 in the adipocyte culture supernatants. In contrast to its suppressive effects on adiponectin secretion, IL-18 stimulated PAI-1 secretion from the adipocytes (Fig. 2*C*), indicating the suppression of adiponectin by IL-18 to be a selective effect. Because adiponectin suppresses PAI-1 expression (32), our results suggest that the effect of IL-18 on PAI-1 expression is either direct or due to reduced adiponectin expression following IL-18 treatment.

We next investigated the effects of IL-18 on adiponectin mRNA expression. IL-18 suppressed the net levels of adiponectin transcripts in the adipocytes, a response that was also blocked by the IL-18 neutralizing antibodies and IL-18BP:Fc chimera (Fig. 2*D*). To determine whether the decreased mRNA levels were due to reduced transcription or to post-transcriptional events, we studied the effects of IL-18 on adiponectin

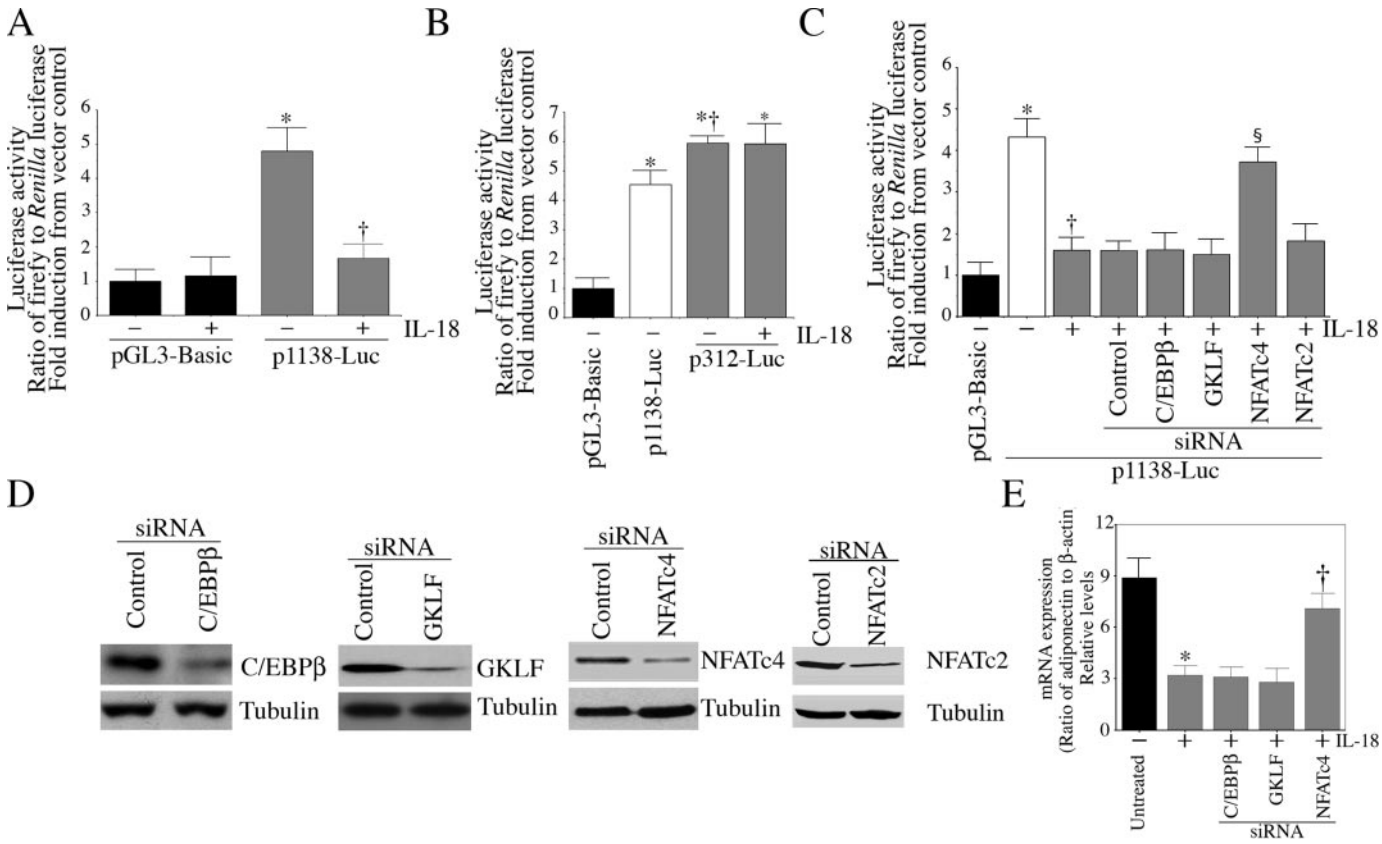

FIGURE 3. **IL-18 suppresses adiponectin transcription in NFATc4-dependent manner.** *A*, IL-18 suppresses adiponectin promoter-reporter activity. Adipocytes transiently transfected with full-length adiponectin promoter were treated with IL-18 (10 ng/ml for 12 h). Co-transfection with pRL-TK vector served as a control for transfection efficiency. *, $p < 0.001$ *versus* IL-18-treated pGL3-Basic, †, $p < 0.01$ *versus* p1138-Luc alone. *B*, deletion construct (p312-Luc) lacking C/EBP, GKLF, and NFAT binding sites failed to respond to IL-18-mediated suppression. *, $p < 0.001$ *versus* pGL3-Basic, †, $p < 0.05$ *versus* p1138/+30. *C*, knockdown of NFATc4, but not C/EBPβ, GKLF, or NFATc2, reverses IL-18-mediated suppression of adiponectin promoter-reporter activity. *, $p < 0.001$ *versus* pGL3-Basic, †, $p < 0.001$ *versus* p1138-Luc alone, §, $p < 0.01$ *versus* IL-18-treated p1138-Luc. *D*, knockdown of C/EBPβ, GKLF, NFATc2, and NFATc4 were confirmed by Western blotting. α-Tubulin served as a control. *E*, knockdown of NFATc4 reverses IL-18-mediated suppression of adiponectin mRNA expression. Adipocytes were transfected with NFATc4 siRNA and were treated with IL-18 48 h post-transfection. Adiponectin mRNA expression was analyzed by RT-qPCR. Actin served as a control. *, $p < 0.001$ *versus* untreated, †, $p < 0.01$ *versus* IL-18 by ANOVA.

