Signal Transduction Pathways Involved in Rheumatoid Arthritis
Synovial Fibroblast Interleukin-18-induced Vascular Cell Adhesion
Molecule-1 Expression

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Vascular cell adhesion molecule (VCAM-1) has been implicated in interactions between leukocytes and connective tissue, including rheumatoid arthritis (RA) synovial tissue fibroblasts. Such interactions within the synovium contribute to RA inflammation. Using phosphoinositide 3-kinase (PI3-kinase) inhibitor LY294002 and Src inhibitor PP2, we show that interleukin (IL)-18-induced ERK1/2 activation is Src kinase-dependent. Antisense (AS) c-Src oligonucleotide (ODN) treatment reduced IL-18-induced ERK1/2 expression by 32% compared with control, suggesting an upstream role of Src in ERK1/2 activation. AS c-Src ODN treatment also inhibited Akt expression by 74% compared with sense control. PI3-kinase inhibitor LY294002 or AS PI3-kinase ODN inhibited Akt expression. AS c-Src ODN inhibited Akt phosphorylation, confirming Src is upstream of PI3-kinase in IL-18-induced RA synovial fibroblast signaling. IL-18 induced a time-dependent activation of c-Src, Ras, and Raf-1, suggesting this signaling cascade plays a role in ERK activation. IL-18 directly activated Src kinase by more than 4-fold over basal levels by enzymatic assay. Electrophoretic mobility shift assay showed that activator protein-1 (AP-1) is activated by IL-18 through ERK and Src but not through PI3-kinase. In an alternate pathway, inhibition of IL-1 receptor-associated kinase-1 (IRAK-1) with AS IRAK reduced IL-18-induced expression of nuclear factor κB (NFκB). Finally, IL-18-induced cell surface VCAM-1 expression was inhibited by treatment with AS ODNs to c-Src, IRAK, PI3-kinase, and ERK1/2 by 57, 43, and 41%, and 32% compared with control sense ODN treatment, respectively. These data support a role for IL-18 activation of three distinct pathways during RA synovial fibroblast stimulation: two Src-dependent pathways and the IRAK/NFκB pathway. Targeting VCAM-1 signaling mechanisms may represent therapeutic approaches to inflammatory and angiogenic diseases characterized by adhesion molecule up-regulation.

Interleukin-18 (IL-18)1 is a proinflammatory cytokine associated with various pathological conditions including rheumatoid arthritis (RA). IL-18 induces the release of Th1 cytokines by T cells and macrophages and also stimulates the production of inflammatory mediators, such as chemokines by synovial fibroblasts or nitric oxide by macrophages and chondrocytes (1, 2). Additionally, we have shown that IL-18 acts upon endothelial cells to induce angiogenesis and cell adhesion (3, 4). IL-18 is mainly produced by activated macrophages, whereas the IL-18 receptor (IL-18R) is expressed on T lymphocytes, natural killer cells, macrophages, neutrophils, and chondrocytes (1, 5, 6). The IL-18R complex is composed of two protein chains α and β. The IL-18Rα is the extracellular binding domain of the IL-18R complex, whereas the IL-18Rβ is the signal transducing chain. When IL-18 binds to the IL-18R, it induces the formation of an IL-1R-associated kinase (IRAK)/TNF receptor-associated factor 6 (TRAF-6) complex that subsequently activates nuclear factor κB (NFκB) in Th1 cells (7) and in EL4/6.1 thymoma cells (8). IL-18 also operates through IRAK-independent pathways that remain to be elucidated. For instance, IL-18 mediates interferon-γ (INF-γ) production by the mitogen-activated protein kinases p38 and p42/44, also known as ERK1/2 (9).

Phosphatidylinositol 3-kinase (PI3-kinase) is a lipid kinase that consists of catalytic (p110) and regulatory (p85) subunits. PI3-kinase catalyzes the phosphorylation of the inositol phospholipids at position 3 to generate phosphatidylinositol 3-phosphates, phosphatidylinositol 3,4-biphosphates, and phosphatidylinositol 3,4,5-triphosphates. These phosphorylated lipid products act as second messengers, activating protein kinases such as Akt (also known as protein kinase B). PI3-kinase is activated by a large spectrum of cytokines, growth factors, and hormones (10). This activation of PI3-kinase is generally regulated by receptor tyrosine kinase and non-receptor tyrosine kinase (NRTK). PI3-kinase can also be activated by G-protein-coupled receptors or by the small GTPase Ras (11, 12). PI3-

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1 The abbreviations used are: IL, interleukin; RA, rheumatoid arthritis; VCAM-1, vascular adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; NFκB, nuclear factor κB; IRAK, interleukin-1 receptor-associated kinase-1; JNK, c-Jun N-terminal kinase; AP-1, activator protein-1; STAT, signal transducer and activator of transcription; PDTC, pyrroldine dithiocarbamate; PI3-kinase, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NRTK, nonreceptor tyrosine kinase; MeSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; RIPA, radioimmunoprecipitation; ODN, oligonucleotide; S, sense; AS, antisense; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbent assay; PTK, protein-tyrosine kinase; IL-18R, IL-18 receptor; TNF, tumor necrosis factor; TRAF, TNF-receptor-associated factor 6; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PI, phosphatidylinositol; RBD, Ras binding domain.

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kinase has been implicated as a key signaling molecule for transcription factor activation, protein synthesis, angiogenesis, and cell adhesion (13, 14). We recently showed that the PI3-kinase inhibitor LY294002 inhibited RA synovial fibroblast IL-18-induced VCAM-1 expression by 50% when used alone and by 85% when used with the NfκB inhibitor pyrrolidine dithiocarbamate (2). These results suggested the existence of at least two independent pathways involved in IL-18-induced adhesion molecule expression.

Further investigation of IL-18-induced signaling mechanisms revealed involvement of Src, an NRTK that functions in ligand-induced cellular responses, such as leukocyte survival, adhesion, migration, and proliferation. Src has also been implicated in various cancers and in bone resorption (15). Activation of Src requires phosphorylation at its activation site Tyr-418 concomitantly with decreased phosphorylation at its negative regulatory site Tyr-529; regulation also involves phosphorylation on other residues such as Tyr-215. c-Src activation by tyrosine kinase receptors leads to translocation from the plasma membrane to the cytoskeleton, where Src interacts with a host of proteins that orchestrate cell-matrix adhesion and cell migration (16). Here we found that IL-18 directly activates Src with rapid kinetics, and Src activation appears to be an early event common to the PI3-kinase/Akt and ERK1/2 pathways.

