We previously reported tumor necrosis factor-α (TNF) modulates transcriptional and post-transcriptional down-regulation of macrophage scavenger receptor (MSR) (Hsu, H. Y., Nicholson, A. C., and Hajjar, D. P. (1996) J. Biol. Chem. 271, 7767–7773); however, TNF-mediated signaling mechanisms are unknown. Here, we demonstrate that ligation of TNF receptor stimulates activity of p21-activated protein kinase (PAK) and mitogen-activated protein kinases (MAPK) as follows: ERK, JNK, and p38 in murine macrophage J774A.1 cells. Upon activation of protein kinases (PK), TNF rapidly increases MSR message and protein; later it markedly reduces MSR expression. Studies using PK inhibitors and dominant negative constructs demonstrate phosphatidylinositol 3-kinase/Rac1/PAK/JNK and phosphatidylinositol 3-kinase/Rac1/PAK/p38 pathways contribute to important roles in the late stage of TNF down-regulation of MSR expression and taking up of OxLDL. Alternatively, the PKC/MEK1/ERK pathway in the early stage plays a significant role in up-regulation of the MSR gene. By using anti-TNF-R1 agonist antibody, we further confirm TNF-R1-mediated MAPK in regulation of MSR. Furthermore, in MSR gene promoter-driven luciferase reporter assays with TNF, PKC activator increases, but antioxidant N-acetylcysteine, PK inhibitors, and dominant negative constructs decrease luciferase activity in MSR gene promoter-transfected cells. Our current results show the first evidence of crucial roles for TNF-mediated MAPK pathways in the transcriptional regulation of MSR gene and increase MSR expression; in contrast, with TNF longer treatment the pathways down-regulate MSR and foam cell formation probably via post-transcriptional process.

Tumor necrosis factor-α (TNF), a 17-kDa polypeptide cytokine, is derived from the TNF precursor, a 26-kDa membrane-bound polypeptide via processing of TNF-converting enzyme (2). TNF is produced mainly by activated mononuclear phagocytes, including activated macrophages and T cells (3–5), in response to inflammation, infection, and various environmental stimulations. TNF exerting a wide array of biological effects is known to be mediated through two distinct surface binding sites, a type I receptor (TNF-R1, p55–60 kDa) expressed on all cell types, and a type II receptor (TNF-R2, p75–80 kDa) expressed primarily on immune cells and macrophages (6). These two receptors bind TNF with high affinity but differ in their intracellular domains and thus mediate distinct cellular responses.

Ligation of TNF receptor stimulates signal transduction pathways involving PI 3-kinase, PKC, MEK1, Rac1/Cdc42, PAK, ERK, JNK, and p38, etc. in various cells (7–9). The role of TNF receptor-associated factors in TNF-mediated transducing signals has been extensively examined. For example, an important molecule known as TNF receptor-associated factor 2 (TRAF2), one of the more than 15 distinct proteins, has been identified that indirectly associated with the cytoplasmic domains of the TNF-R1 via TRADD. During the past 5 years, it has been reported that TRAF2 involves a battery of TNF-mediated signal transductions and cellular functions. Specifically, at least four proteins are associated with the cytoplasmic domains of TRAF2 that mediate rapid activation of MAPKs including JNK (10), p38 (11), and transcription factor NF-κB (12). In addition, another important signal transduction cascade including PKC, MEK1, and ERK can be activated through another TNF receptor-associated protein, FAN. Moreover, FAN binds to TNF-R1 at amino acids 307–321 and activates neutral sphingomyelinase activity (13). Although TNF-R1 and TNF-R2 are independently activated in TNF signalings, the large majority of TNF-induced signaling events can be mediated solely by TNF-R1 (14). Both types of TNF receptor are expressed on murine macrophage J774A.1 cells (15), and we are interested in investigating the biological function and molecular mechanism for TNF receptor-mediated signaling pathways in macrophages relevant to atherosclerosis.

Inflammatory cytokine TNF derived from activated macrophage cell surface receptors induces the production of various cytokines, chemokines, and adhesion molecules, and promotes an inflammatory response. It has been shown that TNF stimulates the expression of a number of proinflammatory genes in cells, including iNOS, TNF, and IL-1β. TNF also enhances the transcription of a number of other genes, including IL-12, IL-6, and IL-8. In addition, TNF induces the expression of several adhesion molecules, such as E-selectin, ICAM-1, and VCAM-1. These adhesion molecules are crucial for the recruitment of leukocytes to inflamed sites.

In summary, our findings provide new insights into the role of TNF in regulating MSR expression and foam cell formation. This study highlights the importance of understanding the signaling pathways involved in TNF-mediated regulation of MSR and foam cell formation. Further research is needed to elucidate the molecular mechanisms underlying these processes and to develop potential therapeutic strategies for the prevention and treatment of atherosclerosis.
phages plays an important role in the development of atherosclerosis (5, 16–18). Specifically, TNF involved in the fatty-streak formation of atherogenesis directly and indirectly leads to the formation of advanced, complicated lipid-laden lesions of atherosclerosis. Atherosclerotic related vascular diseases are the major causes of morbidity and mortality in the industrialized countries of the world (19). Extensive epidemiological studies in the development of human atherosclerosis, particularly the pleiotropic process in atherogenesis, remains not fully defined (16, 18). The plasma lipoproteins, particularly modified forms of low density lipoproteins (LDL) including oxidized LDL (OxLDL), are mainly accumulated in macrophage-derived foam cells adjacent to atherosclerotic plaque (20). Massive accumulation of foam cells in intimal blood vessels is recognized as the main damage-generating source and one of the main reasons for the development of atherosclerosis (16, 18, 22, 23); in addition to acting as the principal, inflammatory mediator of cells in the atherosclerotic plaque, they also function as scavenger cells of OxLDL. Following their migration from the blood and transformation into lipid-laden foam cells, monocyte-derived macrophages play a multifaceted role in lesion development (16, 18, 21, 24, 25). Although the mechanisms by which macrophages transformed into foam cells are not fully characterized, both macrophages and macrophage-derived foam cells overexpress macrophage scavenger receptors class A (MSR) type I and II that mediate high affinity binding and internalization of OxLDL (24–26). In vivo and in vitro studies indicate MSR aberrantly taking up OxLDL, leading to lipid-accumulated macrophages and further transforming into foam cells (18). Overexpression of MSR on macrophage-derived foam cells taking up oxidized LDL in human atherosclerotic lesion has been observed (18, 25–27), implicating the receptor plays a critical role in the development of atherosclerosis.

Both TNF production and MSR overexpression in activated macrophages and macrophage-derived foam cells and the effect of TNF on MSR expression and function during atherosclerosis remain less known and studied. Specifically, the autocrine effect of TNF on MSR including activation of the signaling molecule, protein kinase, and the biological reaction related to atherosclerosis have yet to be investigated. In our previous studies, we demonstrated that TNF down-regulates MSR gene expression and protein via transcriptional and post-transcriptional processes (1). In addition, the studies of cycloheximide, a protein synthesis inhibitor in reversal of the destabilizing effects of TNF on MSR message, suggest TNF-induced expression of protein(s) that accelerated MSR mRNA degradation (1).

In this report, we examined further TNF-mediated signal transductions in the regulation of MSR expression and function related to foam cell formation. Specifically, the objective of this study was to evaluate systematically the activity of TNF-mediated MAPKs (ERK, JNK, and p38) during TNF stimulation of murine macrophage J774A.1 cells; moreover, the role of these MAPKs in the regulation of MSR expression and function were dissected. By using specific antagonism of the PKC and MEK1 with calphostin C and PD98059, respectively, in the PKC/MEK1/ERK pathway, we found that the two inhibitors blocked TNF-induced MSR expression at an early stage of TNF incubation. However, they had no reversal effect on TNF inhibition of MSR expression and function at late stage. In contrast, dominant negative mutant of JNK and specific antagonism of p38 with SB203580 reversed TNF reduction of MSR expression and function in foam cell formation. The findings support the involvement of JNK and p38 in regulating the reduction of MSR expression followed by ligation of the TNF receptors. In addition, by using a specific anti-mouse TNF-R1 agonist anti-body (anti-TNF-R1 Ab), we further demonstrated that anti-TNF-R1 Ab effectively regulates MSR gene expression as well as stimulates MAPK activity (ERK, JNK, and p38) which is similar to the activity of TNF ligation-mediated transducing signals. Collectively, our current results and our previous report (1) together with the findings that TNF-R1 protects against atherosclerotic lesion development in the TNF-R1-null (p55-null) mice (17) strongly support the concept that TNF-mediated signal transduction pathways play important roles in regulation of MSR, which are relevant to the development of atherogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**

Murine macrophage J774A.1 cells were obtained from the ATCC (Manassas, VA). In this paper, the murine macrophage J774A.1 cell is referred as J774A.1.1 cell. Human blood monocyte-derived macrophages used here were isolated from the blood of healthy persons obtained from Taiwan Blood Bank (Taipei, Taiwan).