transcription by nuclear run-on assay and on mRNA stability using an actinomycin D pulse. Adipocytes were treated with IL-18 for 6 h, followed by an actinomycin D pulse for up to 6 h. Adiponectin mRNA was quantified by RT-qPCR. As shown in Fig. 2*E*, the calculated half-lives of adiponectin mRNA in the untreated controls and IL-18-treated cells were essentially identical, suggestive of similar degradation rates. In contrast, nuclear run-on assays revealed suppressed adiponectin gene transcription following IL-18 treatment (Fig. 2*F*). Thus together, these results indicate that (i) IL-18 selectively inhibits adiponectin expression, (ii) reduced adiponectin expression is not due to cell death (Fig. 1*C*), and (iii) IL-18-mediated suppression of adiponectin expression is due to reduced gene transcription (Fig. 2).

IL-18 Suppresses Adiponectin Promoter-Reporter Activity via NFATc4—We next investigated the effect of IL-18 on adiponectin promoter activity. Adipocytes were transiently transfected with the full-length adiponectin promoter-reporter construct (p-1138-Luc) and treated with IL-18. IL-18 markedly attenuated adiponectin promoter activity, with a significant reduction of firefly luciferase activity ($p < 0.001$) compared with controls. Previous studies have identified a repressor region (−472 to −313) in the mouse adiponectin gene promoter that contains a number of responsive elements (28). To determine whether this region is also responsible for the suppressive effect of IL-18, we generated a deletion construct (p312-Luc), which lacks this region, and tested it in transfection assays. In the unstimulated cells, the activity of this construct was significantly higher than the larger construct containing the repressor region ($p < 0.05$ *versus* p1138-Luc) (Fig. 3*B*). Furthermore, treatment with IL-18 failed to inhibit the reporter activity of this construct, suggesting that the repressor region between −472 and −312 bp may also mediate responsiveness to IL-18. The repressor region of the mouse adiponectin promoter contains binding sites for NFAT (−363 to −344), gut-enriched Kruppel-like factor (GKLF, −409 to −395), and C/EBP (−462 to −451), among others.