There is precedence for the involvement of transcription factor activation in IL-18-mediated immune and inflammatory functions. For instance, IL-18 activates AP-1 and NfκB in the Jurkat T cell leading to IL-2 expression (17). We therefore examined involvement of the PI3-kinase and ERK1/2 signaling pathways in AP-1 activation. Because many cytokine-mediated functions, including IL-18-induced IFN-γ expression, are regulated via NfκB activation (18, 19), its significance in IL-18-induced VCAM-1 expression was examined. One approach for determining the role of the NfκB pathway is by way of IRAK involvement, because IRAK is known to be associated with NfκB upon IL-18 stimulation (20, 21). Furthermore, IRAK was initially cloned and characterized as a kinase associated with the IL-1 receptor (22), suggesting its importance to IL-18 signaling as IL-18 is a member of the IL-1 family.

Because antisense oligodeoxynucleotides (ODNs) offer a potential gene therapy strategy to block transcription or translation of specific genes, antisense ODNs to relevant signaling molecules were employed to examine RA synovial fibroblast signaling events following IL-18 stimulation. Ultimately, the treatment effect of antisense ODN on IL-18-induced VCAM-1 expression implicated specific signaling molecules involved in RA synovial fibroblast VCAM-1 expression. The ability of these antisense ODNs to inhibit IL-18-induced activation of the transcription factor NfκB and downstream VCAM-1 expression was also assessed.

We examined the signal transduction mechanisms by which IL-18 induces VCAM-1 expression in RA synovial fibroblasts. Our findings demonstrate that IL-18 induced VCAM-1 expression through Src kinase, PI3-kinase/Akt, and ERK1/2 pathways. The important role of the IRAK/NfκB pathway in VCAM-1 expression was also elucidated. Finally, we describe a new signaling cascade involving Src/Ras/Raf/ERK/AP-1 in IL-18-stimulated RA synovial fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human IL-18 (specific activity 4.1 × 10⁴ units/mg) was purchased from Peprotech (Rocky Hill, NJ). Orthovannadate, para-nitrophosphosphate, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, dimethyl sulfoxide (Me₂SO), IGEPA-C 630, protein A- and G-agarose, pertussis toxin, and phosphatidylinositol were bought from Sigma. Protease inhibitor mixture tablets were obtained from Roche Molecular Biochemicals. Modified radioimmunoprecipitation (RIPA) lysis buffer was prepared according to Upstate Biotechnology, Inc., protocol, with final concentrations being Tris-HCl (50 mM, pH 7.4), Nonidet P-40 (1%), NaCl (150 mM), EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), aprotinin/leupeptin/pepsstatin (1 μg/ml each), NaF (1 mM). The anti-Src-agarose beads, clone G11, a monoclonal mouse anti-human phospho-ERK1/2 antibody, and the Ras activation detection kit were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), as was Src kinase and the Src kinase assay kit, including Src kinase reaction buffer (SrcRB), Src substrate peptide, Src mammalian ATP mixture, and P81 phosphocellulose paper. The radioisotope [γ-³²P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life Sciences. Phosphoric acid (0.75%) diluted from 85% stock solution and trisodium citrate acid was from Sigma. LY294002, PD98059, SB203580, and PD98059 were purchased from Calbiochem. Monoclonal mouse anti-human VCAM-1, clone 4B9 that recognizes domain 1 of VCAM-1, was a generous gift from Dr. Roy Lobb (Biogen, Cambridge, MA); mouse IgG1 antibody (negative control) was purchased from Coulter Clone (Hialeah, FL); goat anti-mouse PE (Jackson ImmunoResearch) was used as secondary antibody for flow experiments. Mouse monoclonal anti-phospho-nuclear factor activation (clone 4G10), mouse monoclonal anti-human phospho-ERK1/2 antibody, and the Ras activation detection kit were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit anti-human phospho-Src antibody (Tyr(P)⁴¹⁸) and polyclonal rabbit anti-human phospho-Raf-1 (Tyr(P)²⁵⁹) and Tyr(P)²⁵⁹ antibody were obtained from BIOSOURCE International (Camarillo, CA). Monoclonal anti-phospho-Src antibody (Src Tyr(P)⁴¹⁸) was purchased from Cell Signaling Technology (Beverly, MA) and BIOSOURCE International. Mouse polyclonal anti-human IRAK antibody was obtained from BD PharMingen, and rabbit polyclonal anti-human NFκB p65 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-tubulin antibody was obtained from Oncogene Research Products (Boston, MA). Goat anti-rabbit IgG horseradish peroxidase-conjugated antibody was purchased from Sigma. Protein estimation reagents (BCA kit) were from Pierce. Enhanced chemiluminescence Western blotting detection reagents and sheep anti-mouse IgG horseradish peroxidase-conjugated antibody were purchased from Amersham Biosciences. LipofectAMINE and LipofectAMINE Plus™ Reagents were obtained from Invitrogen.

**Cell Culture**—Fibroblasts were isolated from synovium obtained from RA patients meeting the American College of Rheumatology criteria for RA who had undergone total joint replacement surgery or synovectomy (23). Fresh synovial tissues were minced and digested in solution of dispase, collagenase, and DNase. Cells were used at passage 5 or older, at which time they were a homogeneous population of fibroblasts. Synovial fibroblasts were grown in 175-mm tissue culture flasks (Falcon, Franklin Lakes, NJ) at 37 °C, in a humidified atmosphere with 5% CO₂. Upon confluence, cells were passaged by brief trypsinization as described previously (24).

**Cell Lysis and Immunoblotting**—RA synovial fibroblasts were plated onto 6- or 10-cm Petri dishes (Falcon) at 1 × 10⁸ cells/ml and allowed to adhere for 24 h at 37 °C in 5% CO₂ atmosphere. Alternatively, for ODN experiments, fibroblasts were plated at 4 × 10⁶ cells/ml in 6-well plates. Fibroblasts were serum-starved for at least 14 h before stimulation with IL-18 (10 ng/ml) for 0, 5, 10, and 20 min. At the end of each period, supernatants were gently aspirated, and fibroblasts were lysed in extraction buffer containing 100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₃PO₄, 2 mM Na₂VO₃, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (1 tablet/10 ml). For experiments with signaling inhibitors, RA synovial fibroblasts were preincubated with the respective inhibitor for 60–120 min before activation with IL-18. For the ODN experiments the cells were treated as detailed and lysed similarly. Nuclei were pelleted (1250 × g at 4 °C for 5 min), and supernatants of different samples were collected for determination of protein content using a BCA protein assay kit. Cell lysates were mixed 1:1 with Laemmli’s sample buffer, boiled for 5 min, and then centrifuged at 10,000 × g for 10 min. Equal amounts (or 15 μg) of each sample was subjected to 10% SDS-PAGE. Separated proteins were electrophoretically transferred from the gel onto nitrocellulose membranes using a semi-dry transblotting apparatus (Bio-Rad). To block nonspecific binding, membranes were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Blots were incubated in respective primary antibody in TBST + 5% milk at 4 °C overnight. After washing with TBST, blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10,000) or with goat anti-rabbit IgG (1:10,000) for 1 h at room temperature. An ECL detection system (Amersham Biosciences) was used to detect specific protein bands. Differential bands were then...
scanned and quantitated using the software UN-SCAN-IT version 5.1 (Silk Scientific, Orem, UT). Blots were subsequently stripped and re transfomed with antibody to tubulin to determine relative band densities.