**Materials**

Disposable tissue culture materials were purchased from Corning Glass. Medium RPMI 1640, t-glutamin, penicillin, streptomycin, fetal bovine serum (FBS), LipofectAMINE PLUS, reagent, and BRL 100-bp DNA ladder were purchased from Life Technologies, Inc. EGTA, leupeptin, aprotinin, and DNA molecular weight markers were obtained from Roche Molecular Biochemicals. Sodium orthovanadate, HEPES, NaCl, glycerol, Triton X-100, MgCl2, phenylmethylsulfonyl fluoride, phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (fraction V), trypsin blue, curcumin, and N-acetylcycteine (NAC) were purchased from Sigma. For cell free cell formation assay, Oil Red O, hematoxylin, formaldehyde, and propylene glycol were also purchased from Sigma. 2,7′-dichlorofluorescein diacetate (DCFH) was obtained from Molecular Probes, Inc. (Eugene, OR). Immobilon® polyvinylidene difluoride membrane was purchased from Millipore Inc. (Bedford, MA). DuPont Western blot Chemiluminescence Reagent, Renaissance®, a non-radioactive light-emitting system, and 10 Ci/mmol (γ-32P)ATP were purchased from PerkinElmer Life Sciences. 3′,5′-cyclic GMP was purchased from PROtech Technology Co. (Taipei, Taiwan). GeneAmp® RNA PCR kit for RT-PCR amplification was purchased from PerkinElmer Life Sciences. For growth factors and antibodies, human recombinant TNF and polyclonal goat anti-mouse TNF-R1 agonist antibodies were obtained from R & D Systems (Minneapolis, MN); the concentration used in this work was 150 units/ml. Rabbit polyclonal anti-macrophage scavenger receptor-A antibody, which recognizes the collagen-like domain of MSR protein, was generated by the synthetic peptide for polyclonal antibody, and a gift of hSRII anti-macrophage scavenger receptor-A peptide antibody from Dr. T. Kodama (University of Tokyo, Japan) was used as a positive control. Monoclonal antibody anti-C36 was purchased from Sigma. Anti-PAR, rabbit polyclonal IgG, anti-rabbit IgG-HRP, anti-mouse IgG-HRP, and protein G plus-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The kinase assay kits, p44/42 MAP Kinase Assay Kit, SAPK/JNK Assay Kit, and p38 MAP Kinase Assay Kit were purchased from New England Biolabs, Inc. (Beverly, MA). For the protein kinase inhibitors, PD98059 was from New England Biolabs; calphostin C and herbimycin A were from Calbiochem; and wortmannin was from Sigma. For oligonucleotides, primers for MSR-A type I (MSR), manganese superoxide dismutase (MnSOD), and glyceraldehyde-phosphate dehydrogenase (GAPDH) were synthesized from local MD Bio. Inc. (Taipei, Taiwan). The dominant negative JNK construct (DN-JNK) was a gift from Dr. Michael Karin (University of California, San Diego, CA) (28). The dominant negative Rac1 construct (DN-Rac1) was a gift from Dr. S. Bagrodia (Cornell University, Ithaca, NY) (29).

**Isolation of LDL and Preparation of OxLDL**

Human LDL (d = 1.019–1.063 g/ml) was prepared as described (1) and occasionally screened for peroxides and endotoxins. The preparation for OxLDL followed the published method as described previously (1). Briefly, LDL (about 2.5 mg of protein) in 137 mM NaCl, 2.7 mM KCl, 7.9 mM sodium phosphate, pH 7.4, and 60 μg/ml kanamycin (buffer A) was incubated with 5 μM CuSO4 at 37 °C for 48 h. OxLDL was dialyzed against 150 mM NaCl and 0.3 mM EDTA (pH 7.4) at 4 °C for another 48 h and then filtered through a 0.45-μm Millipore membrane.
RNA Isolation
Total RNA was isolated from J774A.1 cells by using RNeasy® C&T as per manufacturer’s instruction, quantitated by AgNano and Ag2000, stored as 1 μg/μl at −80 °C, and used within 1 week. The isolated RNA was monitored by the intactness of some constitutively expressed gene GAPDH.

RT-PCR Amplification
The RT-PCR was used as described (1) to detect the expression of MSR and MnSOD, respectively, in J774A.1 cells, as follows. RT-PCR was mixed with 0.5 μl of 50 units/μl murine leukemia virus, 4 μl of 2.5 mM dNTP, 0.5 μl of 20 units/μl RNase inhibitor, 1 μl of 10× reaction buffer, 2 μl of 25 mM MgCl₂, 0.5 μl of 100 mM DTT, and 0.5 μl of 50 μM random hexamer (PerkinElmer Life Sciences). The reaction was performed at 42 °C for 30 min, at 99 °C for 5 min, and then cooled down to 4 °C for 30 min or overnight, and the product cDNA was stored at −20 °C for future PCR use.

PCR—1.5 μl of cDNA was mixed with 1.25 μl of 10× reaction buffer, 0.5 μl of 2.5 mM dNTP, 0.8 μl of 25 mM MgCl₂, 0.125 μl of 100 mM DTT, 0.25 μl of 10 pmol/μl 5′-primer and 3′-primer, and 0.125 μl of 5 units/μl AmpliTaq, then water was added to 12.5 μl for the reaction. The condition for MSR are as follows: for step 1, 94 °C, 2 min; for step 2, 94 °C, 20 ± 5; 93 °C, 20 °C, 2 min, for 35 cycles; for step 3, 72 °C, 10 min. The condition for MnSOD are as follows: for step 1, 94 °C, 2 min; for step 2, 94 °C, 2 min; 56 °C, 1 min; 72 °C, 1 min, for 35 cycles; for step 3, 72 °C, 10 min. The products from the PCR were examined by either 1 or 2% agarose gel electrophoresis with ethidium bromide (EtBr), and normalized by comparison to RT-PCR of mRNA of GAPDH, a constitutively expressed gene. Each EtBr-stained band was quantified using ImageQuant® software from a PhosphorImager® from Molecular Dynamics (Sunnyvale, CA).

Assay of Macrophage Scavenger Receptor Protein on Cell Membrane
The method for isolation of membrane protein is described elsewhere (31) with modifications. Briefly, J774A.1 cells were grown in 100-mm plates to 80% confluency, washed with PBS twice, and 0.5 ml of collection buffer (CB: 20 mM HEPES, 250 mM sucrose, 5 mM EGTA, 5 mM EDTA, 2 μg/ml aprotonin, 1 μg/ml pepstatin A, 2 μg/ml leupeptin) was added to each plate. Cells were scraped by rubber policeman and collected into a 1.5-ml tube and broken by the sonication method (on, 3 s; off, 2 s, for 5 min). The broken cells were centrifuged at 100,000 × g for 1 h, and we saved the pellet but discarded the supernatant. 300 μl of cell lysis buffer (CB: +1 Triton X-100) was added to the pellet, mixed well, and put on and ice bath at 4 °C for 30 min. The lysed pellet was centrifuged at 100,000 × g for 30 min, and the supernatant of membrane protein containing scavenger receptor protein was collected and stored at −80 °C for further Western blotting analysis.

Western Blotting Analysis
The methods for immunoblotting are described previously (32). Briefly, cell lysates of J774A.1 cells (25 μg of protein or as indicated) were solubilized in 10 or 2% SDS-PAGE buffer. After electrophoresis, proteins on the gel were transferred to Immobilon® polyvinylidene difluoride membrane via wet transfer blotting system (Blot Electro- phoretic Transfer Cell, Bio-Rad; transfer buffer: 25 mM Tris base, 192 mM glycine, 0.1% SDS, and 20% methanol at 250 mA for 100 min). The membranes were blocked with blocking buffer (PBS + 0.1% Tween 20 + 1% bovine albumin, Sigma A-7030) at room temperature for 1–2 h with slow shaking. Then the membranes were immunoblotted with primary antibody, rabbit polyclonal anti-macrophage scavenger receptor antibody (dilution ratio 1:1,000 with 1% bovine albumin). After blotting, the membranes were washed in washing buffer (PBS + 0.1% Tween 20, without 1% bovine albumin) once for 15 min followed by two washes for 5 min. The membranes were blocked again in a new blocking buffer for 1 h at room temperature with slow shaking and then immunoblotted with appropriate secondary antibodies in the same blocking buffer for 1 h at room temperature with slow shaking. The secondary antibody for MSR, a goat anti-rabbit IgG conjugated with horseshadish peroxidase, dilution ratio 1:1,500 with 1% bovine albumin, was used. Afterward, membranes were further washed in washing buffer once for 15 min followed by two washes for 5 min. Protein visualization on each immunoblot was developed and performed with Renaissance®, DuPont Western blot Chemiluminescence Reagent, from PerkinElmer Life Sciences as described previously (32), and manufacturer-provided protocols were followed.