To determine the relative importance of each of these transcription factors in IL-18-mediated suppression of adiponectin promoter, we targeted the expression of each of these transcription factors using siRNA. In the case of NFAT, previous studies have shown that the expression of the NFATc2 and NFATc4 isoforms is increased during the differentiation of 3T3-L1 fibroblasts into adipocytes, with higher levels of NFATc4 than NFATc2 (25). However, no change was reported in NFATc1 and NFATc3 levels during differentiation. Therefore, we tar-

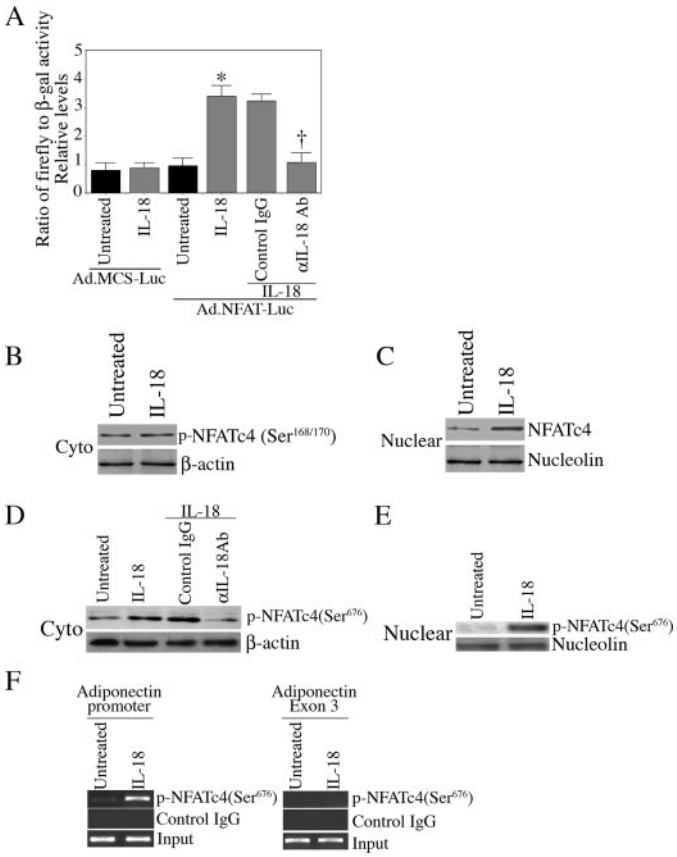

FIGURE 4. **IL-18 induces NFATc4 activation.** *A*, IL-18 induces NFAT-dependent reporter gene activity. Adipocytes were infected with adenovirus expressing NFAT reporter vector for 24 h. Cells were left untreated or treated with IL-18 neutralizing antibodies (10 μg/ml for 1 h) prior to IL-18 addition (10 ng/ml for 12 h). Ad-MCS-Luc served as a control. Ad-β-gal served as an internal control. Firefly and β-galactosidase activities in cleared cell lysates were analyzed as described under "Experimental Procedures." *, $p < 0.001$ *versus* untreated, $p < 0.05$ *versus* IL-18-treated Ad-NFAT-Luc. *B*, treatment with IL-18 increases nuclear total NFATc4 levels, but fails to alter cytoplasmic phospho-NFATc4 ($^{Ser168/170}$) levels (*C*). However, IL-18 increases cytoplasmic (*D*) and nuclear (*E*) phospho-NFATc4 (Ser^{676}) levels. β-Actin and nucleolin served as controls. These experiments were performed at least six times, and representative experiments are shown. *F*, IL-18 increases NFATc4(Ser^{676}) DNA binding to adiponectin promoter *in vivo*. Adipocytes were treated with IL-18 (10 ng/ml for 2 h), and NFATc4(Ser^{676}) binding to DNA was analyzed by ChIP assays using adiponectin gene-specific primers and phospho-NFATc4(Ser^{676}) antibodies.

geted NFATc2 and NFATc4 expression by specific siRNA. Our results demonstrate that knockdown of NFATc4, but not NFATc2, C/EBPβ, or GKLF, reversed IL-18-mediated suppression of adiponectin promoter-reporter activity (Fig. 3*C*). Knockdown of C/EBPβ, GKLF, NFATc4, and NFATc2 was confirmed by Western blotting (Fig. 3*D*). Furthermore, knockdown of NFATc4 reversed IL-18-mediated suppression of adiponectin mRNA expression (Fig. 3*E*). Together, these results indicate that IL-18 suppresses adiponectin expression via NFATc4 (Fig. 3).

