The Pro-atherogenic Cytokine Interleukin-18 Induces CXCL16 Expression in Rat Aortic Smooth Muscle Cells via MyD88, Interleukin-1 Receptor-associated Kinase, Tumor Necrosis Factor Receptor-associated Factor 6, c-Src, Phosphatidylinositol 3-Kinase, Akt, c-Jun N-terminal Kinase, and Activator Protein-1 Signaling*

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We recently demonstrated that the chemokine CXCL16 is expressed in aortic smooth muscle cells (ASMC) and induces ASMC adhesion and proliferation (Chandrasekar, B., Bysani, S., and Mummidi, S. (2004) J. Biol. Chem. 279, 3188–3196). Here we report that interleukin (IL)-18 positively regulates CXCL16 transcription in rat ASMC. We characterized the cis-regulatory region of CXCL16 and identified a functional activator protein-1 (AP-1) binding motif. Deletion or mutation of this site attenuated IL-18-mediated CXCL16 promoter activity. Gel shift, supershift, and chromatin immunoprecipitation assays confirmed AP-1-dependent CXCL16 expression. CXCL16 promoter-reporter activity was increased by constitutively active c-Fos and c-Jun and decreased by dominant negative or antisense c-Fos and c-Jun. Src kinase inhibitors PP1 and PP2, phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002, Akt inhibitor, the c-Jun N-terminal kinase (JNK) inhibitor SP600125, antisense JNK and dominant negative MyD88, interleukin-1 receptor-associated kinase (IRAK)-1, IRAK4, and phosphatidylinositol 3-kinase inhibitor all attenuated IL-18-mediated AP-1 binding and reporter activity, CXCL16 promoter-reporter activity, and CXCL16 expression. Thus IL-18 induced CXCL16 expression via the MyD88 → IRAK1-IRAK4-TRAF6 (tumor necrosis factor receptor-associated factor 6) → c-Src → PI3K → Akt → JNK → AP-1 pathway. Importantly, IL-18 stimulated ASMC proliferation in a CXCL16-dependent manner. These data provide for the first time a mechanism of IL-18-mediated CXCL16 gene transcription and CXCL16-dependent ASMC proliferation and suggest a role for IL-18-CXCL16 cross-talk in atherogenesis and re-stenosis following angioplasty.

Atherosclerosis is an inflammatory disease responsible for considerable morbidity and mortality in Western societies. Both proinflammatory cytokines and chemokines play a central role in atherogenesis. The cross-talk between cytokines and chemokines amplifies the inflammatory cascade, resulting in the development and progression of atherosclerosis (1–3). IL-18 is a proinflammatory and pro-atherogenic cytokine. Several lines of evidence suggest that it plays a critical role in the generation of atherosclerotic plaque. IL-18 expression is detected in human atherosclerotic lesions (4, 5), and administration of IL-18 aggravates atherosclerosis in mice (4–8). Furthermore, the generation of atherosclerotic lesions is reduced in IL-18-deficient apolipoprotein E null mice (9), suggesting that IL-18 may play an important role in atherogenesis. Most of the IL-18 effects in atherogenesis are thought to be mediated via the induction of IFN-γ (6, 7, 9, 10).

It was recently reported that intraperitoneal administration of IL-18 can induce the expression of the chemokine CXCL16 in atherosclerotic lesions and spleens of SCID (severe combined immunodeficiency)/apoE null mice (6). CXCL16, a recently discovered transmembrane chemokine (11, 12), is a member of the non-ELR (absence of glutamic acid-leucine-arginine motif before the first conserved cysteine) CXC chemokine subfamily. Unlike other members of this subgroup, it is structurally similar to CX3CL1 (fractalkine (13)), containing four distinct domains; that is, a chemokine domain tethered to the cell surface via a mucin-like stack, which in turn is attached to transmembrane and cytoplasmic domains. As a transmembrane protein it acts as an adhesion molecule, and upon cleavage by ADAM10 (14, 15) it acts as a chemokine. It attracts CXCR receptor 6 (CXR6) expressing T, NK, and NKT cells to the sites of inflammation.

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flammation and injury (16–20). CXCL16 also functions as a scavenger receptor for phosphatidylserine and oxidized lipoprotein (hence, the name SR-PSOX (21)). By enhancing the uptake of oxidized low density lipoprotein, CXCL16 promotes foam cell formation. We recently demonstrated that CXCL16 induces SMC proliferation (22). SMC proliferation is a hallmark of atherosclerosis and restenosis after angioplasty (23–26). Furthermore, a positive correlation was shown between mutations in the gene and the severity of coronary artery stenosis (27), suggesting that CXCL16 may play a causal role in the development and progression of atherosclerosis.