**PI3-Kinase Assay**—RA synovial fibroblasts (8 × 10⁵ cells) were plated on 10 cm-dishes in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin. Once cells were 80%–90% confluent, they were further incubated in serum-free RPMI 1640 for at least 14 h. RA synovial fibroblasts were then stimulated with IL-18 (10 nM) for 0, 10, and 20 min at 37°C. At the end of the incubation, cell lysate was prepared. Protein content of each sample was quantitated using a BCA protein assay kit and normalized according to the protein concentration. 500 µl of each sample in 500 µl of lysis buffer was incubated overnight at 4°C with 5 µl of rabbit anti-PI3-kinase antibody directed against the 85-kDa regulatory subunit. 60 µl of protein A-agarose conjugate (50% slurry in PBS) was added to each sample and further incubated for 1 h at 4°C. The immunoprecipitates were collected by centrifugation at 14,000 × g for 10 s. The immunoprecipitates were then washed 3 times with buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1 mM sodium orthovanadate) containing 1% nonionic detergent IGE-PAL CA-630, followed by 3 washes with buffer B (0.1 M Tris-HCl, pH 7.4, 5 mM lithium chloride, and 0.1 mM sodium orthovanadate) and 3 washes with TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA) containing 0.1 mM sodium orthovanadate. To each sample, the following reagents were added sequentially: 50 µl of TNE, 10 µl (20 µg) of phosphatidylinositol (PI, in 10 mM Tris-HCl, pH 7.4 containing 1 mM MgCl₂), and 10 µl of 100 µM MgCl₂. The PI reaction was initiated with addition of 5 µl of [γ-³²P]ATP. The reaction mixture was incubated at 37°C for 15 min with continuous agitation. The reaction was stopped by addition of 20 µl of 6 N HCl. Radiolabeled lipid was extracted from the reaction sample by addition of 160 µl of CH₂Cl₂/MeOH (1:1), vortexing, and separating the organic and aqueous phases by centrifugation for 10 min at 14,000 × g. 50 µl of radiolabeled lipid containing the lower organic phase were spotted onto oxalate-treated TLC plates (Fisher) and developed in CHCl₃/MeOH/H₂O/NH₄OH (6:47:11.3:2). TLC plates were dried and autoradiographed.

**Ras Activation Assay**—Ras activation was used as a Ras activation kit (Upstate Biotechnology, Inc.). RA synovial fibroblasts were stimulated with IL-18 (10 nM) for different times. At each time point, synovial fibroblast extracts were prepared with cell lysis buffer, and protein content in each sample was quantitated. The Ras activation assay involved two steps. In the first step, the activated form of Ras was immunoprecipitated from 800 µg of each synovial fibroblast lysate sample with an immobilized Raf-1-Ras binding domain (Raf-1-RBD) and assayed by Western blotting as described above. The presence of activated Ras in samples was then detected by probing with a specific mouse monoclonal anti-Ras antibody (1 µg/ml). Different bands were then scanned and quantitated using an imaging densitometer.

**Src Kinase Activation Assay**—Src kinase was first immunoprecipitated with agaroase beads conjugated with anti-Src antibody (mouse monoclonal antibody (MAb)). RA synovial fibroblasts were plated 10⁵ cells on 10-cm culture dishes, adhered overnight in complete media, serum-starved overnight, and stimulated with IL-18 (10 nM) for 10 min. Cells were washed in cold PBS and lysed with 0.5 ml of RIPA buffer. The total cell lysate was diluted to 1 µg/µl with PBS, and 1 mg was mixed with 4 µg (8 µl) of anti-Src antibody-conjugated agaroase beads and gently rocked at 4°C for 2 h. Beads were washed by microcentrifuging (5 s at 14,000 × g), the supernatant was drained, and beads were then washed (3 times) with ice-cold PBS. Samples containing agaroase beads, Src antibody, and Src protein in complex were then used to determine Src kinase activation.

Direct activation of Src was examined with a Src kinase assay kit (Upstate Biotechnology, Inc.) to measure the ability of activated Src to act on known substrate. Stock solutions necessary for the assay were prepared as follows: Src substrate peptide (600 µM, diluted in SrcRB), purified Src kinase (p60 c-Src, 20 units/10 µl/test), [γ-³²P]ATP (PerkinElmer Life Sciences, 1 mCi/100 µl, 3000 Ci/mmol, further diluted to 1 µCi/µl with Src manganese/ATP mixture), and 0.075% phosphoric acid (diluted from 5% with PBS). Substrate peptide (10 µl, 150 µM final concentration) was added to 10 µl of SrcRB, to which was added 10 µl of Src (1000 c-Src, 20 units/10 µl), immunoprecipitated with ODN (200 µg minimum) and 10 µl of diluted [γ-³²P]ATP (10 µCi) in a microcentrifuge tube. The reagent mixture was incubated for 10 min at 30°C with agitation. To precipitate peptides, 20 µl of 40% trichloroacetic acid was added to each mixture and incubated at room temperature for 5 min. Onto the center of 8’1 phosphocellulose paper squares, 25 µl of each sample was spotted. Squares were washed with 0.75% phosphoric acid (5 times for 5 min) and acetone (once for 3 min). Samples were read in a scintillation counter, and counts/min of immunoprecipitated enzyme samples were compared with counts/min of the background control samples (no enzyme). This assay was performed with fibroblasts from 4 different RA donors.

**Preparation of Oligonucleotides and Lipofection of RA Synovial Fibroblasts**—Sequences of the ODNs employed in this study are listed in Table I. Antisense ODNs were selected for sequence target to c-Src (25), IRAK-1 (26), PI3-kinase (27, 28), and ERK1/2 (29). The corresponding sense ODN was used as control for each antisense ODN. The ODNs were synthesized and purified by the Northwestern University Biotechnology Laboratory and modified with phosphorothioate. Lipofection-encapsulated ODNs were prepared using LipofectAMINE and LipofectAMINE Plus Reagent from Invitrogen. Each ODN was reconstituted to 0.4 µg/µl concentration in double distilled water and then diluted in Opti-MEM media as detailed. For transient transfection of cells, ODN/plus reagent-LipofectAMINE complex was prepared with 40 µg of ODN/100 µl of serum-free Opti-MEM with 2.5 µl of Plus reagent and 4 µg of LipofectAMINE Reagent/100 µl of serum-free Opti-MEM and incubation at room temperature for 30 min, followed by dilution with another 800 µl of Opti-MEM serum-free medium for an additional 15-min incubation. RA synovial fibroblasts were plated at 90% confluency at 4 × 10⁵ cells per well on 6-well plates or per 6-cm dish and allowed to adhere in RPMI, 10% FBS, 1% penicillin and streptomycin. After attachment, cells were then treated with 5 µM of antisense or sense ODN by incubation with 1 ml of ODN/plus reagent/ LipofectAMINE complex for 5 h, followed by media change to complete RPMI, 10% FBS, 1% penicillin and streptomycin overnight. The media were also changed to serum-free RPMI, 1% penicillin and streptomycin for 8–10 h prior to stimulation with IL-18 (10 nM, 10 min, for the Western blot and Src kinase experiments; 5 nM, 8 h, for VCAM-1 expression by flow cytometry experiments).