IL-18 Induces NFAT Activation, Nuclear Translocation, and DNA Binding—Next we investigated whether IL-18 induces NFAT activation as assessed by the adenoviral transduction with a reporter vector containing four repeats of NFAT binding sites. Ad-MCS-Luc served as a control. IL-18 significantly stimulated NFAT-dependent reporter gene activity (Fig. 4*A*), and this was blocked by IL-18 neutralizing antibodies. We next investigated if IL-18 increases nuclear NFATc4 levels. The results in Fig. 4*B* show that treatment with IL-18 did indeed increase NFATc4 levels in the nucleus of the adipocytes. NFATc4 is present in the cytoplasm in an inactive state due to phosphorylation at Ser$^{168/170}$. Because dephosphorylation at Ser$^{169/170}$ results in its activation and nuclear translocation (33, 34), we investigated whether IL-18 alters pNFATc4 (Ser$^{168/170}$) levels in cytoplasm. Surprisingly, we found that levels of phospho-NFATc4(Ser$^{168/170}$) in the cytoplasm were not altered following IL-18 treatment (Fig. 4*C*). Although phosphorylation at Ser$^{168/170}$ keeps NFATc4 in an inactive state and localized to the cytoplasm, it has been recently demonstrated that phosphorylation at Ser^{676} promotes its activation and nuclear translocation (25). Therefore, we investigated whether IL-18 induces NFATc4 phosphorylation at Ser^{676}. Using phospho-specific antibodies and Western blotting, we observed that IL-18, but not neutralized IL-18, induced phosphorylation of NFATc4 at Ser^{676} (Fig. 4*D*) resulting in its nuclear translocation (Fig. 4*E*). Furthermore, using ChIP assays, we observed that treatment with IL-18 also induced the binding of NFATc4(Ser^{676}) to the adiponectin promoter (Fig. 4*F*). Together, these results indicate that (i) IL-18 induces NFAT activation, nuclear translocation, and binding to the adiponectin promoter and (ii) IL-18-mediated NFATc4 activation, and DNA binding is not dependent on dephosphorylation at Ser$^{168/170}$, but rather on phosphorylation at Ser^{676} (Fig. 4).

IL-18 Induces NFATc4 Phosphorylation and Adiponectin Expression via ERK—Various MAPKs have been shown to regulate the phosphorylation status of NFAT transcription factors (33–35). Therefore, we investigated whether IL-18-induced NFATc4 phosphorylation at Ser^{676} is mediated by MAPKs. Our results show that, although inhibition of p38 MAPK by SB, or JNK by SP, failed to modulate (Fig. 5*A*), inhibition of ERK by PD markedly attenuated IL-18-mediated NFATc4 phosphorylation at Ser^{676} (Fig. 5*B*). In addition, knockdown of ERK1/2 also inhibited IL-18-mediated NFATc4 phosphorylation at Ser^{676} (Fig. 5*C*); knockdown of ERK1/2 was confirmed by Western blotting (Fig. 5*D*). Our results also show that IL-18 induced ERK phosphorylation (Fig. 5*E*) and kinase activity (Fig. 5*F*), and pretreatment with the MEK inhibitor U0126 or the ERK inhibitor PD attenuated ERK phosphorylation (Fig. 5*G*) and kinase activity (Fig. 5*H*). Moreover, PD and ERK1/2 knockdown, but not SB or SP, reversed IL-18-mediated suppression of adiponectin promoter-reporter activity (Fig. 6*A*), mRNA expression (Fig. 6*B*), and secretion (Fig. 6*C*). Together, these results indicate that IL-18 suppresses adiponectin expression via ERK-dependent NFATc4 phosphorylation at Ser^{676} (Figs. 5 and 6).