Taken together, these studies suggest that IL-18 may induce SMC proliferation through a CXCL16-mediated mechanism. In this study we tested this hypothesis directly and delineated the mechanisms by which IL-18 induces CXCL16 expression in SMC. IL-18 induced CXCL16 expression through an AP-1-dependent mechanism, and this induction involves MyD88, IRAK, TRAF6, c-Src, PI3K, Akt, and JNK. More importantly, IL-18 induced SMC proliferation in a CXCL16-dependent manner. IL-18-mediated CXCL16 expression was independent of the classical inflammatory cytokines IL-1β, TNF-α, and IFN-γ. These results ascribe a previously unrecognized role for IL-18 in SMC proliferation and suggest that IL-18 and CXCL16 cross-talk may lead to the amplification of an inflammatory cascade in vessel wall-promoting atherosclerosis.

MATERIALS AND METHODS

Cell Culture—Non-transformed rat aortic smooth muscle cells (ASMC) were a generous gift from Dr. Sergei N. Orlov (University of Montreal, Montreal, Canada) and have been previously described (28). ASMC were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 100 units/ml each of penicillin and streptomycin. The cells were passaged when confluent. For cytokine-stimulation experiments, ASMC were grown until 70–80% confluent, then the medium was replaced with Dulbecco’s modified Eagle’s medium plus 0.5% bovine serum albumin. After overnight culture recombinant rat IL-18 (rrIL-18; #521-RL-025/CF, R&D Systems, Minneapolis, MN) was added to the indicated doses and for the indicated time periods.

Cytokine Stimulation—Recombinant IL-18 preparations contained < 1 pg/ml of endotoxin as determined by the LAL method (manufacturer’s technical data sheet, R&D Systems). To determine any possible contribution of endotoxin to the IL-18-mediated effects, ASMC were treated simultaneously with 10 μg/ml polymyxin B (#P9392, Sigma). In the inhibition studies, before the addition of IL-18 ASMC were treated with either 100 μM LT294092 for 1 h (PE850-2H16-1, N-cyclopentyl-1-[2-(1H-pyrazol-3-yl)pyrazol-4-yl]-1H-pyrazole-5-carboxamide), 1.2 kilobases of the 5′-flanking region of the gene was amplified from Clontech, Palo Alto, CA) to amplify complete CXCL16 gene sequences in GenBank™ (NW_047334). This contained 1.2 kilobases of the 5′-flanking region and the 1.2 kilobases of the 5′-untranslated region of CXCL16 gene and the severity of coronary artery stenosis (27), suggesting that CXCL16 may play a causal role in the development and progression of atherosclerosis.

To investigate IL-18-induced apoptosis, ASMC were cultured in complete medium until ~70% confluent. The medium was replaced with Dulbecco’s modified Eagle’s medium plus 0.5% bovine serum albumin. After overnight culture recombinant rat IL-18 (rrIL-18; #521-RL-025/CF, R&D Systems, Minneapolis, MN) was added to the indicated doses and for the indicated time periods.

Cytokine Stimulation—Recombinant IL-18 preparations contained < 1 pg/ml of endotoxin as determined by the LAL method (manufacturer’s technical data sheet, R&D Systems). To determine any possible contribution of endotoxin to the IL-18-mediated effects, ASMC were treated simultaneously with 10 μg/ml polymyxin B (#P9392, Sigma). In the inhibition studies, before the addition of IL-18 ASMC were treated with either 100 μM LT294092 for 1 h (PE850-2H16-1, N-cyclopentyl-1-[2-(1H-pyrazol-3-yl)pyrazol-4-yl]-1H-pyrazole-5-carboxamide), 1.2 kilobases of the 5′-flanking region of the gene was amplified from Clontech, Palo Alto, CA) to amplify complete CXCL16 gene sequences in GenBank™ (NW_047334). This contained 1.2 kilobases of the 5′-flanking region and the 1.2 kilobases of the 5′-untranslated region of CXCL16 gene and the severity of coronary artery stenosis (27), suggesting that CXCL16 may play a causal role in the development and progression of atherosclerosis.

To investigate IL-18-induced apoptosis, ASMC were cultured in complete medium until ~70% confluent. The medium was replaced with Dulbecco’s modified Eagle’s medium plus 0.5% bovine serum albumin, and the cells were incubated for 48 h. IL-18 was added at 25 ng/ml and incubated for 24 h. Cells were harvested, and the cytokine levels of mononucleosomes and oligonucleosomes were assayed by enzyme-linked immunosensor (Cell Death Detection ELISA™ kit, Roche Applied Science (30, 31)). As a positive control, S-nitroso-N-acetylimidicillin (SNAP, #487910, EMD Biosciences, Inc., La Jolla, CA) was added to a final concentration of 500 μM for 24 h (28). Apoptotic cell death was also analyzed using the annexin V fluorescein isothiocyanate apoptosis detection kit (Oncogene Research Products, San Diego, CA). Flooding cells were added and added to the separated adherent cells for the assay. Cells were counterstained with propidium iodide and analyzed by flow cytometry.

Cell Proliferation Assays—Cell proliferation was determined by [3H]thymidine incorporation (31). To confirm the role of CXCL16 in IL-18-mediated ASMC proliferation, ASMC were transfected as above with a mixture of CXCL16 siRNAs (5′-AGU UAC UAU GGU GGU ATT-3′, 5′-AGU UAC UAU GGU GGU ATT-3′, 5′-AGU UAC UAU GGU GGU ATT-3′) and incubated for 48 h before the addition of IL-18.