**Flow Cytometry**—For the chemical inhibition experiments, RA synovial fibroblasts were plated onto 6-cm Petri dishes (Falcon) at 1 × 10⁵ cells. For the chemical inhibition experiments, RA synovial fibroblasts were plated onto 6-cm Petri dishes (Falcon) at 1 × 10⁵ cells.**TABLE I**

| ODN          | Sequence (5'-3') |
|--------------|-----------------|
| Anti sense IRAK-1 | CCC CCC GCC CAT GCC TGC |
| Sense IRAK-1    | GCA GCC ATG GCC GGG GGG |
| Anti sense c-Src | GTA CTT GCT TTT GCT GCT CAT CCC CAT |
| Sense c-Src     | ATG GGG AGC AGC AAG AGC AAG CCC |
| Anti sense PI3-kinase | GTA CTT GTA CCC CTC AGC ACT CAT |
| Sense PI3-kinase | AGT AGC GGT GAG GGG TAC CAG TAC |
| Anti sense ERK1/2 | GGG GCC GCC GGC GCC AT |
| Sense ERK1/2    | ATG GCC GGC GCC GGC GC |

**Fig. 1.** IL-18 activates PI3-kinase in RA synovial fibroblasts. Serum-starved cells were stimulated for the indicated times with IL-18 (10 nM). Extracts were subjected to immunoprecipitation with a rabbit antibody against the p85 subunit of PI3-kinase. PI3-kinase activity was determined using an in vitro kinase assay as described under "Experimental Procedures." Products were separated by thin layer chromatography. Activity of PI3-kinase is presented as production of phosphatidylinositol phosphate (PIP), indicated by arrows. Experiments were repeated two times with essentially identical results.
IL-18 (10 nM) for 8 h. Inhibitors (LY294002, PP2, SB203580, and PD98059) or control in RPMI 1640, 2% fetal calf serum before stimulation with IL-18 (5 nM).

For the ODN experiments, RA synovial fibroblasts were pertussis toxin (100 ng/ml) for 12 h before stimulation with IL-18 (10 nM). Cells were plated and treated as detailed above prior to IL-18 (5 nM) stimulation for 8 h. Cells were harvested with a cell scraper and transferred to a fluorescence-activated cell sorting (FACS) tube (BD PharMingen). Cells were then treated with mouse anti-VCAM-1 or isotype-matched control (5 μg/ml) as primary antibody followed by incubation for 30 min with PE-conjugated goat anti-mouse antibody (1.5 μg/ml). Samples were washed twice with PBS, 1% FBS, and then fixed with 1% paraformaldehyde. Samples were assayed using an Epics XL-MCL flow cytometer (Beckman Coulter). Prior to data acquisition, the PE channel was standardized using fluorescent beads (Rainbow Beads, Spherotech, Libertyville, IL). Isotype-matched control values were subtracted from the test results.

Cellular ELISA—RA ST fibroblasts were plated on 96-well tissue culture plates in RPMI 1640, 10% fetal calf serum and adhered for 14 h. Cells were preincubated with specific inhibitors or MeSO vehicle control in RPMI 1640, 2% fetal calf serum before stimulation with IL-18 (5 nM) for 8 h. Inhibitors (LY294002, PP2, SB203580, and PD98059) or vehicle MeSO were applied to cells for 60 min, and then cells were stimulated with IL-18 (5 nM) for 12 h. Cell viability was judged by trypan blue exclusion and was >90%. RA synovial fibroblasts were successively fixed in 3.7% formalin in PBS and blocked in PBS, 1% bovine serum albumin, 5% goat serum for 15 min. After successive incubations in mouse anti-human VCAM-1 or isotype-matched control for 2 h, goat anti-mouse IgG peroxidase-conjugated antibody was added for 1 h, and the ELISA was developed with tetramethylbenzidine substrate. The reaction was stopped with 1 N H2SO4 before reading at 450 nm with a Bio-Rad model 550 microplate reader.

Electrophoretic Mobility Shift Assay—RA synovial fibroblasts (1 x 10⁶) were grown on 10-cm dishes (Falcon) and starved for more than 12 h in serum-free RPMI 1640, 1% penicillin/streptomycin, before stimulation with 10 nM of IL-18 for 0, 1, 2, and 4 h. For experiments using specific inhibitors, cells were pretreated for 2 h before stimulation with IL-18 (10 nM). Cytoplasmic and nuclear extracts were prepared as described previously (4), and 10 μg of nuclear extract from each sample was incubated with 1.5 μg of poly(dI-dC) and 47 fmol of 32P-end-labeled ODN probe containing the DNA-binding site for AP-1 (Promega, Madison, WI). Cold ODN competition control was performed using labeled and unlabeled ODNs at a ratio of 1:2 and 1:4. Protein-DNA complexes were resolved on 4% polyacrylamide gels and visualized by autoradiography.

Statistical Analysis—Data were analyzed using the Wilcoxon rank order test, and p values less than 0.05 were considered statistically significant.

RESULTS

IL-18 Promotes PI3-kinase Activation—We have shown previously (4) that the PI3-kinase inhibitor LY294002 (10 μM) inhibits RA synovial fibroblast VCAM-1 expression. These results suggested PI3-kinase might be involved in IL-18-induced VCAM-1 expression. To confirm activation of PI3-kinase by IL-18, we employed an in vitro kinase assay using phosphatidylinositol (PI) as a substrate (Fig. 1). IL-18 (10 nM) stimulation cells/ml and allowed to adhere overnight, and cells were pretreated with pertussis toxin (100 ng/ml) for 12 h before stimulation with IL-18 (10 nM) for 8 h. For the ODN experiments, RA synovial fibroblasts were plated and treated as detailed above prior to IL-18 (5 nM) stimulation for 8 h. Cells were harvested with a cell scraper and transferred to a fluorescence-activated cell sorting (FACS) tube (BD PharMingen). Cells were then treated with mouse anti-VCAM-1 or isotype-matched control (5 μg/ml) as primary antibody followed by incubation for 30 min with PE-conjugated goat anti-mouse antibody (1.5 μg/ml). Samples were washed twice with PBS, 1% FBS, and then fixed with 1% paraformaldehyde. Samples were assayed using an Epics XL-MCL flow cytometer (Beckman Coulter). Prior to data acquisition, the PE channel was standardized using fluorescent beads (Rainbow Beads, Spherotech, Libertyville, IL). Isotype-matched control values were subtracted from the test results.