DISCUSSION

Here we demonstrate for the first time that IL-18 suppresses adiponectin expression in adipocytes via a novel signal transduction pathway involving ERK-dependent NFATc4 phosphorylation. These results suggest that the inverse relationship observed between IL-18 and adiponectin in various chronic inflammatory conditions may not be coincidental, but rather, has a causal component. Therefore, targeting IL-18 expression may enhance adiponectin expression and blunt disease progression.

During the past 20 years, there has been a steady increase in obesity in the U.S. and other developing countries as a result of increased caloric intake and sedentary lifestyle. In addition, the number of individuals with morbid obesity is increasing due to the accumulation of excessive amounts of fat in both adipose (subcutaneous and visceral) and non-adipose tissue. Of note, obesity is associated with insulin resistance, type II diabetes, coronary artery disease, hypertension, dyslipidemia, metabolic syndrome, and increased mortality. Although adipose tissue serves as an energy reserve, it also acts as an endocrine gland secreting various adipokines, such as leptin, resistin, and adiponectin, and these mediators in turn affect energy metabolism. Adiponectin is secreted exclusively by adipose tissue (1, 2, 36), but paradoxically, obesity is associated with decreased circulating adiponectin levels (3, 4). However, expression levels of other adipokines such as leptin, tumor necrosis factor-α, and PAI are not altered, and in fact their levels are increased during obesity (5, 6), suggesting that obesity is selectively associated with decreased adiponectin levels. Plasma adiponectin declines during early phases of obesity and persist at these low levels.

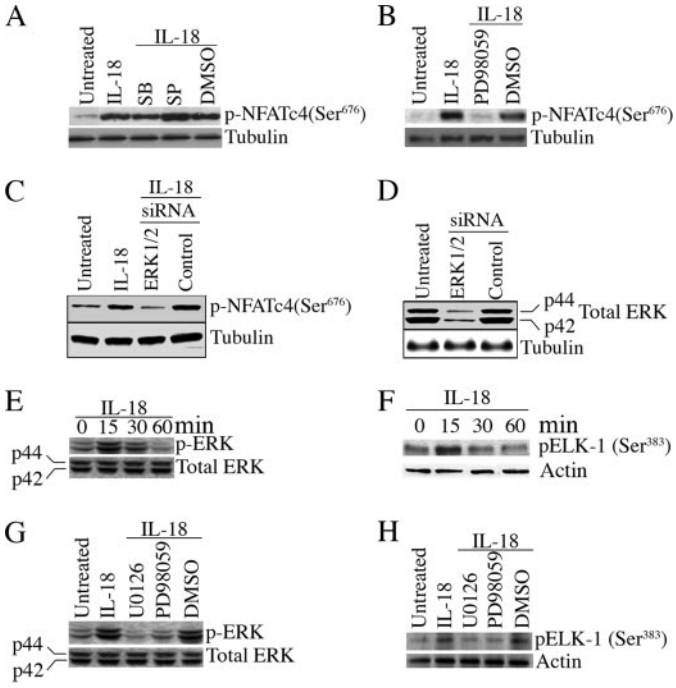

FIGURE 5. **IL-18 suppresses adiponectin expression via ERK1/2-dependenet NFATc4 phosphorylation.** *A*, IL-18-induced NFATc4 (Ser^{676}) phosphorylation is not dependent on p38 MAPK and JNK. Adipocytes were treated with the p38 MAPK inhibitor SB or the JNK inhibitor SP for 1 h prior to IL-18 addition (10 ng/ml for 1 h). Phospho-NFATc4(Ser^{676}) levels were analyzed in cleared cell lysates by Western blotting. Tubulin served as an internal control. A representative of six experiments is shown. *B* and *C*, IL-18 induces NFATc4(Ser^{676}) phosphorylation via ERK1/2. Adipocytes were treated with the ERK1/2 inhibitor PD (*B*) or ERK1 and 2 siRNA (*C*) prior to IL-18 addition. Phospho-NFATc4(Ser^{676}) levels were analyzed as in *A*. *D*, knockdown of ERK1/2 was confirmed by Western blotting. Tubulin served as an internal control. *E*, IL-18 induces ERK activation. Adipocytes treated with IL-18 for various time periods were analyzed for total and phospho-ERK levels by Western blotting. *F*, IL-18 induces ERK kinase activity. Adipocytes treated as in *E* were analyzed for ERK activation by immunocomplex kinase assays. *G* and *H*, PD inhibits IL-18-mediated ERK activation. Adipocytes were treated with PD for 1 h prior to IL-18 addition. ERK activation was analyzed by Western blotting using activation-specific antibodies (*G*) and immunocomplex kinase assays (*H*).