5′ Rapid Amplification of cDNA Ends (5′ RACE)—The 5′ ends of rat CXCL16 mRNA transcripts were determined by 5′ RACE using ASMC total RNA. Total RNA was isolated using the TRIzol reagent (Invitrogen) and treated with RNase-free DNase to eliminate any genomic DNA contamination. We used the SMART RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA) to amplify and to delete the CXCL16 transcripts following the manufacturer’s instructions. Successful PCR was carried out using the rat CXCL16-specific antisense primers, 5′-TCA TTC GTC TCT GTG CTT GGT TGT-3′ and 5′-TTC CTC GGG TCC CAA GAT GCT TTC-3′, and the sense primers provided in the kit. The products of the second PCR amplification were gel-purified and cloned into the pCR2.1-TOPO vector (Invitrogen). The nucleotide sequence of four independent clones was determined.

Cloning of the 5′-Flanking Sequence of Rat CXCL16 and Vector Construction—A blast search of the GenBank™ data base identified rat CXCL16 gene sequences in GenBank™ (NW_047334). This contained in tandem the open reading frame of CXCL16 and the 5′-untranslated region (UTR) sequences that we identified by 5′-RACE. Approximately ~1.2 kilobases of the 5′-flanking region of the gene was amplified from rat genomic DNA using the sense primer 5′-CTG ACA TAA GGA CTC AGG TCT CTG-3′ and the antisense primer 5′-TCA GTA GGA TCC AGT CAT G-3′ and cloned into the pcR2.1-TOPO vector. A series of nested deletions was generated using the sense primers 5′-gag cct GGA AAG TGA ATG TAT GGT GTG TTT-3′, 5′-gag ctt AAA GGC TGC CTG AGA GAG TAA-3′, and 5′-gag cct AAG AGC ACA TGA TAA CTT CAG CAG-3′. All the sense primers contained a SacI restriction site at the 5′ end (lowercase). The antisense primer (5′-ctg gag TTA GTA GGA TCC AGT CAG-3′) contained a Xhol restriction site. The PCR products were cloned into pcR2.1-TOPO and subcloned into the pG3L-Basic reporter vector (Promega). The deletion construct S5 was generated by annealing sense (5′-gag ctt TAC CTC TGA GGA TTT GGT CTT TGT CTA CAT GAG CAG TAT GCT ACT-3′) and antisense oligonucleotides, digesting with SacI and Xhol, and inserting into pG3L-Basic. The 5′-flanking sequence of the CXCL16 gene was analyzed by MatInspector Professional™ software to identify the potential binding sites for various transcription factors. The nucleotide sequences of the rat CXCL16 5′-flanking region and the mRNA have been deposited with the GenBank™ under the accession numbers DQ025527 and DQ025528, respectively.
Site-directed Mutagenesis—Mutation of the AP-1 binding site in the S2 reporter vector construct was performed by site-directed mutagenesis using the QuikChange kit (Stratagene), the sense primer 5'-AACCGGATTCTATACACCAAGGATGCG-3'; the antisense primer 5'-CGCATTTTAGGTATCAATGATTTTGT-3' (mutated nucleotides are in lowercase). Mutation was confirmed by nucleotide sequencing.

Cell Transfection and Reporter Assays—ASM C were transfected with 3 μg of the CXCL16 reporter constructs and 100 ng of the control Renilla luciferase vector pRL-TK (Promega) using Lipofectamine® (22, 27, 30, 31). 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. The transfection efficiency of rat ASM C (~32%) was determined using pEGFP-N1 vector (BD Biosciences Clontech (28)). All plasmids were purified using EndoFree Plasmid Maxi kit (Qiagen).

To determine whether IL-18 induces AP-1 activation in ASM C, we used pAP-1-Luc (PathDetect® AP-1 Cis-reporting System, Stratagene), an inducible AP-1 reporter vector containing seven repeats of the AP-1 enhancer element. ASM C were co-transfected with pAP-1-Luc and pRL-TK and after 24 h of incubation were treated with the indicated doses of IL-18. pEGFP-Luc, a vector that encodes a fusion of an enhanced green fluorescent protein and luciferase under the regulation of the CMV promoter (pCMV-SPORT6) was used as a control.