Assay of ERK, JNK, and p38 in TNF-treated J774A.1 Cells with Protein Kinase Inhibitors

Cell Line Preparation—Cells were incubated with TNF or with TNF and additional chemicals, e.g., curcumin (inhibitor of PI-3 kinase), curcumin (inhibitor of JNK), and SB203580 (inhibitor of p38) as described. At the indicated times, cells were washed twice with ice-cold PBS (without Ca²⁺ and Mg²⁺) and harvested in cell lysis buffer (LB: 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 205 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were left on ice for 10 min and scraped into a 1.5-ml tube, shaken for 15 s, and centrifuged (12,000 × g) at 4 °C for 10 min. The protein concentration of supernatant was determined, and the rest of the supernatant was saved at −80 °C for various MAPK activity assays.

Immunoprecipitation—The methods for immunoprecipitation were as described previously with some modification (32) or as described in the New England Biolabs protocols. Briefly, cell lysates stored at −80 °C as described above were normalized for protein concentration as follows: 1) for ERK activity, 200 μg of protein samples; 2) for JNK activity, 250 μg of protein samples; 3) for p38 activity, 200 μg of protein samples, respectively, and at various times were diluted with LB to 20 μg, incubated with the corresponding antibody as follows: 1) 15 μl of immobilized phospho-p44/42 MAPK (i.e., ERK, Thr-202, and Tyr-204 monoclonal antibody, 50% slurry); 2) 20 μl (2 μg) of e-Jun (1–89) fusion protein beads; and 3) 20 μl of resuspended immobilized phospho-p38 MAPK (Thr-190/Tyr-182) monoclonal antibody under shaking at 4 °C for 1 h. The next day the immunocomplex was washed three times with 1× Western lysis buffer (LB: 25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 μM Na₃VO₄, 10 mM MgCl₂) and then resuspended in 1× KB for the specific kinase activity assay.

In Vitro MAP Kinase Activity Reaction—The manufacturer-provided protocols were followed. Briefly, 1) for ERK activity, we resuspended the immunocomplex with 50 μl of KB in the presence of 200 μM cold ATP and 2 μg of Elk-1 fusion protein at 30 °C for 30 min; 2) for JNK activity, we resuspended the active JNK-c-Jun complex with 50 μl of KB in the presence of 100 μM cold ATP at 30 °C for 30 min; 3) for p38 activity, we resuspended the immunocomplex with 50 μl of KB in the presence of 200 μM cold ATP and 2 μg of ATP-2 fusion protein and incubated at 30 °C for 30 min. The individual reaction was terminated by addition of 25 μl of 3× sample buffer (SB: 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue, 5% 2-mercaptoethanol).

Analysis of MAP Kinase Activity—The activity was determined by Western blotting analysis and the chemiluminescence method as described above. Specifically, 1) for ERK activity, the Elk-1 phosphorylation was measured by Western blotting of nonradioactive labeled samples using phospho-Elk-1 (Ser-383) antibody. 2) For JNK activity, the phosphorylation of c-Jun at Ser-63 was measured by Western blotting of nonradioactive labeled samples using phospho-c-Jun (Ser-63) antibody. 3) For p38 activity, the phosphorylation of ATP-2 at Thr-71 was measured by Western blotting of nonradioactive labeled samples using phospho-ATP-2 (Thr-71) antibody. The individual reaction was detected by Phototope®-HRP Western Detection System via chemiluminescence. Similarly, the inhibitory effect of a specific protein kinase inhibitor on individual protein kinase was carefully monitored and examined.

Quantification of MAP Kinase Activity—Measurement by Phosphor-Image® of phospho-Elk-1 (Ser-383), of phospho-c-Jun (Ser-63), and of phospho-ATP-2 (Thr-71) for each sample was by using ImageQuant® software from Molecular Dynamics. The relative activity of each treated sample was expressed as comparison with untreated J774A.1 cells (control cells, at t = 0 or at indicated time), i.e., the relative MAPK activity = MAPK activity of treated sample/MAPK activity of untreated sample.

Transient Transfection of DN-JNK and Assay of MSR Expression in DN-JNK upon TNF Treatment

The conditions for growing murine macrophage J774A.1 cells to be transfected were identical to those of regular J774A.1 cells, except cells were sub-passaged the day before transfection, and fresh medium was used. The transient transfection of DN-JNK (at 10 μg of DNA per 100-mm plate) into J774A.1 cells was conducted by using the Lipo-fectAMINE PLUS® reagent (Life Technologies, Inc.) method according to the protocol from the manufacturer. Occasionally, co-transfection with β-galactosidase to monitor the transfection efficiency was performed.
formed, and the assay followed the protocol supplied by Promega. Transfected cells were treated with TNF or various reagents as indicated, and cells were harvested after 24 and 48 h. The MSR expression detected by RT-PCR was measured as described above.

**Assay of p21 PAK Activity in J774A.1 Cells**

A regular radioactive protein kinase assay was used for PAK activity measurement. Since it contains radioactive materials and reactions, all steps were restricted and followed the regulations and rules for radioactive material, as follows.

**Cell Lysate Preparation**—The method in PAK activity was identical to that used under “Assay of MAPK Activity in J774A.1 Cells” as described above.

**Immunoprecipitation**—The method for immunoprecipitation was similar to that used under “Assay of MAPK Activity in J774A.1 Cells” as described with some modifications. A 200–μg protein of cell lysates from various times were diluted with LB to 200 μl, incubated with 2 μg of anti-PAK antibody at 4 °C, under shaking for 8 h, and then 20 μl of protein G was added, incubated at 4 °C, and shaken for additional 2 h. The immunoprecipitated active PAK was microcentrifuged (12,000 × g) at 4 °C for 15 min, and the discarded supernatant and pellet were washed twice with 1× LB and 1× kinase buffer (KB: 25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 10 mM MgCl2), respectively, and then resuspended in 1× KB for kinase activity assay.

**In Vitro PAK Activity Reaction**—The published protein kinase protocol was followed (33). Briefly, we resuspended the immunocomplex with 14.5 μl of KB containing 10 μM cold ATP and 5 μg of myelin basic protein (MBP) with an additional 1 μl of [γ-32P]ATP (5 μCi, 330 μM) and incubation at 30 °C for 15 min. The reaction was terminated by the addition of 25 μl of 3 times SB as above.

**Analysis of PAK Activity**—The PAK activity determined by Western blotting was similar to that used under “Assay of MAPK Activity” as described above with some modifications. Briefly, after finishing electrophoresis, the gel was stained with Coomassie Blue staining solution (0.025% Coomassie Brilliant blue, 40% methanol and 7% acetic acid) for 1 h and then destained with destaining solution (Destain I 40% methanol and 7% acetic acid; Destain II: 5% methanol and 7% acetic acid) overnight, and then the gel was dried.

**Quantification of PAK Activity**—The dried gel was put into PhosphorImager scanning® of STORM 840 (Molecular Dynamic Co., CA) was used to identify the phosphorylated MBP and measure the PAK activity by ImageQuant® software from Molecular Dynamics. The relative activity of each treated sample was expressed as comparison with untreated J774A.1 cells (i.e., control cells, at t = 0 or at the indicated times), for example, relative PAK activity = PAK activity of treated sample/PAK activity of untreated sample.

**Foam Cell Formation Assay**

By using Oil-Red O staining method (34, 35), foam cells derived from J774A.1 cells were detected. Briefly, cells were treated as indicated, and 50 μg/ml OxLDL was added to the tested samples and incubated at 37 °C for 5 h. After incubation, the medium was removed, and cells were fixed with 6% paraformaldehyde in PBS for 30 min, rinsed with propylene glycol for 2 min, and 5 mg/ml Oil Red O was added to cover the cell and left overnight. The next day, Oil Red O stain was removed, and the cells were stained with hematoxylin for 45 s, and the background stain was washed out with 6% paraformaldehyde, and then pictures were taken. We counted ten microscopic fields, and we compared the cell number with and without staining. The foam cell formation number is calculated by the ratio of Oil Red O-stained cells to Oil Red O-nonstained cells; the relative foam cell formation number is the treated (treated) foam cell formation number/the untreated (control) foam cell formation number.