Adiponectin is a ~28-kDa protein consisting of 244 amino acids (37). It has an N-terminal signal sequence, a variable domain, a collagen-like (tail) domain, and a C-terminal globular (head) domain (36). Its expression is regulated at transcriptional, translational, and post-translational levels. Post-translational modification by glycosylation and hydroxylation results in a low molecular weight trimer, a middle molecular weight hexamer, and a high molecular weight multimer (38). A small globular fragment of adiponectin-globular adiponectin can also be detected in the circulation, but at lower levels (39). Although all of these isoforms are detectable in plasma, their differential significance in the pathophysiology of cardiovascular and metabolic diseases is not completely known. In both animals and humans, lower levels of circulating high molecular weight, but not other isoforms, correlate with the onset and progression of metabolic abnormalities, coronary artery disenso, and type II diabetes (37). In addition, weight loss following diet, liposuction, or gastric bypass is also shown to be predominantly due to the high molecular weight isoform (40, 41). The ELISA method used in the present study does not differentiate among the high, middle, and low molecular weight isoforms of adiponectin and does not detect the globular form. Therefore, based on the stud-

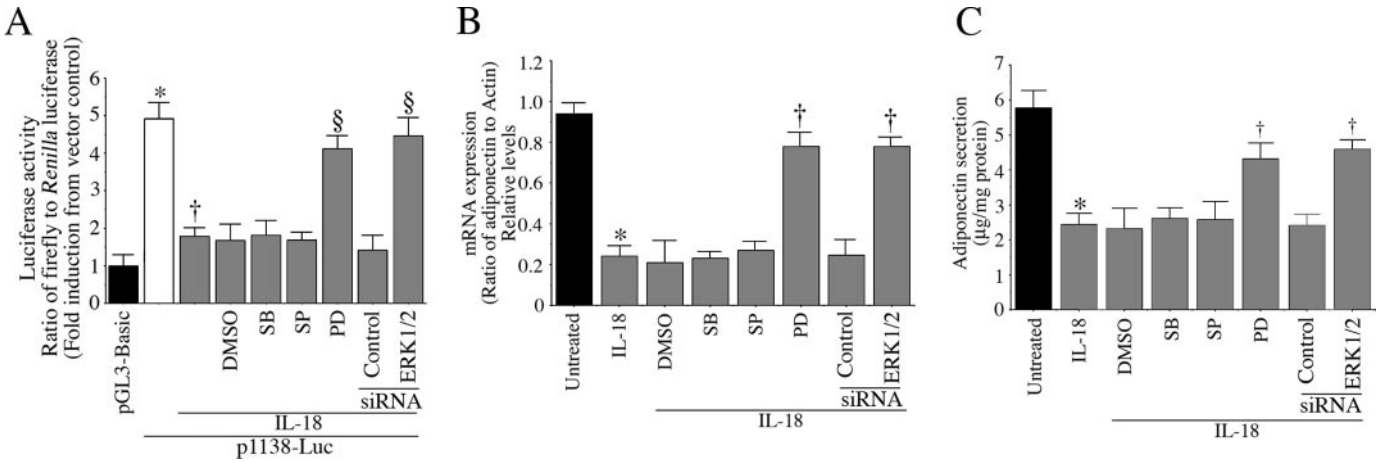

FIGURE 6. **Inhibition of ERK, but not p38 MAPK or JNK, reverses IL-18-mediated suppression of adiponectin expression.** Adipocytes were transfected (*A*) or not (*B* and *C*) with adiponectin promoter-reporter, treated for 1 (SB, SP, or PD) or 48 h (ERK1 and 2 siRNA or control siRNA) followed by IL-18 for 12 h (*A*) or 24 h (*B* and *C*). Adiponectin mRNA expression was analyzed by RT-qPCR (*B*) and its secretion by ELISA (*C*). *A*: *, $p < 0.001$ *versus* pGL3-Basic, †, $p < 0.01$ *versus* p1138-Luc, §, $p < 0.001$ *versus* IL-18 alone or IL-18 + Me_2SO; *B* and *C*: *, $p < 0.001$ *versus* untreated, †, $p < 0.01$ *versus* IL-18 by ANOVA.