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IL-18 Promotes PI3-kinase Activation—We have shown previously (4) that the PI3-kinase inhibitor LY294002 (10 μM) inhibits RA synovial fibroblast VCAM-1 expression. These results suggested PI3-kinase might be involved in IL-18-induced VCAM-1 expression. To confirm activation of PI3-kinase by IL-18, we employed an in vitro kinase assay using phosphatidylinositol (PI) as a substrate (Fig. 1). IL-18 (10 nM) stimulation
IL-18 Activates Phosphorylation of Akt through PI3-Kinase—
The lipid products of PI3-kinase are known to activate serine-/threonine protein kinase Akt through phosphorylation of serine 473 and threonine 308 (30). We therefore examined the ability of IL-18 to induce Akt phosphorylation by probing Western blots of proteins from IL-18-stimulated RA synovial fibroblasts using antibodies to phosphorylated Akt (serine 473). IL-18 induced Akt phosphorylation in a time-dependent manner (Fig. 2, A and B), and after 5 min there was a 4-fold increase in Akt activation, with maximum activation (11-fold) observed at 20 min ($p < 0.05$, $n = 3$). This increase in Akt phosphorylation could be blocked when RA synovial fibroblasts were preincubated with the PI3-kinase inhibitor LY294002 (10 $\mu$m) (Fig. 2, C and D). Pretreatment resulted in $-80 \pm 17\%$ (mean $\pm$ S.E.) decrease in Akt phosphorylation ($p < 0.05$, $n = 3$). The role of PI3-kinase in Akt activation was more specifically demonstrated through inhibition with PI3-kinase AS ODN.

Antisense PI3-Kinase Inhibits RA Synovial Fibroblast Akt Expression—To examine involvement of the Akt pathway in IL-18 signaling through a more specific approach, antisense ODN targeting PI3-kinase and corresponding control sense ODN were used to treat RA fibroblasts with subsequent IL-18 (10 nM) stimulation. Levels of Akt expression were determined by Western blot of total cell lysates. IL-18 (10 nM) stimulated Akt protein expression from nearly undetectable basal levels. To confirm the above data and, more importantly, to demonstrate specificity of inhibition by antisense ODN, treatment with antisense PI3-kinase ODN reduced the level of Akt protein expression almost completely versus treatment with corresponding sense ODN, 100 $\pm$ 17.92 to 1.03 $\pm$ 0.11 ($p < 0.05$, $n = 4$), which is consistent with the expectations for Akt as a target of PI3-kinase-activated signals (Fig. 2, E and F).

IL-18 Activates the Nonreceptor Protein Tyrosine Kinase c-Src—We next investigated signaling pathways involved in PI3-kinase/Akt activation. Because PI3-kinase is generally activated by protein-tyrosine kinase (PTK), we examined PTKs activated by IL-18 using a mouse monoclonal antibody (4G10), which specifically reacts with tyrosine-phosphorylated proteins. IL-18 (10 nM)-induced protein tyrosine phosphorylation in RA synovial fibroblasts in a time-dependent manner, showing maximum activation at 10 and 20 min by Western blot. Two major bands were detected at 50–75- and 35–50-kDa ranges (Fig. 3A). Proteins phosphorylated at tyrosine residues corresponding to molecular mass ranges of 60–65 and 42–44 kDa are Src kinases and MAPK, respectively. Like PI3-kinase, Src kinases are located near the cell membrane, and their activation occurs relatively early in the signaling sequence (31).
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FIG. 4. IL-18 directly activates Src kinase in RA synovial fibroblasts, and antisense c-Src ODN treatment inhibits IL-18-induced Akt phosphorylation. A, serum-starved RA synovial fibroblasts were stimulated for 10 min with IL-18 (5 nM). Cell lysates were prepared with RIPA buffer, immunoprecipitated with anti-Src antibody-conjugated agarose beads, and then assayed for Src kinase activity in the presence of peptide substrate, [γ-32P]ATP and manganese/ATP mixture. Data from five RA donors were averaged and are represented as mean ± S.E., expressed as fold increase relative to nonstimulated control; *, p < 0.05; n = number of donors. B, antisense c-Src ODN inhibits Akt expression. RA synovial fibroblasts were treated with antisense (AS) c-Src ODN or the corresponding control sense (S) ODN and incubated with or without IL-18 (10 nM) for 20 min. Total cell lysates were prepared for equal protein loading and separation by Western blot. Akt was detected with mouse monoclonal anti-human phospho-Akt antibody (\(\text{p-Akt}\)). Data from four RA donors were scanned and analyzed for quantification with the UN-SCAN-IT software, and band intensities for Akt were normalized to tubulin to quantitate relative Akt protein expression and presented as the mean ± S.E.; *, p < 0.05; n = number of donors; S, sense ODN; AS, antisense ODN.

Moreover, it has been demonstrated that c-Src recruits and activates PI3-kinase in NIH3T3 fibroblasts (32). Thus, we examined the effect of IL-18 stimulation on Src activation by Western blot and found that IL-18 induces the phosphorylation of Src in a time-dependent manner (p < 0.05, n = 3). As compared with nonstimulated RA synovial fibroblasts, a 4-fold increase in phosphorylated Src was observed at 10 and 20 min (Fig. 3, B and C).

**IL-18 Activates Src Kinase in RA Synovial Fibroblasts**—A direct role for IL-18 activation of Src was examined by an enzymatic assay. Confluent RA synovial fibroblasts were stimulated with IL-18 (10 nM) or control (media alone) for 20 min; their total lysates were collected and immunoprecipitated to isolate Src; and the Src kinase assay was performed with Src substrate peptide and [γ-32P]ATP. Results represent fold increase in counts/min versus non-stimulated control cells. IL-18 (10 nM) significantly induced Src activation greater than a 4-fold over basal activation (4.35 ± 1.92) (mean ± S.E.) in the four different RA patient samples examined (p < 0.05) (Fig. 4A).

**Antisense c-Src ODN Inhibits RA Synovial Fibroblast Phospho-Akt Expression**—To examine the involvement of the Akt pathway in IL-18 signaling, antisense ODN targeting of the gene for c-Src and corresponding control sense ODN were used to treat cells with subsequent IL-18 (10 nM) stimulation. The level of phospho-Akt expression was determined by Western blot of total cell lysates. Again, IL-18 (10 nM) stimulated phospho-Akt protein expression from nearly undetectable basal (or non-stimulated) levels, similarly to the data in Fig. 2E. Antisense c-Src ODN caused a reduction in the level of phospho-Akt expression, apparently through specific reduction in c-Src (Fig. 4, B and C). Antisense c-Src ODN inhibited phospho-Akt expression by 75%, from 100 ± 15.13 to 25.7 ± 10.96 (mean ± S.E.) (p < 0.05, n = 4). This finding suggests that not only does IL-18-induced phospho-Akt expression occur through the activation of c-Src but that this phospho-Akt expression can be inhibited by c-Src blockade. With the knowledge that Akt is a downstream substrate of PI3-kinase activity, which was confirmed above to validate the use of antisense PI3-kinase ODN, these results showing Akt down-regulation via c-Src ODN treatment support the critical position of Src upstream of PI3-kinase/Akt.