**Calcium Phosphate Transfection and Luciferase Assay**

Human embryonic kidney 293 T cells were sub-passaged, plated to 6-well plates the day before transfection, and fresh medium was used. The calcium phosphate transfection method is described elsewhere (36). Briefly, the plasmids of macrophage scavenger receptor promoter in luciferase reporter gene, such as plasmids HACLDL Xba-A1-luc promoter and HACLDL Xba-A1-luc Enhancer, gifts from Dr. C. Glass, University of California, San Diego (37), were co-transfected with β-galactosidase to monitor the transfection efficiency. Transfected cells were treated with various reagents such as NAC and protein kinase inhibitors and co-transfected with DN-JNK as indicated. The luciferase activities of transfected 293 T cells were measured according to the method as described (36). The β-galactosidase assay followed the protocol supplied by Promega.

**Measurement of TNF-induced Intracellular ROS**

TNF-induced intracellular ROS was measured by a fluorometer, Cytofluor (Millipore, MA), via the method of detecting the intensity of DCFH as described (38). Briefly, J774A.1 cells grown in serum-free phenol red-free RPMI medium for 24 h, and cells were incubated with 10 μM/liter DCFH (Molecular Probes®) at 37 °C for 15 min in the dark, followed by treatment with various reagents, e.g., TNF, NAC, and PMA. The relative fluorescence intensity of fluorophore DCF, which was formed by peroxide oxidation of its nonfluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a Cytofluor. DCFH with culture medium was used as a blank control.

**Protein Assay**

Protein amounts were determined by Bio-Rad protein assay.

**Statistical Analysis**

Statistical differences between the experimental groups were examined by analysis of variance, and statistical significance was determined at p < 0.05. The experiments were conducted three times or as indicated, and all data are expressed as mean ± S.E.

**RESULTS**

**Effect of TNF on MSR Gene Expression and Function on Murine Macrophage J774A.1 Cells**—RT-PCR analyses of expression of MSR mRNA (in this report, MSR indicates MSR class A type I) on murine macrophage J774A.1 cells were examined. For cells treated with TNF (150 units/ml), analyses were conducted between 4 and 8 h, and mRNA of MSR increased more than 2-fold as compared with the control cells. After 12 h, MSR message reduced to 75% of the control cells; after 24 h, it markedly decreased to about 30% of control samples (Fig. 1A). In this report, however, we focused on the effect of TNF on MSR class A type I; a similar alteration trend for MSR class A type II expression in TNF-treated J774A.1 cells was observed (data not shown). Similar results from Northern blot analyses of TNF on MSR message in J774A.1 cells were observed (data not shown) as described previously in human macrophage THP-1 cells (1). Next, we examined the effect of TNF on MSR protein expression on macrophage cell surface. As shown in Fig. 1B, using Western blotting analyses, TNF increased MSR protein expression on cell surface between 4 and 8 h, by 130 and 330%, respectively. Up to 24 or 36 h of TNF treatment, surface MSR protein expression was reduced to 80 and 50% of control cells, respectively. Moreover, the foam cell formation for studying the effect of TNF on MSR function of taking up OxLDL in J774A.1 cells was examined. The cells were loaded with OxLDL (50 μg/ml for 5 h); there were less lipid droplets stained in TNF-treated cells (Fig. 1C-II) than with TNF-untreated control cells (Fig. 1C-I). As in Table I, TNF treatment of lipid-laden foam cells significantly rendered the foam cell formation to 40% of control cells. Taken together these results indicate that TNF affects MSR mRNA and protein expression and MSR function of taking up OxLDL transforming into foam cells in murine macrophage J774A.1 cells.

**TNF Activates ERK in J774A.1 Cells**—To examine the TNF-mediated signal transduction pathways in regulation of MSR gene expression and function, initially we tested whether TNF interacts with MAPK members. Stimulation of J774A.1 cells with TNF led to the phosphorylation of Elk-1 (Fig. 2A), a transcriptional factor, as evidenced by the activation of ERK by TNF. The experiments for time course of TNF-induced ERK activity were conducted. As detected by Western blot analysis with anti-phospho-Elk1, an antibody that specifically recognized the activated, Ser-383-phosphorylated form of Elk 1 by activated ERK (39), ERK activity barely exists in untreated cells. Upon
TNF stimulation, ERK activity was detected around 45 min and reached to the maximal level about 500-fold at 60 min; after 120 min it returned to basal level (Fig. 2, A and B).

Role of Inhibitors of MEK1 and PKC in TNF-regulated MSR Gene Expression and Function—The activity of ERK in TNF-treated J774A.1 cells peaked at 60 min (Fig. 2, A and B); concomitantly, there was more than a 2-fold increase in MSR message between 4 and 8 h of TNF treatment (Fig. 1A). Since MEK1 and PKC are involved in the stimulation of ERK activity (40), we tested the molecular mechanism for TNF-activated ERK in the regulation of MSR. Cells were pretreated with PD98059 or calphostin C, inhibitors of MEK1 or PKC, respectively, for 1 h followed by 4 h of TNF incubation. We found that both inhibitors markedly reduced MSR message (Fig. 3A). Specifically, either PD98059-treated or calphostin C-treated cells contained only about 10% of MSR message of TNF-induced cells (Fig. 3A, sample 2 versus samples 3 and 4). Alternatively, there is no difference in the MSR message between cells incubated with and without PD98059 or calphostin C in the 24-h TNF treatment (Fig. 3B, sample 6 versus samples 7 and 8), indicating MEK1 and PKC involve less TNF down-regulation of MSR in the late stage of TNF treatment. To further test the role of MEK1 and PKC in the MSR function of taking up OxLDL, foam cells derived from J774A.1 cells via aberrantly accumulating OxLDL were examined. The results in foam cell formation study show that in the absence of TNF, both PD98059 and calphostin C do not affect J774A.1 cell-derived foam cells. However, for 24-h TNF treatment, there is no significant difference of foam cell formation between cells with and without these protein kinase inhibitors (Table I).

TNF Activates JNK Activity in J774A.1 Cells—The stress response of J774A.1 cells to TNF by suppression of MSR prompted the TNF-mediated JNK signaling pathway. First, we

**Fig. 1. Effect of TNF on expression of macrophage scavenger receptor in J774A.1 cells.** A, RT-PCR analysis of the expression of MSR mRNA in J774A.1 cells. Total RNA was isolated from cells grown in either the presence or absence of TNF (150 units/ml) for various times as indicated. The ethidium bromide-stained agarose gel with MSR at 432 bp and normalized by comparison to RT-PCR of mRNA of GAPDH, a constitutively expressed gene. Arrows indicate MSR and GAPDH, respectively. All data are expressed as a fold relative to control untreated cells ($t = 0$ or 24 h). The results shown are representative and average three independent experiments ($n = 3$). A similar result of RT-PCR for mRNA of MSR class A type II in J774A.1 cells treated with TNF was found (data not shown). B, Western blotting analysis of MSR protein expression on the surface of TNF-treated J774A.1 cells. Cells were treated with or without TNF for various times as indicated. The proteins isolated from cell membrane were analyzed via Western blot with anti-MSR antiserum. The detailed method is described under “Experimental Procedures.” The indicated arrow on the right side represents MSR protein. All data are expressed as a fold relative to control untreated cells ($t = 0$ h). This result is representative of three independent experiments and averaged. C, effect of TNF on foam cell formation in J774A.1 cells exposed to OxLDL. J774A.1 cells incubated without TNF (left panel, control) or with TNF (right panel, TNF) for 24 h, followed by exposure to OxLDL (50 μg/ml for another 5 h and foam cell formation studies were conducted. Then cells were stained with Oil Red O as described under “Experimental Procedures” and observed under the microscope. The red droplets accumulated in the cells are stained lipids.

**Table I**

| Protein Kinase Inhibitor | Relative Foam Cell Number |
|-------------------------|---------------------------|
| PD98059                 | 0.42                      |
| Calphostin C            | 0.41                      |
| Wortmannin              | 0.45                      |
| Relative Foam Cell Number | 0.93                     |
|                        | 1.0                       |
|                        | 1.05                      |
|                        | 1.07                      |
|                        | 1.04                      |

TNF stimulation, ERK activity was detected around 45 min and reached to the maximal level about 500-fold at 60 min; after 120 min it returned to basal level (Fig. 2, A and B).