In addition to the pharmacological inhibitors described above, ASM C were transfected with rat MyD88 siRNA (sGenOME SMARTpool siRNA, #M-099595-08-00, Dharmacon, Lafayette, CO), dominant negative (dn) IRAK1 in pCl-Neo, dn IRAK4 (1-191) in pDNAHisMaxC (Invitrogen), dn TRAF6 in pRK5 (TRAF6-259-522-FLAG), and dn P38k in pDNAas described previously (30, 31). The corresponding empty vectors served as controls. Inhibitions were confirmed by Western blotting, by treating ASM C with antisense oligonucleotides against JNK1 (5'-CTC TCT GTA GCC GCG CTT GG-3'), and JNK2 (5'-GTC GTC GCG AGC CCA AAG TG-3') in ASM C expressing JNK using site-directed mutagenesis (mutated nucleotides are in lowercase), which amplified a 239-bp segment of the rat CXCL16 gene from 845 to 305 relative to the transcription start site. The rat CXCL16 cDNA was aligned with reverse-transcribed ASM C poly(A)+ RNA using the following primers: sense, 5'-CTT CTT TGG GCT GCT GAC TC-3'; antisense, 5'-GCT GGT CCT AAA CGA TAC AG-3'. The aligned PCR products were subcloned into pCR2.1-TOPO (Invitrogen), and sequenced on both strands. The rate of CXCL16 gene transcription was analyzed by nuclear run-on assays. CXCL16 mRNA stability and protein (or vehicle) treatment was determined after actinomycin D treatment (36).

Analysis of Protein Expression—Protein extraction, Western blotting, autoradiography, and densitometry were performed as described previously (36). Goat anti-mouse CXCL16 antibodies (AF503) and anti-IFN-γ antibodies (AF-585-NA) were obtained from R&D Systems. Cytokine-chemokine in smooth muscle cell proliferation

Cytokine-Chemokine in Smooth Muscle Cell Proliferation

RESULTS

The pluriopotent effects of IL-18 are mediated through a heterodimeric receptor IL-18R comprising a ligand binding subunit IL-18Rα and a signal transducing subunit IL-18Rβ. We confirmed that ASM C express both of these receptors at basal conditions (Fig. 1A), and these results are in agreement with the observations of Gerdes et al. (5). Although quiescent ASM C express low levels of CXCL16, IL-18 induced CXCL16 mRNA expression in a dose- and time-dependent manner. Peak levels of CXCL16 mRNA were detected at a concentration of 25 ng/ml (Fig. 1B). CXCL16 was detected after 30 min of treat-
IL-18 induces CXCL16 expression in rat aortic smooth muscle cells. A, ASMC express IL-18Rα and -β mRNA. Northern blot analysis was performed using 2 µg of poly(A)+ RNA isolated from cultured ASMC. B, dose-dependent induction of CXCL16 mRNA by IL-18. Quiescent ASMC were treated with the indicated concentrations of IL-18 for 2 h, and total RNA was isolated and analyzed for CXCL16 mRNA and 28S rRNA (loading control). C, time course studies. Quiescent ASMC were treated with 25 ng/ml IL-18 for up to 24 h, and total RNA was analyzed for CXCL16 mRNA expression. D, IL-18 induced CXCL16 protein expression. Quiescent ASMC were treated with IL-18 as in C, and cell extracts were analyzed by Western blotting for CXCL16 protein and β-actin. E, IL-18-induced CXCL16 expression was not mediated by endotoxin. Quiescent ASMC were either untreated or treated with polymyxin B vehicle (H2O) or 10 µg/ml polymyxin B (left three lanes) or with 25 ng/ml IL-18, 25 ng/ml IL-18 plus vehicle, or 25 ng/ml IL-18 plus 10 µg/ml polymyxin B (right three lanes) for 2 h. Total RNA was analyzed for CXCL16 expression as before. F, IL-18-induced CXCL16 expression was regulated at the transcriptional level. Quiescent ASMC were treated with 25 ng/ml IL-18 for 2 h, and the nuclear RNA was isolated and analyzed for CXCL16 transcription by the run-on assay. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the loading control, and pCR2.1-TOPO vector served as the internal control. A representative of three independent experiments is shown. G, IL-18-induced CXCL16 expression was not due to increased mRNA stability. Quiescent ASMC were treated with 25 ng/ml IL-18 for 2 h followed by the addition of 5 µg/ml actinomycin D for up to 6 h. Total RNA was isolated and analyzed for CXCL16 expression by Northern blotting as before. Band intensity was determined by densitometry and normalized to the value obtained at 2 h (100%). The results are the mean ± S.E. of four independent experiments.