**IL-18-induced ERK1/2 Activation Is PI3-Kinase-independent but Src-dependent**—Other studies have shown a role for PI3-kinase in signaling ERK activation in a number of cell systems (33, 34). Hence, we looked at the kinetics of ERK activation in IL-18-stimulated RA synovial fibroblasts by Western blot and found a time-dependent response, which was maximal at 20 min (p < 0.05, n = 3) (Fig. 5, A and B). To determine whether ERK1/2 activation was dependent on Src kinases, we studied the effect of the Src kinase inhibitor PP2 on IL-18-induced ERK1/2 activation. PP2 (25 μM) strongly inhibited ERK1/2 activation (Fig. 5C). To assess the role of PI3-kinase in ERK activation by IL-18, cells were preincubated with LY294002, a potent inhibitor of PI3-kinase. LY294002 did not change IL-18-mediated ERK stimulation suggesting PI3-ki-
nase is not involved in the signaling route leading to ERK activation (Fig. 5D).

**Antisense c-Src ODN Treatment Inhibits RA Synovial Fibroblast IL-18-induced ERK1/2 Expression**—IL-18 (10 nM) greatly enhanced ERK1/2 protein expression by about 25–35-fold from basal levels (2.83 ± 1.51 to 3.72 ± 0.1) in RA synovial fibroblasts. To determine whether treatment with antisense ERK1/2 ODN specifically inhibits target gene expression, ERK1/2 protein in antisense or sense ERK1/2 ODN-treated cells stimulated with IL-18 (10 nM) was detected by Western blot analysis. Antisense ERK1/2 ODN inhibited IL-18-induced ERK1/2 protein expression (Fig. 5E). The results showed a mean of 85% inhibition of ERK1/2 protein expression by antisense ERK1/2 ODN, whereas sense ERK1/2 ODN was unable to block this expression in IL-18-stimulated cells, 15.13 ± 7.78 versus 100 ± 9.95 (mean ± S.E., p < 0.05, n = 4) (Fig. 5F). To examine the effect of c-Src inhibition on IL-18-induced ERK1/2 expression in RA synovial fibroblasts, cells were treated with antisense or sense c-Src ODN prior to IL-18 (10 nM) stimulation. Antisense c-Src ODN treatment inhibited ERK1/2 protein expression by 32%, whereas sense c-Src ODN was unable to block ERK1/2 protein expression in IL-18-stimulated cells, 68.43 ± 18.18 versus 100 ± 15.29 (mean ± S.E.) (p < 0.05, n = 4) (Fig. 5, E and F).

**IL-18 Activates the Ras/Raf Pathway**—A number of studies have shown Raf kinase is a predominant MEK kinase which, in turn, is activated by binding to Ras-GTP (35). Src family kinases have also been shown to be linked to MAPK through Ras/Raf kinases (36, 37). To study the activation of Ras/Raf kinases, we first immunoprecipitated activated Ras with a Raf-1-Ras binding domain (RBD) conjugated to agarose beads and subsequently probed with mouse monoclonal anti-Ras antibody. IL-18 stimulation of RA synovial fibroblasts showed a time-dependent activation of the Ras/Raf pathway with maximal activation at 10 and 20 min (p < 0.05, n = 3) (Fig. 6A). We confirmed activation of Raf by IL-18 using an antibody specific for phosphorylated Raf-1 (p < 0.05, n = 3) (Fig. 6B).

**IL-18 Activates AP-1 through ERK but Not PI3-Kinase**—Because in our previous study inhibitors of the transcription factor NF-kB were only able to reduce IL-18-induced VCAM-1 expression by 50%, we explored whether other signaling pathways and transcription factors might be involved in induction of VCAM-1 transcription. AP-1 was an appropriate candidate because AP-1 is activated by PI3-kinase and MAPK and has also been involved in the induction of adhesion molecule gene transcription (38, 39). By using gel shift assays, we show that DNA binding of AP-1 was potently induced by IL-18 at 1 h (Fig. 7A). The specificity of the AP-1-DNA binding complexes was
confirmed by the fact that their formation could be eliminated by the addition of a 4 M excess of unlabeled ODN (Fig. 7B). This AP-1 binding to DNA was completely inhibited in RA synovial fibroblasts pretreated with PD98059, but had no effect in cells pretreated with LY294002 or PDTC, and had a partial effect with PP2 pretreatment (Fig. 7C). PDTC served as a control due to its known absence of effect on AP-1 (40).

Antisense IRAK-1 ODN Inhibits IRAK-1 Gene Expression—Initial studies involved specificity determination to validate that the antisense ODN could inhibit expression of its corresponding protein. To verify that antisense IRAK-1 ODN inhibits target gene expression, IRAK-1 protein in ODN-treated cells stimulated with IL-18 (10 nM) was detected by Western blot analysis. The expression of tubulin was examined as the housekeeping protein, and optical density measurement of the band representing the protein of interest was normalized to that of tubulin to quantitate relative protein expression (Fig. 8). Antisense IRAK-1 ODN inhibited IL-18-induced IRAK-1 protein expression. Western blot analysis showed 76% inhibition of IRAK-1 protein expression by antisense IRAK-1 ODN, whereas sense IRAK-1 ODN was unable to block IRAK-1 protein expression in IL-18-stimulated cells, 24.11 ± 1.23 versus 100 ± 18.1 (p < 0.05, n = 4). To demonstrate further the relative specificity of the antisense IRAK-1 ODN, as a control, cells were also treated with antisense or sense c-Src ODN. c-Src ODNs were also unable to block IRAK-1 protein expression, which was comparable with control (no ODN). As shown, IL-18 clearly up-regulated IRAK-1 expression, whereas basal levels by non-stimulated control cells were not readily detectable in these Western blots.

Antisense IRAK-1 ODN Inhibits RA Synovial Fibroblast IL-18-induced NF-κB Activation—IL-18 (10 nM) significantly stimulated NF-κB activation from basal levels of 25.7 ± 3.4 to 100 ± 7.5, a 4-fold increase. This IL-18-induced NF-κB activation was inhibited by antisense IRAK-1 ODN treatment for 5 h. The expression of NF-κB was decreased by 32.9% from control expression of 100 ± 7.5 to 67.12 ± 6.6 (p < 0.05, n = 4) (Fig. 9). There was no difference between antisense and sense ODN nonstimulated control cells. Again, as a control, cells were also treated with antisense or sense c-Src ODN, neither of which was able to block IL-18-induced NF-κB activation, with similar values of 100 ± 10.8 and 101.75 ± 3.2, although both were increased from basal levels of 32.37 ± 4.4 and 25.44 ± 2.4, respectively.