Role of Inhibitors of MEK1 and PKC in TNF-regulated MSR Gene Expression and Function—The activity of ERK in TNF-treated J774A.1 cells peaked at 60 min (Fig. 2, A and B); concomitantly, there was more than a 2-fold increase in MSR message between 4 and 8 h of TNF treatment (Fig. 1A). Since MEK1 and PKC are involved in the stimulation of ERK activity (40), we tested the molecular mechanism for TNF-activated ERK in the regulation of MSR. Cells were pretreated with PD98059 or calphostin C, inhibitors of MEK1 or PKC, respectively, for 1 h followed by 4 h of TNF incubation. We found that both inhibitors markedly reduced MSR message (Fig. 3A). Specifically, either PD98059-treated or calphostin C-treated cells contained only about 10% of MSR message of TNF-induced cells (Fig. 3A, sample 2 versus samples 3 and 4). Alternatively, there is no difference in the MSR message between cells incubated with and without PD98059 or calphostin C in the 24-h TNF treatment (Fig. 3B, sample 6 versus samples 7 and 8), indicating MEK1 and PKC involve less TNF down-regulation of MSR in the late stage of TNF treatment. To further test the role of MEK1 and PKC in the MSR function of taking up OxLDL, foam cells derived from J774A.1 cells via aberrantly accumulating OxLDL were examined. The results in foam cell formation study show that in the absence of TNF, both PD98059 and calphostin C do not affect J774A.1 cell-derived foam cells. However, for 24-h TNF treatment, there is no significant difference of foam cell formation between cells with and without these protein kinase inhibitors (Table I).

TNF Activates JNK Activity in J774A.1 Cells—The stress response of J774A.1 cells to TNF by suppression of MSR prompted the TNF-mediated JNK signaling pathway. First, we
examined whether TNF activated JNK in J774A.1 cells. Stimulation of cells with TNF led to the activation of JNK with a time course fashion, as determined by Western blot analysis via anti-phospho-c-Jun, an antibody that specifically recognizes the activated, serine 63-phosphorylated form of c-Jun (41, 42). Incubation of cells with TNF gradually increased JNK activity; the maximal level of JNK activation after ~45 min was about 16-fold than that of untreated cells, and after 120 min the activity returned to basal level (Fig. 4, A and B).

DN-JNK Construct Blocks TNF Down-regulation of MSR Gene Expression—To study the role of TNF-mediated JNK in the regulation of MSR, the JNK inhibitor curcumin was used. Preincubating J774A.1 cells with curcumin did not completely inhibit JNK activity (data not shown). Alternatively, by using a genetic approach, experiments of transient transfection of DN-JNK into cells were further conducted. RT-PCR analyses of MSR mRNA were examined for DN-JNK-transfected cells as compared with untreated cells (Fig. 4D, samples 3 and 4 versus sample 1) resulting from the basal level of JNK activity in transfected cells (data not shown). However, there was about 30–40% of MSR mRNA left in mock or un-transfected cells after 24 h of TNF incubation, indicating TNF-induced JNK activity involves down-regulation of MSR expression in late stage of TNF treatment.

TNF Activates p38 Activity in J774A.1 Cells—Next, we examined whether TNF activated p38, another important member of MAPK superfamily in J774A.1 cells. Upon TNF stimulation, the activity of p38 gradually increased as detected by Western blotting analysis with anti-phospho-AKT-2, an antibody that specifically recognizes the activated Thr-71-phosphorylated form of AKT-2 (43). The time course study of TNF-induced p38 activity indicated that around 120 min the activity of p38 in TNF-treated cells was approximately 12-fold as compared with control cells; after 240 min it returned to basal levels (Fig. 5, A and B).

DN-Rac1 Construct Reduces TNF-stimulated Activity of JNK and p38—Activation of Rac1 GTPase will stimulate PAK activity in some cells (29). To dissect the TNF-induced signaling relationship between Rac1 and JNK as well as between Rac1 and p38, experiments for JNK and p38 kinase activity in transient transfection of DN-Rac1 into J774A.1 cells were conducted. The results of JNK and p38 activity in DN-Rac1-transfected cells treated with TNF indicate a 4- and 9-fold lower activity of JNK (Fig. 4C, sample 1 versus sample 2) and p38 (Fig. 5C, sample 1 versus sample 2), respectively, than that of the mock or un-transfected control cells (sample 2) with TNF stimulation.

TNF Activates PAK Activity in J774A.1 Cells—The time course study for activation of PAK by TNF was examined. PAK activity was assayed by using an immunoprecipitated radiolabeled protein kinase assay, and phosphorylation of substrate, myelin basic protein, was used (33). As shown in Fig. 6, A and B, the maximal activation of PAK occurred around 30 min, and after 60 min the PAK activity returned to basal levels. Inhibitor of PI 3-Kinase, Wortmannin, Reduces TNF-regulated Activity of JNK and/or p38 in J774A.1 Cells—As shown above, TNF reduces MSR gene expression and its function of taking up OxLDL. However, the pharmacological inhibitors of PKC and MEK1 lack in blocking TNF down-regulation of MSR in the late stage of TNF treatment, indicating that PKC, MEK1, and the downstream ERK involve less TNF regulation of MSR genes. To dissect further the molecular mechanism for TNF-mediated specific signal transduction in regulation of the MSR gene, we used an inhibitor of PI 3-kinase, wortmannin, to test the role of PI 3-kinase in TNF activation of JNK and p38. J774A.1 cells were pretreated with or without wortmannin for 1 h; followed by incubating with TNF for an additional 45 min (for JNK) or 120 min (for p38), then the activities of JNK and p38 were examined. In the absence of wortmannin, TNF induced JNK activity about 16-fold as compared with control cells (Fig. 7, A and B, lane 3 versus lane 1). Interestingly, pretreatment of cells with wortmannin for 1 h led to TNF failure of inducing JNK activity (Fig. 7, A and B, lane 4 versus lane 3). However, in the absence of TNF, wortmannin slightly increased JNK activity in the cell (Fig. 7, A and B, lane 2). Similarly, in the absence of wortmannin, TNF induced p38 activity about 12-fold as compared with control cells (Fig. 7, C and D, lane 3 versus lane 1). Preincubating cells with wortmannin for 1 h inhibited TNF-induced p38 activity to the basal level (Fig. 7, C and D, lane 4 versus lanes 3 and 1). These results suggest that PI 3-kinase is involved in TNF-mediated JNK and p38 activity.

Inhibitor of PI 3-Kinase, Wortmannin, Blocks TNF-mediated Down-regulation of MSR Gene Expression of MSR Function in Foam Cell Formation—Moreover, the potential roles of upstream PI 3-kinase in TNF-mediated signal transductions in the regulation of MSR gene expression and function were examined. In the absence of TNF, there was no significant differ-
inhibitor of PKC, calphostin C on TNF-regulated MSR gene expression, all the conditions were the same as those of testing PD98059, except GAPDH mRNA. All data are expressed as a fold relative to control untreated cells (Fig. 8). Following by cells incubated with or without PD98059 (50 nM) for 1 h, cells were divided into two groups, TNF-untreated samples and TNF-treated samples as indicated. The treated samples were cells incubated with TNF (150 units/ml) for an additional 4 h or 24 h, respectively. After the treatments as indicated, samples were subjected to RT-PCR analysis as described in Fig. 1 or under "Experimental Procedures." The amount of EthBr-stained MSR at a position of 432 bp in agarose gel was normalized by comparison to RT-PCR of mRNAs of GAPDH. All data are expressed as a fold relative to control untreated cells (n = 4). In the experiments of studying the effect of inhibitor of PKC, calphostin C on TNF-regulated MSR gene expression, all the conditions were the same as those of testing PD98059, except calphostin C (50 nM) was used to replace PD98059. Similar results in the studies of PD98059 and of calphostin C were obtained in four separate experiments (n = 4) and averaged.

![Fig. 3](image)

**Fig. 3.** Effect of protein kinase inhibitors on TNF-regulated MSR gene expression and function in J774A.1 cells. Effect of inhibitor of MEK1, PD98059, on TNF-regulated MSR gene expression in the early stage of TNF treatment (4 h treatment) (A), or in the late stage of TNF treatment (24 h treatment) (B). Followed by cells incubated with or without PD98059 (50 nm) for 1 h, cells were divided into two groups, TNF-untreated and TNF-treated samples as indicated. The treated samples were cells incubated with TNF (150 units/ml) for an additional 4 h or 24 h, respectively. After the treatments as indicated, samples were subjected to RT-PCR analysis as described in Fig. 1 or under "Experimental Procedures." The amount of EthBr-stained MSR at a position of 432 bp in agarose gel was normalized by comparison to RT-PCR of mRNAs of GAPDH. All data are expressed as a fold relative to control untreated cells (Fig. 3). In the experiments of studying the effect of inhibitor of PKC, calphostin C on TNF-regulated MSR gene expression, all the conditions were the same as those of testing PD98059, except calphostin C (50 nm) was used to replace PD98059. Similar results in the studies of PD98059 and of calphostin C were obtained in four separate experiments (n = 4) and averaged.

ence of MSR mRNA between cells pretreated with and without inhibitor of PI 3-kinase, wortmannin (Fig. 8, sample 3 versus sample 1). In the presence of TNF, as expected, TNF decreased MSR mRNA; in contrast, wortmannin successfully blocked TNF down-regulation of the MSR message, resulting in restoration of the MSR message (Fig. 8, lane 2 versus lane 4). Alternatively, there is no difference of MSR messages between TNF-treated cells with wortmannin preincubation and TNF-untreated control cells (Fig. 8, lane 4 versus lane 1). To examine further the role of PI 3-kinase in TNF-regulated MSR function, experiments for foam cell formation were conducted. For J774A.1 cells pretreated with wortmannin for 1 h prior to 24-h TNF incubation followed by OxLDL loading, the result of measuring foam cells revealed that in the presence of TNF more relative foam cell number (0.93) transformed from wortmannin-pretreated cells than that of wortmannin-untreated control cells (0.42) (Table I). The results of the wortmannin preincubation study indicate that activation of PI 3-kinase involves TNF-mediated down-regulation of MSR expression and of MSR function in taking up OxLDL subsequently related to foam cell formation.