Although CXCL16 was strongly induced in the ASMC after IL-18 treatment, it is not evident from the above experiments whether this was dependent on the up-regulation of other proinflammatory cytokines such as IL-1β, TNF-α, or IFN-γ, all of which have been shown previously to induce CXCL16 expression in fibroblasts, endothelial cells, and SMC (14, 15, 39, 40). To investigate whether IL-18 induces CXCL16 expression directly or its up-regulation is mediated via induction of IL-1β, TNF-α, or IFN-γ, we used antisense oligonucleotides (AS ODN) to suppress their expression. Our results indicate that treatment with AS ODN directed against IL-1β, TNF-α, or IFN-γ all failed to modulate either the low basal or the marked IL-18-induced, CXCL16 mRNA expression level (Fig. 2A). However, the AS ODN significantly inhibited lipopolysaccharide/IL-18-mediated corresponding cytokine secretion (data not shown). To rule out the role of preformed cytokines in IL-18-mediated CXCL16 induction, ASMC were incubated with neutralizing antibodies to either IL-1β, TNF-α, or IFN-γ for 1 h before IL-18 addition. Again, these treatments all failed to modulate IL-18-mediated CXCL16 mRNA expression (Fig. 2B). Normal goat IgG, used as a control, also failed to modulate IL-18-mediated CXCL16 expression (data not shown). The ability of these antibodies to neutralize their target cytokines was verified in transient transfection assays in which ASMC transected with either NF-κB (pNF-κB-Luc) or IFN-γ-activating sequence (pGAS-Luc) reporter constructs were treated with the antibodies before incubation with their respective recombinant proteins (rrIL-1β, 100 pg/ml; rrTNF-α, 100 pg/ml; rrIFN-γ, 10
Because IL-18 strongly induced CXCL16 gene transcription (Fig. 1F), we sought to determine the cis-trans interactions that lead to its increased expression. To this end, we determined the genomic structure of rat CXCL16 gene and defined the cis-elements that are responsible for its induction by IL-18. As a preliminary step, we performed extensive blast searches to identify full-length mouse and human CXCL16 transcripts, for which cap-based libraries are available. Through this bioinformatics approach, we identified several mouse CXCL16 transcripts obtained from ASMC that were either untreated (Fig. 4A) or treated with IL-18 for different lengths of time (Fig. 4B–D). Once again, knockdown of IFN-γ had no effect on the induction of CXCL16 mRNA by IL-18 (D, fifth lane).