ies by others (37, 40, 41), we hypothesize that the observed decrease in adiponectin following IL-18 treatment was due mainly to a reduction of the high molecular weight isoform. In the future, we will test this possibility *in vitro* in adipocytes and *in vivo* in relevant mouse models when the appropriate reagents become available.

Adiponectin exerts both anti-inflammatory and anti-atherogenic effects. Kumada *et al.* have reported that adiponectin acts as a modulator of the inflammatory response in the vascular wall (42). It affects various cell types involved in atherogenesis. For example, it inhibits monocyte-endothelial adhesion, endothelial cell apoptosis, transformation of macrophages into foam cells, and smooth muscle cell proliferation (8–10). Importantly, plasma adiponectin levels show an inverse correlation with coronary artery disease (43) and serve as a risk factor. Administration of globular adiponectin protected *ob*/*ob* mice from diabetes and apoE$^{-/-}$ mice from atherosclerosis (44). In contrast, mice deficient in adiponectin have increased neointimal proliferation after injury and increased atherosclerosis (45), further emphasizing the anti-apoptotic and anti-atherosclerotic role of adiponectin.

While adiponectin levels decrease, systemic levels of various pro-inflammatory cytokines increase in obesity. IL-18 is a pro-inflammatory and proatherogenic cytokine that induces the expression of other pro-inflammatory cytokines and adhesion molecules (16, 17). It acts either directly or synergizes with other cytokines to induce vascular inflammation. It localizes in human atherosclerotic lesions (18), and circulating IL-18 levels, which are increased in acute coronary syndromes, are predictive of future cardiovascular events. A positive correlation between serum IL-18 levels and carotid intimal-medial thickening has also been demonstrated (16). Administration of IL-18 aggravates atherosclerosis in mice (46). Moreover, atherogenesis is reduced in IL-18-deficient apoE$^{-/-}$ mice (22), suggesting a causal role for IL-18 in the development and progression of atherosclerosis.

An inverse correlation has been demonstrated between systemic adiponectin and IL-18 levels in various chronic inflammatory conditions, including obesity and coronary artery disease. Straczkowski *et al.* have demonstrated an association between increased serum IL-18 concentration and hypoadiponectinemia in obesity (11), whereas Giugliano *et al.* have reported increased circulating IL-18 and decreased adiponectin in obese women that was reversed following liposuction (47). Vilarrasa *et al.* have recently reported that high levels of circulating IL-18 and significantly low levels of adiponectin in morbidly obese patients revert toward normal with weight loss following gastric bypass surgery (48). However, it has not been clear whether this inverse correlation is causal or coincidental. Here we demonstrate that IL-18 suppresses adiponectin secretion from adipocytes through the down-regulation of adiponectin gene transcription and mRNA expression.

Our data demonstrate that IL-18 suppresses adiponectin expression via ERK-dependent phosphorylation of the NFATc4 transcription factor. The NFAT family of proteins comprises 5 members, NFATc1-c5, whose activation is regulated by calcineurin, a Ca^{2+}-dependent phosphatase (33, 34). NFATc1 through -c4 function as transcription factors and regulate various genes. They play a role in the differentiation of pre-adipocytes to adipocytes. Both c1 and c3 were detected at basal conditions in preadipocytes, and their levels were not altered during differentiation to mature adipocytes (25). In contrast, the low basal levels of c2 and c4 in preadipocytes showed a dramatic increase during the differentiation process, with high levels detected in the fully differentiated cells (25). Our results show that knockdown of NFATc4, but not NFATc2, significantly attenuates IL-18-mediated adiponectin gene transcription and mRNA expression. Therefore, NFATc4 appears to be the predominant member of the NFAT family that mediates IL-18 signaling in adipocytes.