**TNF Induces Intracellular ROS and Increases mRNA of MnSOD in J774A.1 Cells**—For pretreatment of cells with DCFH, rapid induction of intracellular ROS releasing was detected upon stimulation with TNF or PMA (as a positive control), respectively (Fig. 9A). In contrast, preincubation of cells with NAC, an effective antioxidant prior to TNF or PMA treatment, results in a significant decrease of the relative ROS, i.e., fluorescence intensity by scavenging free ROS from TNF- or PMA-stimulated cells (Fig. 9A) at both 30 and 60 min, respectively. Moreover, to investigate cellular defensive protection system against the TNF-induced intracellular ROS including activation of endogenous antioxidant enzyme (44), we demonstrated that TNF rapidly up-regulates MnSOD message around 2 h (280%) and extending to 24 h (350%) as compared with control cells detected by RT-PCR (Fig. 9B).

**Involvement of ROS, PKC, MEK1, JNK, and p38 in MSR Gene Promoter-driven Luciferase Reporter Gene Activity Mediated by TNF Stimulation**—We investigated the role of individual signaling molecules such as ROS, PKC, MEK1, JNK, and p38 in luciferase reporter activity driven by MSR gene promoter in 293 T cells. As shown in Fig. 10, comparing with untreated cells, TNF increases MSR gene promoter-driven luciferase activity, but NAC significantly decreases TNF-mediated specific luciferase activity by 50%. In the absence of TNF, PKC activator, PMA stimulates the luciferase activity about 2-fold; in contrast, PKC inhibitor, calphostin C, significantly decreases the luciferase activity induced by TNF. Moreover, PD98059 (MEK1 inhibitor) and transfection of DN-JNK markedly decrease TNF-induced MSR gene promoter-driven luciferase activity by 40 and 30%, respectively, although SB203580 (p38 inhibitor) slightly reduces the luciferase activity (Fig. 10).

**Role of Anti-TNF-R1 Ab in Regulation of MSR Gene Expression, in Stimulation of MAPK Activity, and in MnSOD Message**—To support further the evidence regarding the biological relevance issue in the effect of ligation of TNF-mediated transducing signals on the regulation of MSR, we used a polyclonal goat anti-mouse TNF-R1 agonist antibody (anti-TNF-R1 Ab) to delineate the role of TNF-R1 (p55) and the importance of the receptor-mediated specific signaling pathways in the regulation of scavenger receptor in J774A.1 macrophages. Specifically, we conducted the experiments of anti-TNF-R1 Ab and focused on the following: 1) validation of the TNF-R1 regulation of MSR expression; 2) investigation and illumination of the TNF-R1-mediated signal transduction pathways in induction of MAPK activity (ERK, JNK, and p38); and 3) TNF-R1-related MnSOD expression. As shown in Table II, for incubation of J774A.1 cells with anti-TNF-R1 Ab instead of TNF for the indicated times, the trends of increasing and decreasing MSR message are compatible to that of TNF-treated cells as reported above (Fig. 1A). Moreover, cells treated with anti-TNF-R1 Ab, the TNF-R1-induced activity of MAPK including ERK, JNK, and p38, were measured, and the maximum activity and time for these MAPKs (Table II) are similar to MAPK activity of TNF-treated cells (Figs. 2A, 4A, and 5A). In addition, the trend of increasing MnSOD message in anti-TNF-R1 Ab-treated cells (Table II) is compatible to that of TNF-treated cells (Fig. 9B). In contrast, the preimmune serum has no effect on the message of MSR and MnSOD and MAPK activity (data not shown).

**DISCUSSION**

The current results of studying TNF inhibition of MSR mRNA and protein expression in murine macrophage J774A.1 cells are comparable with the previous demonstration of TNF inhibition of MSR in PMA-differentiated human THP-1 macrophages (1), indicating that the effect of TNF on MSR is species-independent. It is known that PMA stimulates PKC activity and induces human suspension THP-1 monocytes to differen-
Briefly, whole cell lysates (250 μg of protein) were incubated with 2 μg of c-Jun-(1–89) fusion protein beads at 4 °C for 24 h. The active complex of JNK-c-Jun fusion protein beads was centrifuged at 4 °C for 10 min, the pellet washed twice with 1× lysis buffer and 1× kinase buffer, respectively, then resuspended in 1× kinase buffer. The kinase reactions were performed in the presence of 100 μM cold ATP and 2 μg of ATP-2 fusion protein at 30 °C for 30 min. The phosphorylation of c-Jun at Ser-63 was measured by Western blotting of nonradioactive labeled samples using phospho-c-Jun (Ser-63) antibody and Phototope®-HRP Western Detection System via chemiluminescence.

The detailed method is described under "Experimental Procedures" or in the New England Biolabs instruction manual. B, histograms represent quantification by PhosphorImager® of phospho-ATF-2 (Thr-71) of each sample by using ImageQuant® software from Molecular Dynamics for TNF-stimulated p38 activity. All data of relative activity are expressed as comparison with untreated cells (t = 0, activity of control cells defined as 1). Similar results were obtained in four separate experiments and averaged. C, effect of DN-Rac1 transfection on reduction of TNF-stimulated p38 activity in J774A.1 cells. Cells were transiently transfected with DN-Rac1 as described under "Experimental Procedures." After 24 h of transfection, cells were treated with TNF for 45 min, and JNK activity of the treated cells was analyzed as described above. Similar results were obtained in three separate experiments and averaged.

FIG. 4. Time course of TNF-activated c-Jun NH2-terminal kinase (JNK) activity in J774A.1 cells. A, analysis of JNK activity of TNF-treated J774A.1 cells. The JNK-induced phosphorylation of c-Jun was measured by quantitative Western blotting using phospho-c-Jun (Ser-63) antibody and Phototope®-HRP Western Detection System. Briefly, whole cell lysates (250 μg of protein) were incubated with 2 μg of c-Jun-(1–89) fusion protein beads at 4 °C for 24 h. The active complex of JNK-c-Jun fusion protein beads was centrifuged at 4 °C for 10 min, the pellet washed twice with 1× lysis buffer and 1× kinase buffer, respectively, then resuspended in 1× kinase buffer. The kinase reactions were performed in the presence of 100 μM cold ATP and 2 μg of ATP-2 fusion protein at 30 °C for 30 min. The phosphorylation of c-Jun at Ser-63 was measured by Western blotting of nonradioactive labeled samples using phospho-c-Jun (Ser-63) antibody and Phototope®-HRP Western Detection System via chemiluminescence. The detailed method is described under "Experimental Procedures" or in the New England Biolabs instruction manual. B, histograms represent quantification by PhosphorImager® of phospho-ATF-2 (Thr-71) of each sample by using ImageQuant® software from Molecular Dynamics for TNF-stimulated p38 activity. All data of relative activity are expressed as comparison with untreated cells (t = 0, activity of control cells defined as 1). Similar results were obtained in four separate experiments and averaged. C, effect of DN-Rac1 transfection on reduction of TNF-stimulated p38 activity in J774A.1 cells. Cells were transiently transfected with DN-Rac1 as described under "Experimental Procedures." After 24 h of transfection, cells were treated with TNF for 45 min, and JNK activity of the treated cells was analyzed as described above. Similar results were obtained in three separate experiments and averaged.