Fig. 2. IL-18-mediated CXCL16 expression is independent of IL-1β, TNF-α, and IFN-γ. A, quiescent ASMC were transfected with 10 μg IL-1β, TNF-α, or IFN-γ antisense oligonucleotides and incubated for 16 h before the addition of 25 ng/ml IL-18. Cells were harvested at 2 h, and total RNA was isolated and analyzed for CXCL16 expression by Northern blotting. A representative of three independent experiments is shown. B, to exclude the role of preformed cytokines in IL-18-mediated CXCL16 expression, quiescent ASMC were incubated with IL-1β, TNF-α, or IFN-γ neutralizing antibodies (5 μg/ml) followed by the addition of 25 ng/ml IL-18 for 2 h, and CXCL16 expression was analyzed as in A. C and D, to confirm that the IL-18 induction of CXCL16 expression was independent of IFN-γ, ASMC were transfected with either IFN-γ or control siRNAs and incubated for 48 h. The cells were then untreated or treated with 25 ng/ml IL-18 for 3 h before isolation of total cell protein. Knock-down of IL-18-induced IFN-γ protein expression was confirmed by Western blotting (C, fifth lane). Knock-down of IFN-γ had no effect on the induction of CXCL16 mRNA by IL-18 (D, fifth lane).
FIG. 3. Genomic organization and potential regulatory elements of rat CXCL16 gene. A, the CXCL16 gene is located on rat chromosome 10 and is composed of 5 exons (boxes, roman numerals) and 4 introns (straight lines, Arabic numbers and circled). The positions of the ATG start codon and the TAG stop codon are indicated. Regions of the exons encoding the open reading frame are shown in black. The lengths of the exonic
regions that encode the open reading frame are shown in bold, and the lengths of the introns are in italics. Numbering is relative to the ATG start codon. The regions of the exons that encode the five domains (Sig, signal peptide; Chemokine, mucin stalk; TM, transmembrane; Cyt, cytoplasmic) of the CXCL16 protein are also shown. B, the 5′-flanking sequence of rat CXCL16 (lowercase) was analyzed by MatInspector Professional, and several potential transcription factor binding motifs that can mediate the inflammatory response are identified (in red and overlined with arrows; core sequences shown in large lowercase). Nucleotide residues in green encode the beginning of the open reading frame. The 5′-end of the transcript expressed in rat ASMCD was verified by 5′RACE. The nucleotide sequences of the forward primers (S1–S4) and the reverse primer (AS, in the 5′UTR region) used to prepare the deletion constructs are underlined. C, identification of the IL-18-responsive region in the 5′-flanking region of the rat CXCL16 gene. A deletion series of the 5′-flanking region of the rat CXCL16 gene in the pGL3-Basic reporter vector was co-transfected with the pRL-TK vector into rat ASMCD, and firefly and Renilla luciferase activities were determined after 24 h of incubation. The results are the mean ± S.E. of six independent experiments.
FIG. 4. IL-18 induces AP-1 DNA binding activity and AP-1-dependent reporter activity. A, IL-18 induces AP-1 DNA binding activity in ASMC. EMSA for AP-1 DNA binding activity was carried out using the AP-1 DNA binding sequence from the CXCL16 promoter, and nuclear extracts from quiescent ASMC or ASMC treated with 25 ng/ml IL-18 for the indicated times. Competition experiments (lanes 1 and 2) were performed with nuclear protein extracts from ASMC treated with IL-18 for 1 h. Arrows denote specific DNA-protein complexes. B, supershift assays. Nuclear extracts from ASMC treated with IL-18 for 1 h were incubated with anti-c-Fos, c-Jun, -JunD, -JunB, or isoform-specific control IgG antibodies, and EMSA was performed as in A. Supershifted complexes are indicated by the white arrow. C, chromatin immunoprecipitation analysis of the CXCL16 AP-1 binding site. Quiescent ASMC were untreated or treated with IL-18 for 1 h, then cross-linked chromatin was prepared and immunoprecipitated with or without antibodies (Ab) to c-Fos before amplification of the CXCL16 gene region containing the AP-1 site. Specific DNA sequences in the chromatin immunoprecipitated by c-Fos antibody are shown (lanes 2 and 4). Amplification of the input DNA is shown in lanes 5 and 6. D, IL-18 increases transactivation by AP-1. The pAP-1-Luc reporter plasmid was co-transfected with the control pRL-TK Renilla luciferase plasmid into ASMC, and after 24 h, the transfected cells were incubated with antibody to IL-18 or control IgG for 1 h followed by the addition of 25 ng/ml IL-18 for 7 h. Firefly and Renilla luciferase activities were determined, and the luciferase activity was normalized to the Renilla values. The data represent the mean ± S.E. of four independent experiments. *, p < 0.001 (versus untreated); †, p < 0.01 versus IL-18-treated transfected cells. E, the AP-1 site in the rat CXCL16 promoter mediates IL-18-induced transactivation. The AP-1 site in the pCXCL16-S2 promoter construct was mutated by site-directed mutagenesis from TGACTcaAATCC to TGACTtgAATCC, and the wild-type and mutant constructs were transfected and assayed as in D. *, p < 0.001 versus S2-mut AP-1. IRF, interferon regulatory factor. F, IL-18 increases transactivation by NF-κB. The pNF-κB-Luc reporter plasmid was transfected and assayed as in D. The data represent the mean ± S.E. of four independent experiments. *, p < 0.001 (versus untreated); †, p < 0.01 versus IL-18-treated pNF-κB-1-Luc transfected cells.
were treated with IL-18 for 10 min. PI3K lipid kinase assays were performed in p85 immunoprecipitates (28, 31, 43). Fig. 7A shows significantly increased levels of phosphatidylinositol 3-phosphate (P13P) in ASMC treated with IL-18, and treatment with the PI3K-specific inhibitors wortmannin and LY294002 inhibited PI3K-mediated phosphatidylinositol 3-phosphate formation. This induction of PI3K activity could also be blocked specifically by pretreating the cells with antibody to IL-18 before the addition of IL-18 (data not shown). Akt is one of the downstream substrates for PI3K. Western blot analysis using anti-phospho Akt (Thr308) antibodies, which is one of the downstream substrates for PI3K. Western blot analysis using activation-specific antibodies. JNK kinase activity was also determined. The results show that IL-18 rapidly induced p38 MAPK phosphorylation at Tyr172, with increased levels of phospho-p38 MAPK seen at 15 min (Fig. 8A, upper panel). These levels remained high throughout the 120-min study period. However, total p38 MAPK levels remained unchanged after treatment (Fig. 8A, lower panel). Similarly, IL-18 increased phospho-ERK levels without affecting total ERK levels (Fig. 8B). IL-18 also increased phospho-JNK levels (Fig. 8C, upper panel) and JNK kinase activity (Fig. 8D, lower panel). Pretreatment with SB203580, PD98059, and SP600125 inhibited IL-18-induced p38 MAPK (Fig. 8E), ERK (Fig. 8F), and JNK (Fig. 8G) activation, respectively. However, inhibition of JNK by SP600125 was the only treatment to significantly attenuate IL-18-induced CXCL16 expression (Fig. 8H). Together, these results indicate that IL-18 induces p38 MAPK, ERK, and JNK and investigated whether these might mediate IL-18-induced CXCL16 expression. Quiescent ASMC were treated with IL-18, and the levels of total and phosphorylated p38 MAPK, ERK, and JNK were semiquantitated by Western blotting using activation-specific antibodies. JNK kinase activity was also determined. The results show that IL-18 rapidly induced p38 MAPK activation, with increased levels of phospho-p38 MAPK at 15 min (Fig. 8A, upper panel). These levels remained high throughout the 120-min study period. However, total p38 MAPK levels remained unchanged after treatment (Fig. 8A, lower panel). Similarly, IL-18 increased phospho-ERK levels without affecting total ERK levels (Fig. 8B). IL-18 also increased phospho-JNK levels (Fig. 8C, upper panel) and JNK kinase activity (Fig. 8D, lower panel). Pretreatment with SB203580, PD98059, and SP600125 inhibited IL-18-induced p38 MAPK (Fig. 8E), ERK (Fig. 8F), and JNK (Fig. 8G) activation, respectively. However, inhibition of JNK by SP600125 was the only treatment to significantly attenuate IL-18-induced CXCL16 expression (Fig. 8H). Together, these results indicate that IL-18 induces p38 MAPK, ERK, and JNK activation in ASMC, but it is the activation of JNK and not p38 MAPK or ERK that plays a prominent role in IL-18-mediated CXCL16 expression in ASMC.