Signaling Mechanisms Involved in RA Synovial Fibroblast VCAM-1 Expression—To examine signaling pathways involved in RA synovial fibroblast VCAM-1 production, we first tested the effect of different concentrations of inhibitors of PI3-kinase, Src kinase, and ERK on IL-18-induced VCAM-1 expression by cell ELISA. The advantage of cell ELISA to examine the effect of inhibitors is the requirement of only a small number of cells. Thus, we were able to compare the effect of several concentrations of different inhibitors concurrently on IL-18-induced VCAM-1 expression. In the literature, effective concentrations used for the specific inhibitors tested are between 10 and 100 μM. The PI3-kinase inhibitor (LY294002) and the Src inhibitor

![Time-dependent Ras and c-Raf activation in RA synovial fibroblasts stimulated with IL-18.](image)

**A** Time-dependent Ras and c-Raf activation in RA synovial fibroblasts stimulated with IL-18. Serum-starved RA synovial fibroblasts were stimulated with IL-18 for various time points as indicated. Cells were extracted with cell lysis buffer, and the protein content in each sample was estimated. Each sample (100 μg) was immunoprecipitated with a Raf-1-RBD-agarose conjugate and subsequently subjected to 10% SDS-PAGE. A, the presence of activated Ras in the samples was then detected by probing with a specific mouse monoclonal anti-Ras antibody (1 μg/ml). B, phospho-c-Raf (“p-raf-1”) was detected by direct Western blot analysis. Blots were stripped and reprobed with mouse anti-human tubulin. CD, in each case, blots were scanned and analyzed for quantification, and data from three RA donors were averaged and are represented as the mean ± S.E., expressed as fold increase with respect to nonstimulated (NS) cells. *, p < 0.05 versus time 0; n = number of donors.
PP2 at 25 μM significantly decreased IL-18-induced VCAM-1 expression by 32 and 35% (0.329 ± 0.06 OD for Me2SO versus 0.223 ± 0.04 OD for LY294002, and 0.212 ± 0.03 OD for PP2, each value representing the mean ± S.E., p < 0.05). The inhibition was greater with 50 μM of these inhibitors, 43 and 46%, respectively (data not shown). Interestingly, an inhibitor of ERK (PD98059) at these concentrations did not have an effect on IL-18-induced VCAM-1 expression measured by cell ELISA, which appeared to suggest the lack of ERK involvement, but results of inhibition with antisense ODN described below showed otherwise. Because studies have suggested that G proteins can activate PI3-kinase and Src (41, 42), we also examined the potential role of G proteins in IL-18-induced VCAM-1 expression. RA synovial fibroblasts were pretreated with the Go/i protein inhibitor, pertussis toxin, before stimulation with IL-18. The expression of VCAM-1 on RA synovial fibroblasts was measured by FACS analysis and was not different for cells in the presence or absence of pertussis toxin (data not shown).

We further examined the effect of inhibiting these signaling pathways by transfecting the RA synovial fibroblasts with antisense or sense ODN to block gene translation/transcription directly through targeting c-Src, PI3-kinase, IRAK, and ERK1/2. The ODN-treated cells were stimulated with IL-18 (5 nM) for 8 h and analyzed by flow cytometry to examine the treatment effect on VCAM-1 surface expression. Results represent expression of VCAM-1 after antisense ODN treatment relative to control sense ODN treatment (Fig. 10). Antisense c-Src ODN treatment resulted in a 57% decrease in VCAM-1 expression (43.23 ± 9.61% versus 100% for control sense ODN, p < 0.05, n = 4). Antisense IRAK ODN treatment reduced VCAM-1 expression by 43% (57.13 ± 10.66% versus 100% for control sense ODN, p < 0.05, n = 5). Likewise, treatment with antisense ERK1/2 ODN and antisense PI3-kinase ODN resulted in a 41 and 32% decrease in VCAM-1 expression compared with control sense ODN (59.41 ± 14.48 and 68.14 ± 16.07% versus 100% control, respectively, p < 0.05, n = 5 for each).

**Fig. 7.** Time-dependent AP-1 DNA binding activity in IL-18-stimulated RA synovial fibroblasts and inhibition of AP-1 nuclear activation by PD98059. A, RA synovial fibroblasts were stimulated with IL-18 (10 nM) for various time points as indicated. Nuclear extracts were prepared and analyzed for AP-1 binding activity by electrophoretic mobility shift assay. AP-1 denotes the specific AP-1-DNA complexes. B, competition experiments used 2- and 4-fold molar excess of unlabeled ODN in nuclear extracts of RA synovial fibroblasts stimulated for 1 h with 10 nM IL-18. C, serum-starved RA synovial fibroblasts were pretreated with specific inhibitors LY294002, PD98059, PP2 (25 μM), or Me2SO vehicle control for 2 h before stimulation with IL-18 (10 nM) for 1 h. For PDTC, cells were pretreated for 8 h at 300 μM. Nuclear extracts were analyzed by EMSA. Experiments were repeated two times with essentially identical results. LY corresponds to LY294002, PD to PD98059, PDTC to pyrrolidine derivative of dithiocarbamate; M = molar.

**Fig. 8.** Antisense IRAK-1 ODN inhibits IL-18-induced IRAK-1 protein expression. A, RA synovial fibroblasts were treated with antisense (AS) IRAK-1 ODN or the control sense (S) ODN and incubated with or without IL-18 (10 nM) for 20 min. Lysates were prepared with lysis buffer, and equal amounts of total protein were separated by 10% SDS-PAGE and transferred to nitrocellulose. IRAK-1 was detected with mouse polyclonal anti-human IRAK antibody. B, data from four RA donors were scanned and analyzed for quantification with the UN-SCAN-IT software, and band intensities for IRAK-1 were normalized to tubulin and presented as the mean ± S.E.; *, p < 0.05; n = number of donors. Antisense and sense c-Src ODN treatment effect on IRAK-1 was analyzed similarly as shown.
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Because the PI3-kinase inhibitor partially blocked IL-18-induced VCAM-1 expression, we hypothesized IL-18 could stimulate PI3-kinase activity. Indeed, IL-18 potently stimulated PI3-kinase activity with rapid kinetics. The serine-threonine protein kinase Akt is one of the major targets of PI3-kinase-generated signals (43). In our experiments, we showed that Akt becomes phosphorylated after IL-18 stimulation and that the PI3-kinase-specific inhibitor LY294002 could inhibit such stimulation. This was confirmed by nearly complete abrogation of IL-18-induced Akt activation through antisense ODN treatment.