Monocytes and in J774A.1 cells (data not shown). Although the surface markers of macrophages have been used, it is still difficult to monitor the percentage and degree of differentiation in the tested THP-1 macrophages. With the obstacles of highly stimulated PKC activity and the uncertainty of differentiation in THP-1 macrophages, we initially encountered difficulty in the time course study of TNF-mediated activity of protein kinases and further in studies of the effect of protein kinases on MSR regulation. In contrast, there is a basal level of PKC activity in the fully differentiated resting macrophage J774A.1 cells with consistent MSR expression. Additional advantages of using J774A.1 cells in this study are the facts that property of TNF receptor (15) and study of lipid accumulation (30) have been well characterized.
The regulatory mechanisms of scavenger receptor expression and activity in macrophage and smooth muscle cells (SMC) have been reported in response to TNF (1, 46, 47), bacterial lipopolysaccharide (48, 49), interferon-γ (50), transforming growth factor-β family (51), lipoproteins (52), α-tocopherol (53), all-trans-retinoic acid, dexamethasone (45), PMA, oxidative stress (54), PKC (55), etc. Nevertheless, there is less information about the autocrine effect and inflammatory activity of TNF on macrophages; moreover, the molecular mechanism by which TNF-mediated transducing signals in macrophages relate to atherosclerosis is undefined. Our previous study showed that TNF reduction in MSR mRNA half-life was inhibited by cycloheximide implying that new protein synthesis was necessary and suggesting that TNF induced expression of protein(s) which accelerated MSR mRNA degradation (1). To mimic the autocrine effect of TNF on activated macrophages or foam cells adjacent to the atherosclerotic lesion, in this study, we intended to examine TNF-mediated signal transduction in the absence of cycloheximide. To simplify further the complicated effect of macrophage-derived multiple cytokines in atherosclerotic lesions, we focused on the effect of TNF on atherogenesis-relevant gene MSR in macrophages. In this report, we are the first to investigate systematically TNF-mediated signal transduction pathways including role of protein kinase and the specific member in MAPK superfamily in regulation of MSR expression and function related to foam cell formation. TNF-mediated protein kinases in the regulation of MSR activity were monitored by several indicators at different levels including transcriptional regulation of MSR gene promoter-driven luciferase activity, RT-PCR analysis of MSR message, MSR surface protein expression, and OxLDL taking up foam cell formation. Our data indicated TNF initially up-regulates MSR expression and later down-regulates MSR activity in J774A.1 cells. Specifically, in time course studies, TNF rapidly increases mRNA and protein expression of MSR in the early stages; by increasing incubation times, TNF markedly decreases MSR expression and function in the late stage. The TNF time-dependent effects in regulation of the MSR gene result from diversity of MAPK mediated by TNF receptor-associated pleiotropic properties. TNF-induced MAPKs including JNK and p38 play diverse roles in early and late stage signaling pathways reflecting TNF receptor-mediated multiple biologic activities such as regulation of downstream MSR gene or initiation of apoptosis-related caspase activity.²

The major findings are as follows: 1) during TNF stimulation in J774A.1 cells, TNF-dependent protein kinases lead to the activation of MAPKs ERK, JNK, and p38; including the activation of upstream protein kinases PKC, MEK1, PI-3 kinase, and PAK at different times is necessary for the regulation of MSR expression. Similar results of MAPK activation by TNF have been observed in our study of fully differentiated blood-derived human primary macrophages² as reported in mouse bone marrow-derived macrophages (56). 2) The blockade of ERK and JNK activation in the early stage of TNF treatment and the reduction of TNF-induced MSR message probably result from the decreased transcriptional regulation of the MSR gene. 3) Alternatively, in the late stage of TNF treatment, blockade of TNF-mediated JNK and p38 activity, but not ERK, with specific protein kinase inhibitors or with transfection of dominant-negative mutants almost completely inhibit the TNF-mediated reduction of MSR activity and restored MSR message to the untreated cells. The inhibition of TNF reduction of MSR expression and function in the late stage appears to be regulated by a post-transcriptional mechanism (see below). Collectively, our data suggest that the coordination of signalings arising from TNF receptor at different times is necessary for up-regulation and down-regulation of scavenger receptor gene expression and function in macrophages. Similarly, it has been demonstrated that a model of early and late stage in TNF-dependent activation of JNK and p38 signaling pathways regulates TNF receptor-mediated functions (57).

Induction of several signal transduction pathways in cells upon ligation of TNF has been reported. To probe the role of TNF-induced protein kinases including MAPK in regulation of scavenger receptor expression and foam cell formation in macrophages, we utilized various specific pharmacological an-

² H.-Y. Hsu and Y.-C. Twu, unpublished data.
agonists such as calphostin C, PD98059, wortmannin, curcumin, and SB203580 that inhibit the phosphorylation of PKC (32, 58), MEK1 (59), PI-3 kinase (60), JNK (61, 62), and p38 (63), respectively. In TNF-treated macrophage J774A.1 cells, it is interesting to investigate whether there is a coincidence that TNF-stimulated activity of ERK and JNK resulted in increase of MSR mRNA and surface protein expression at early stage of TNF treatment. As it is well known that MEK1 and PKC are involved in the stimulation of ERK activity (40), we first examined the role of MEK1 and PKC in TNF regulation of MSR function. Our results show that PD98059 (MEK1 inhibitor) and calphostin C (PKC inhibitor) effectively reduced TNF activation of MSR in the early stage of TNF treatment (Fig. 3A). In contrast, when cells were incubated with TNF for 24 h, there was no difference of MSR expression and foam cell number in the cells with or without treatment of PD98059 or of calphostin C (Fig. 3B and Table 1). These results indicate TNF-mediated PKC/MEK1/ERK pathway plays differential roles in the regulation of MSR gene and function at different stages of TNF treatment. The results from pretreatment of cells with PD98059 or calphostin C markedly decreased MSR promoter-driven luciferase activity evidence of involvement of MEK1 and PKC in transcriptional regulation of MSR gene (see below). In the presence of TNF, there was a significant restoration of MSR mRNA in 24- and 48-h DN-JNK-transfected mutants as compared with the mock or un-transfected control cells (Fig. 4D), although the JNK antagonist curcumin slightly restored MSR mRNA in TNF-treated cells (data not shown). The results of experiments of DN-JNK-transfected mutants indicate TNF-mediated JNK down-regulates MSR expression and activity.

Upstream signaling molecules Rac1 and Cdc42 that activate JNK activity have been reported (64, 65). Additionally, the PKC family of protein kinases is one of the main targets and interacts with the GTPases of Rac1 and Cdc42 (66). Activation of PKC-mediated signalings by these GTPases (67) leads to induction of JNK and p38 activity but less to ERK activity (29). Here, we demonstrated that incubation of J774A.1 cells with TNF quickly stimulated PAK activity, followed by increasing activity of JNK and p38 comparable with various cell lines (9, 56, 68). Transfection of DN-Rac1 construct inhibits TNF-stimulated JNK and p38 activity (Figs. 4C and 5C); alternatively, the constitutively activated Rac1 mutants expressed more JNK and p38 activity than that of mock. These results support that the level of Rac1 and Pak is at the upstream in the TNF-mediated signal cascades additionally and confirm the role of Rac1 and Pak in the regulation of MSR. Moreover, to obtain specific indication that JNK, p38, Pak, Rac1, and their upstream signal molecule PI 3-kinase are involved in TNF regulation of MSR, we further investigated the role of PI 3-kinase in JNK and p38 activity as well as in the regulation of MSR messagge and taking up OxLDL. Our data showed that preincubation of cells with wortmannin, a PI 3-kinase inhibitor followed by TNF treatment, resulted in decrease activity of JNK (Fig. 7A and B) and p38 (Fig. 7C and D) and subsequently blocked TNF reduction of MSR expression (Fig. 8). Taken together, our results establish the important signaling network inter-relationship between PI 3-kinase, Rac1, and Pak among JNK and p38 in the TNF-mediated signaling path-
The effect of TNF on MSR function of taking up OxLDL via experiments of J774A.1 cell-derived foam cell formation was performed. The reduction of foam cell number in TNF-treated J774A.1 cells to 40% of control untreated cells suggests that the decrease of lipid-laden cells result from TNF down-regulation of scavenger receptors in taking up OxLDL (Fig. 11). The reduction effect of TNF on the MSR gene in taking up OxLDL is comparable to the recent demonstration of a 3-fold decrease of PI 3-kinase/Rac1/PAK/p38, which regulating MSR expression (Fig. 11).

**Fig. 9.** TNF induces intracellular ROS and activates mRNA of MnSOD in J774A.1 cells. A, TNF and PMA induce ROS in macrophage J774.1 cells. Cells grown in serum-free phenol red-free RPMI medium for 24 h, incubated with 10 μmol/liter DCFH (Molecular Probes, Inc.) at 37 °C for 15 min in the dark, then incubated with N-acetylcysteine (NAC, 1 mM or 10 mM) for 30 min, followed by treatment with TNF (150 units/ml) or PMA (100 nM) for additional 30 or 60 min, respectively. The relative fluorescence intensity of fluorophore DCF was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a Cytofluor. DCFH with fresh culture medium was used as a blank control. All data of relative ROS are expressed as comparison with control untreated cells (ROS activity of control cells defined as 1). B, effect of TNF on MnSOD mRNA expression in J774.1 cells. RT-PCR analysis of the expression of MnSOD mRNA in J774A.1 cells. Total RNA was isolated from J774A.1 cells grown in serum-free media in the presence or absence of TNF (150 units/ml) for various times. Ethidium bromide-stained agarose gel with MnSOD and normalized by comparison to RT-PCR of mRNA of GAPDH. All data are expressed as a fold relative to control untreated J774A.1 cells (t = 0 h). The detailed method is described under “Experimental Procedures.” The results shown are representative of three independent experiments and averaged.