NFAT proteins contain both nuclear export and localization sequences. In unstimulated cells, these sequences remain either masked or overt based on the phosphorylation status of specific serine residues in their regulatory domain (33, 34). Although phosphorylation at the serine residues exposes the nuclear export signal, dephosphorylation of the nuclear localization signal results in translocation of the protein to the nucleus, leading in turn to NFAT-dependent gene transcription. Phosphorylation at Ser$^{168/170}$ retains NFATc4 in the cytoplasm, and dephosphorylation results in its activation and nuclear translocation. Our results show that, although treatment with IL-18 results in increased levels of NFATc4 in the nucleus, it does not significantly alter p-NFATc4(Ser$^{168/170}$) levels in the cytoplasm, suggesting that the increased nuclear NFATc4 is not due to dephosphorylation of NFATc4 at Ser$^{168/170}$, but rather to an alternative mechanism. Further investigation revealed that IL-18 induces NFATc4 phosphorylation at Ser^{676}, because treatment with IL-18 increased cytoplasmic and nuclear levels of p-NFATc4Ser^{676}, as well as its *in vivo* binding to the adiponectin promoter. Our results also show that the mechanism of IL-18 induced NFATc4 phosphorylation at Ser^{676} is via ERK activation. This is the first report to demonstrate that a pro-inflammatory cytokine, IL-18 in particular, induces NFATc4 phosphorylation at Ser^{676} resulting in its activation. In support of these observations, Yang *et al.* have demonstrated phosphorylation of NFATc4 at Ser^{676} in *in vitro* kinase assays and shown this to enhance its transactivation potential (25).

We demonstrated that IL-18 suppresses adiponectin expression in adipocytes *in vitro* via ERK1/2-dependent NFATc4 phosphorylation. However, we have not yet investigated the mechanisms involved in IL-18-mediated ERK activation in adipocytes. IL-18 has been shown to be a potent inducer of ERK activation in rheumatoid arthritis synovial fibroblasts (49), and IL-18 mediates ERK activation via c-Src, Ras, and Raf-1. We have previously demonstrated that IL-18 signals via MyD88, IRAK4, IRAK1, TRAF6, and c-Src, leading to MAPK activation (50). Studies are in progress to determine whether similar mechanisms are operative in adipocytes.

Although obesity in humans was shown to be associated with increased IL-18 and decreased adiponectin (11), and reversed by weight loss (47), the mechanisms involved in IL-18-mediated adiponectin suppression *in vivo* have not been investigated. It has been previously reported that systemic levels of pro-inflammatory cytokines (*e.g.* tumor necrosis factor) are increased in adiponectin null mice (51). Furthermore, adiponectin has been shown to suppress cytokine expression as

well as cytokine signaling *in vitro* in both immune and non-immune cells (52). Conversely, other studies, in addition to ours, show that cytokines inhibit adiponectin transcription and translation (53, 54). Importantly, a single high fat meal has been shown to enhance systemic IL-18 and inhibit adiponectin levels in humans (55), and a prolonged high fat diet appears to reduce systemic adiponectin levels and increases pro-inflammatory cytokine levels in mice (56). Thus these studies point to an inverse relationship between cytokine and adiponectin expression that may be an important factor in the pathophysiology of a number of metabolic diseases. Our *in vitro* studies described in this report delineate one potential mechanism that may be responsible for this inverse relationship. However, this may be only one of a number of mechanisms operating *in vivo* where the situation is likely to be far more complex. Nevertheless, in future studies, we intend to investigate the effect of a high fat diet on IL-18 and adiponectin expression in mice and determine if the resulting relationship is mediated via ERK1/2-dependent NFATc4 phosphorylation in adipose tissue. Furthermore, these studies will include intervention by administering IL-18 neutralizing antibodies and IL-18BP to determine the effects of IL-18 directly.

Collectively, these data provide the first evidence that IL-18 selectively suppresses adiponectin expression via ERK-dependent NFATc4 phosphorylation. Our results indicate that the inverse correlation observed between IL-18 and adiponectin during various pathological conditions may be causal, and not coincidental. Therefore targeting the suppression of IL-18 expression offers a novel therapeutic strategy that may attenuate disease progression via restoration of adiponectin levels.

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