Interestingly, IL-18-induced Src phosphorylation was temporally correlated with PI3-kinase and Akt activation. Experiments using antisense c-Src ODN further confirmed the up-stream position of Src in this pathway. Our data indicate that the initial event in the IL-18 response is Src activation, which leads to activation of PI3-kinase and ERK1/2. Our observations appear to support this hypothesis because the Src kinase inhibitor PP2 strongly inhibits IL-18-induced ERK activation, and inhibition with c-Src antisense ODN significantly reduced ERK1/2 expression. Moreover, the Src inhibitor PP2 and the PI3-kinase inhibitor LY294002 have very similar effects on IL-18-induced VCAM-1 expression, suggesting that Src and PI3-kinases belong to the same signaling cascade. However, ERK1/2 and PI3-kinase/Akt are probably independent of each other, based on the failure of the PI3-kinase inhibitor to alter IL-18-mediated ERK1/2 activation.

Our results are consistent with several lines of evidence suggesting that the ERK1/2 and PI3-kinase signaling pathways do not cross-talk (54, 55). Kalina et al. (9) reported previously that IL-18 activates ERK1/2 in the natural killer cell...
FIG. 11. A schematic model of signaling pathways involved in IL-18-induced VCAM-1 expression in RA synovial fibroblasts. IL-18 directly activates Src kinase, which in turn activates two different and independent pathways Ras/Raf-1/ERK and PI3-kinase/Akt. PI3-kinase mediates IL-18-induced VCAM-1 transcription but not likely via AP-1. PI3-kinase probably interacts with other IL-18-inducible signals such as IRAK/TRAF-6/NFκB to induce a full expression of IL-18-induced VCAM-1. Up-regulation of IL-18-induced VCAM-1 involves ERK1/2, PI3-kinase, c-Src, and IRAK. IL-18 stimulation leads to IRAK recruitment, subsequent NFκB activation, and translocation to the nucleus. Shown are regulators of NFκB: TRAF-6, NFκB inducing kinase (NIK), inhibitory kB kinase (IKK), and inhibitory kB (IκB). Dashed arrows correspond to the mechanisms that remain to be clarified, and complete arrows correspond to the known mechanisms.

We next investigated downstream events of PI3-kinase/ Akt and ERK1/2 activation. Our previous work (4) suggested that transcription factors other than NFκB existed that may be involved in IL-18-induced VCAM-1 expression. AP-1 was of particular interest for three reasons. First, AP-1 is activated by PI3-kinase and ERK (45, 62, 63). Second, AP-1 regulates the transcription of adhesion molecules (38, 39). Third, AP-1 is an important transcription factor in RA synovial fibroblasts (64). The data showing that IL-18 induces AP-1 binding activity made this hypothesis plausible and is in accord with previous studies reporting AP-1 activation by IL-18 in different cell types (17, 65, 66). Although the EMSAs only demonstrate AP-1 binding and cannot provide information on its phosphorylation status, our data using specific inhibitors suggest that, unlike PI3-kinase, ERK1/2 regulates AP-1 activation in IL-18-stimulated synovial fibroblasts. For PI3-kinase, the signaling event downstream of Akt remains unanswered. STAT3 might be an interesting candidate because IL-18, c-Src, and PI3-kinase have all been shown to stimulate STAT3 (9, 67, 68). Interestingly, the nuclear translocation of STAT4 is strictly dependent on the presence of IL-18 for IL-12/IL-18-induced IFN-γ-mediated nitric oxide production by mouse peritoneal macrophages (69).

Finally, we showed that PI3-kinase is involved in IL-18-induced VCAM-1 expression. In the literature, there are conflicting reports regarding the effect of PI3-kinase on adhesion molecule expression. Radisavljevic et al. (70) showed that vascular endothelial growth factor up-regulates ICAM-1 expression via a PI3-kinase/Akt pathway in endothelial cells. In contrast, using the same stimulus and a similar cell type, Kim et al. (71) reported an up-regulation of ICAM-1, VCAM-1, and E-selectin expression by PI3-kinase inhibition. The experimental conditions including the different concentrations and types of PI3-kinase inhibitors used by these and other authors may explain this discrepancy. In our hands, blocking PI3-kinase with LY294002 had a repressive effect on IL-18-induced RA synovial fibroblast VCAM-1 expression using two different techniques (FACS and cell ELISA). More specific inhibition with PI3-kinase antisense ODN treatment was confirmatory. These results are consistent with the known properties of PI3-kinase and VCAM-1. Indeed, we and others (13, 14, 72, 73) have implicated these two molecules in cell adhesion and angiogenesis. Moreover, we showed in recent reports (4) that IL-18 mediates leukocyte-fibroblast adhesiveness and stimulates endothelial cell tube formation in vitro and in vivo (3).

Hence, it is possible that PI3-kinase may be a key signaling molecule responsible for IL-18-induced VCAM-1 synthesis, and subsequently cell adhesion and angiogenesis.

As with PI3-kinase, selective ERK1/2 inhibition with antisense ODN treatment decreased IL-18-induced VCAM-1 expression. However, ERK inhibitor PD98059 did not show significant inhibition, which perhaps was due to the lack of ERK inhibition at the concentrations used without affecting viabil-
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ity. Thus, the use of antisense ODN to block signaling molecules specifically may be more appropriate for investigating these pathways and for developing potential therapeutic strategies, at least in the case of ERK1/2. In addition, antisense ODNs may be an alternative to avoid the toxicity that prevents these chemical inhibitors from becoming optimal therapeutics. Taken together, our observations support the perspective that specifically targeting PI3-kinase (71), or perhaps ERK1/2, may represent a valid approach to blocking adhesion molecule expression, cell adhesion, and angiogenesis. Furthermore, that c-Src has a signaling role in IL-18-induced VCAM-1 expression is consistent with the findings that c-Src activation by IL-18 lies upstream to the induction of PI3-kinase and ERK1/2, both of which we showed to affect VCAM-1 expression.

Additionally, the IRAK/NFκB pathway, distinct from the Src-dependent pathways, appears to play a significant role in RA synovial fibroblast VCAM-1 expression. Indeed, antisense IRAK ODN treatment inhibited IL-18-induced VCAM-1 expression. This finding is in accordance with the fact that the IRAK/NFκB signaling pathway has been implicated in various IL-18 functions. IL-18 binding to its receptor induces IRAK recruitment, and IRAK activation leads to nuclear translocation of NFκB and subsequent expression of immune response genes, such as IFN-γ in Th1 cells (20). This NFκB activation is diminished in fibroblasts from IRAK-deficient mice, although additional IL-1 and IL-18 cytokine responses are independent of the IRAK pathway (21), which could explain Src-mediated pathways. IRAK is reportedly necessary for NFκB and JNK activation in response to IL-18, as in the mutant cell line I1A, which lacks IRAK. In addition, IRAK recruitment and phosphorylation, in addition to IRAK recruitment and phosphorylation, of which we showed to affect VCAM-1 expression. This pathway has been implicated in various IL-18 signaling in rheumatoid arthritis synovial fibroblasts.

The essential role of NFκB was shown by Kojima et al. (21), who demonstrated that NFκB activation by blocking PI3-kinase with antisense ODN.

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