MyD88, an adaptor molecule, links IL-18 receptor to IRAK and mediates IL-18 signal transduction (46). In fact, MyD88-deficient mice exhibited defective IL-18 signaling (46), and the MyD88-IRAK-TRAF6 module has been shown to be essential in IL-18-mediated PI3K activation (38). Therefore, we investi-
gated whether IL-18 induces AP-1 activation via MyD88, IRAK, and TRAF6 in ASMC. ASMC were transiently transfected with MyD88 siRNA (knockdown of MyD88 was confirmed by Western blotting, data not shown), dominant negative IRAK1, IRAK4, or TRAF6. Cells transfected with control siRNA or the corresponding empty vectors served as controls. Cells were co-transfected with either AP-1 reporter or CXCL16 promoter-reporter vector. Knockdown of MyD88 or expression of dominant negative IRAK1, IRAK4, and TRAF6, but not the controls, all inhibited IL-18-mediated AP-1 reporter activity (Fig. 9A), CXCL16 promoter-reporter activity (Fig. 9B), and CXCL16 mRNA expression (Fig. 9C). Furthermore, the induction of AP-1 DNA binding activity was also inhibited (data not shown). These studies indicate that MyD88, IRAK1, IRAK4, and TRAF6 mediate IL-18-induced AP-1 activation and CXCL16 expression.

We have demonstrated that IL-18 induces PI3K-dependent Akt kinase activity in ASMC (Fig. 7C). However, it is not known whether PI3K and Akt play a role in IL-18-mediated AP-1 activation. Therefore, quiescent ASMC were treated with PI3K- and Akt-specific inhibitors before the addition of IL-18. PI3K lipid kinase assays were performed as described under “Materials and Methods.” The bottom panel shows immunoblot analysis of the same samples with anti-p85 antibody. DMSO, Me2SO, PI3P, phosphatidylinositol 3-phosphate. B, IL-18 induced PI3K-dependent Akt activation. Quiescent ASMC were treated with the PI3K-specific inhibitors wortmannin or LY294002 or the Akt inhibitor before the addition of 25 ng/ml IL-18. Total and phospho-Akt levels were determined by Western blotting. C, IL-18 induced PI3K-dependent Akt kinase activity. Quiescent ASMC were treated as in B, and phosphorylated glycogen synthase kinase (GSK-3α/β) was determined by Western blotting.
phosphorylation. ASMC were treated with 10 mM PD98059 for 1 h before the addition of 25 ng/ml IL-18. Total and phospho-ERK levels were determined by Western blotting. A representative blot from three independent experiments is shown.

Because IL-18 induced CXCL16 expression in ASMC, 2) IL-18-mediated CXCL16 expression was independent of IL-1β, TNF-α, and IFN-γ, 3) CXCL16 expression was regulated mainly at the transcriptional level, 4) IL-18 induced CXCL16 expression in AP-1-dependent manner, 5) JNK, but not p38 MAPK or ERK, played a role in IL-18-mediated CXCL16 induction, 6) IL-18 mediates CXCL16 expression via a MyD88 → IRAK → TRAF6 → c-Src → JNK → CXCL16 pathway.
Pi3K → Akt → JNK → AP-1-signaling pathway, and 7) IL-18 was mitogenic for ASM, inducing their proliferation in a CXCL16-dependent manner.

A number of proinflammatory cytokines has been shown to induce CXCL16 expression in human vascular cells, including IFN-γ, TNF-α, IL-12, and IL-15 (11, 12, 14, 15, 32, 40). Among these cytokines, IFN-γ has been reported to be the most potent inducer of CXCL16 in cultured ASM, increasing CXCL16 mRNA expression and both the secretion and surface expression of the protein (39). TNF-α and IFN-γ synergistically induce CXCL16 expression in endothelial cells (14). IFN-γ also induces CXCL16 expression in both primary non-transformed human monocytes and the monocytic cell line THP-1 (10). Furthermore, in vivo administration of IFN-γ increases CXCL16 expression in the atherosclerotic lesions in normal chow-fed apoE-null mice (10). Thus, IFN-γ is a potent inducer of CXCL16 both in vivo and in vitro. However, the molecular mechanisms involved in IFN-γ-mediated CXCL16 expression are not known. Within the 1636-bp fragment of the 5’-flanking region of the rat CXC16 gene (Fig. 3), we identified several potential transcription factor binding sites, including those for interferon regulatory factor and AP-1. Thus, we speculate that IFN-γ, which is known to regulate gene transcription through these factors (48), may also regulate CXCL16 expression by interferon regulatory factor and AP-1 in IFN-γ mediated CXCL16 induction in ASM.

IFN-γ has been indirectly implicated in IL-18-mediated CXCL16 induction in vivo (10). IL-18 induces the maturation of naïve CD4+ cells into IFN-γ-producing Th1 effector cells (49), and IFN-γ is a potent inducer of CXCL16 in a variety of cell types (14, 39). Sustained administration of IL-18 to apoE-null mice was shown to substantially increase the size of the spontaneously occurring atherosclerotic lesions compared with mice receiving vehicle alone (7). These pro-atherogenic effects were ablated in mice null for apoE and IFN-γ (7), suggesting that IFN-γ may mediate IL-18 effects. However, the results from the present study clearly demonstrate that IFN-γ is not necessary for IL-18-mediated CXCL16 induction in ASM. Using neutralizing antibodies, antisense oligonucleotide, and siRNA-mediated knockdown, we have demonstrated that IL-18 induces CXCL16 expression in ASM in an IFN-γ independent manner. IFN-γ independent effects of IL-18 have also been shown in other cell systems. Udagawa et al. (50) have shown that IL-18 inhibits osteoclast formation in vivo, and neutralization of IFN-γ failed to attenuate these inhibitory effects. Similarly, IL-18 induced activation and proliferation of natural killer cells in both wild type and IFN-γR knock-out mice (51), demonstrating the IFN-γ-independent effects of IL-18. Furthermore, our results also showed that IL-18 induces CXCL16 expression independent of IL-1β and TNF-γ, two other proinflammatory cytokines known to induce CXCL16 expression (15, 39), indicating that IL-18 is potent and direct inducer of CXCL16 expression in ASM.

Our results further show that IL-18 induces AP-1 activation and CXCL16 expression via c-Src, PI3K, Akt, and JNK activation. Mores et al. (41) have previously demonstrated that IL-18 induces vascular cell adhesion molecule expression in rheumatoid arthritis synovial fibroblasts via direct activation of Src. Using Src antisense oligonucleotides, these authors found that Src lies upstream of PI3K and Akt in the signaling pathway. In fact, Beraud et al. (52) have shown recruitment and activation of PI3K by Src. In ASM, IL-18 induced PI3K-dependent Akt...
activation, and inhibition of Src, PI3K, and Akt attenuated IL-18-mediated AP-1 activation and CXCL16 expression, indicating that IL-18 induces CXCL16 expression in c-Src, PI3K, and Akt-dependent signaling.

Proinflammatory cytokines induce AP-1 activation via p38 MAPK and JNK. IL-18 is a potent activator of p38 MAPK, ERK, and JNK activation, and all three stress-activated protein kinase/MAPKs have been shown to play a role in AP-1

activation, and inhibition of Src, PI3K, and Akt attenuated IL-18-mediated AP-1 activation and CXCL16 expression, indicating that IL-18 induces CXCL16 expression in c-Src, PI3K, and Akt-dependent signaling.
Using pharmacological inhibitors, we show here that IL-18 induced phosphorylation and activation of all three stress-activated protein kinase/MAPKs in ASMC. However, JNK, but not p38 MAPK or ERK, appears to be involved in IL-18-mediated CXCL16 induction. Although the inhibition of p38 MAPK and ERK lowers CXCL16 expression by 18 and 20%, respectively, inhibition of JNK attenuated CXCL16 expression by 46%. Furthermore, IL-18 induced c-Jun phospho-
Сylation at Ser63 and Ser73, and inhibition of JNK attenuated IL-18-mediated AP-1 activation and CXCL16 expression. Together, these results indicate that IL-18 promotes endothelial cell migration and angiogenesis in vivo independent of TNF-α (54). In contrast, IL-18 inhibits tumor growth by suppressing angiogenesis (55). IL-18 induces melanoma cell proliferation via induction of stem cell factor (45). In the present study we demonstrated that IL-18 induces SMC proliferation in a CXCL16-dependent manner. IL-18-mediated SMC proliferation is significantly inhibited by CXCL16 knockdown. However, knockdown of CXCL16 together, these results indicate that IL-18 induces CXCL16 expression via JNK and to a limited extent via p38 MAPK and ERK.

Unlike in endothelial cells (30), IL-18 failed to induce ASMC death. In fact, IL-18 induces ASMC proliferation. This is the first report demonstrating the pro-mitogenic effects of IL-18 on ASMC. Effects of IL-18 on cell survival and proliferation are cell type-dependent. IL-18 promotes endothelial cell migration and angiogenesis in vivo independent of TNF-α (54). In contrast, IL-18 inhibits tumor growth by suppressing angiogenesis (55). IL-18 induces melanoma cell proliferation via induction of stem cell factor (45). In the present study we demonstrated that IL-18 induces SMC proliferation in a CXCL16-dependent manner. IL-18-mediated SMC proliferation is significantly inhibited by CXCL16 knockdown. However, knockdown of CXCL16 did not completely block IL-18-mediated ASMC proliferation, suggesting that IL-18 might also induce other pro-survival and pro-mitogenic factors in ASMC.

Together, our results indicate that IL-18 induces CXCL16 expression in aortic smooth muscle cells via MyD88 → IRAK → TRAF6 → c-Src → PI3K → Akt → JNK → AP-1 signaling. Our results also demonstrated for the first time that IL-18 is mitogenic for ASMC in a CXCL16-dependent manner. These results suggest a role for IL-18-CXCL16 cross-talk in atherosclerosis and in restenosis after angioplasty and provide novel targets to reduce/attenuate atherosclerosis.

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