**Fig. 10.** Involvement of ROS, PKC, MEK1, JNK, and p38 in MSR gene promoter-driven luciferase activity mediated by TNF stimulation in 293 T cells. The studies of relative transcriptional activity of MSR gene promoter in 293 T cells were using calcium phosphate method to transfect with MSR gene promoter construct (such as plasmids HACLDL Xba-A1-luc promoter and HACLDL Xba-A1-luc Enhancer) for 24 h as described under “Experimental Procedures.” After 24 h, transfected cells were treated with NAC, PMA (PKC activator), various inhibitors of protein kinases, e.g. calphostin C, PD98059, and SB203580 for PKC, MEK1 and p38, respectively, for 30 min as indicated. In addition, cells were co-transfected with MSR gene promoter construct and DN-JNK construct for 24 h. Occasionally, co-transfection of β-galactosidase plasmid (Promega) with MSR gene promoter construct into cells to monitor the transfection efficiency was performed. The treated cells were initially incubated with TNF for 4 h, and then MSR gene promoter-driven luciferase activity assays were performed as described under “Experimental Procedures.” All data of relative luciferase activity are expressed as comparison with control untreated cells (luciferase activity of control cells defined as 1). The experiments were performed three times and averaged.
J774A.1 macrophages were incubated with anti-TNF-R1 Ab (titer, 1:4000) at various times as indicated, and TNF treatment of cells was as described in figure legends. The detailed method and calculation of MSR message, MAPK activity, and MnSOD message expression are described under “Experimental Procedures.”

### TABLE II

**Comparison between polyclonal goat anti-mouse TNF-R1 agonist antibody (anti-TNF-R1 Ab) and TNF regarding their effects on MSR message, stimulation of MAPK activity, and MnSOD message expression**

| Anti-TNF-R1 Ab | TNF |
|----------------|-----|
| MSR message expression | |
| (4-h incubation) | 3.54 ± 0.10 | 3.23 ± 0.01 |
| (24-h incubation) | 0.35 ± 0.03 | 0.33 ± 0.02 |
| MAPK activity (maximum) | |
| ERK (90 min) | 546 ± 30 | 550 ± 25 |
| JNK (45 min) | 16.2 ± 3.4 | 17.5 ± 2.5 |
| p38 (120 min) | 14.6 ± 1.8 | 13.4 ± 2.1 |
| MnSOD message expression (24-h incubation) | 3.83 ± 0.14 | 3.48 ± 0.03 |

**FIG. 11. Proposed TNF-mediated signal transduction in the regulation of macrophage scavenger receptor and other macrophage functions.**

increase in taking up and degradation of modified LDL via MSR for cultured peritoneal macrophages from tumor necrosis factor-receptor1 (TNF-R1) knock-out (p55-null) mice than that in wild-type mice (17). It is likely that TNF normally down-regulates MSR activity presumably via TNF receptor-mediated signaling pathways. But in the absence of TNF-R1 (p55-null), MSR is not down-regulated any more in p55-null mice. Thus, in p55-null mice, there is an accumulation of foam cells due to increased production and/or activity of MSR, indicating that TNF-R1-mediated signal transduction is important in protection against atherosclerotic-like reactions, at least in lipid/cholesterol-fed mice. This is suggestive of a response mediated by TNF-R1 with little if any requirement for the binding of TNF to TNF-R2 (see below). Interestingly, we did not observe a significant difference in the number of foam cell formation between the TNF-treated and un-treated macrophages preincubated with a monoclonal antibody blocking CD36 (data not shown), although there is the possibility involving CD36 taking up OxLDL (69).

We hope to conduct further additional experiments and to provide supporting evidence regarding the biological relevance issue in the effect of ligation of TNF-mediated and TNF-R1-mediated transducing signals on the regulation of MSR as well as related reactions on macrophages. In agreement with the issues and molecular mechanism approach, we used a polyclonal goat anti-mouse TNF-R1 agonist antibody (anti-TNF-R1 Ab) to determine if the anti-TNF-R1 Ab could mimic TNF activity, to delineate the type of TNF receptor involving TNF ligation-mediated signalings, and to further illuminate the effect of TNF-R1-mediated specific signal transduction pathways on the regulation of MSR as well as related reactions. As shown above, “Results” and Table II, the key role of TNF-R1 in mediated induction of MAPK (ERK, JNK, and p38) activity in J774A.1 cells by the experiments with agonist anti-TNF-R1 Ab at a higher dilution whose results are consistent to those obtained with TNF-treated J774A.1 cells and some data derived from TNF-R1 knock-out mice is as reported (70, 71). The use of mutant mice lacking TNF-R1 (p55) or TNF-R2 (p75) or both facilitates studies directed at understanding the effect that TNF has demonstrated (17, 70–73). Our additional results from studies of agonist anti-TNF-R1 Ab-mediated MAPK in regulation of MSR and foam cells in J774A.1 macrophages further demonstrate the biologically relevant function of TNF-R1 on MSR genes in macrophages. The evidence that TNF-R1 knock-out mice induced larger atherosclerotic lesions than those of control mice by more than 2-fold in feeding with a high cholesterol diet further strengthened the role of TNF-R1 in the regulation of lipid accumulation (17). These findings indicate under normal conditions the importance of TNF-R1-mediated signals on MSR function related to regulation of atherogenesis. The parts of regulation of MSR expression and foam cell formation are in agreement with those obtained with TNF-R1 knock-out mice as previously reported (17), although we did not examine the effect of TNF receptor knock-outs on the induction of MSR here. In addition, new data show that only the TNF-R1 signaling pathway is important for this action of down-regulation of MSR activity; in contrast, TNF-R2 knock-out (p75-null) mice do not show changes in atherosclerosis as compared with wild-type mice. These results suggest that at least TNF-R1 and TNF-R2 must have different signaling pathways, and also the MSR is susceptible to TNF-R1 but not TNF-R2 pathways. Indeed, in our current studies by using agonist anti-TNF-R1 Ab, it specifically activated TNF-R1 but not TNF-R2 as expected (17, 70–73). Moreover, the data in Table II indicate TNF-mediated signal transductions as well as regulation of MSR and MnSOD most likely contribute from the activated TNF-R1-mediated specific signalings. Hence, we demonstrated that the decreasing foam cell formation (number) in TNF-treated cells (Table I and Fig. 1C), resulting from TNF down-regulation of MSR activity, might explain the reason for increased macrophage lipid accumulation in the p55 null mice mainly due to the disappearance of regular TNF-R1-mediated signal transductions and the effects on down-regulation of MSR activity. Taken together our results and other accumulated reports support the current evidence regarding the effect of TNF-R1 involving specific MAPK signaling pathways on the regulation of MSR and illuminate the important biological relevance of the TNF receptor regulating MSR in macrophages.

The findings of the present study raise one important question of how activation of ERK, JNK, p38, and their upstream

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3 R. LeBoeuf, personal communication.
regulators serves to initiate MSR gene regulation in response to early stage and late stage of TNF treatment. Specifically, during stimulation with TNF, we are interested in molecular mechanisms including trans-acting factors that mediate regulation of MSR gene. The transcription factors relevant to the regulation of scavenger receptor gene expression in macrophage differentiation (37, 48, 74) and in smooth muscle cell (SMC) (46, 47, 54, 55) have been studied. The transcription factor-binding sites for activating protein 1 (AP-1)/ETS between the sequence of −67 and −50 bp and region IV in the 5'-flanking region of human MSR gene promoter have been identified (37, 74). The AP-1/ETS-binding element is critical for MSR expression upon PMA-induced macrophage differentiation (1, 74). The results from Pitas and colleagues (54) reported the requirement of AP-1/c-Jun and CCAAT/enhancer-binding protein (C/EBP) in the transcriptional activation of the receptor expression in SMC. In SMC, the condition of increasing MSR expression is the same as induction of AP-1/c-Jun binding activity in electrophoretic mobility shift assays of cell nuclear extracts interacting with the −67- and −50-bp-specific sites on MSR gene promoter (54). The similarity of scavenger receptor expression in macrophages and SMC indicating transcriptional activation of receptor gene in response to transcription factor AP-1 and JNK activity is cell-independent.

The cis elements involved in the activation of MSR expression by TNF have not been rigorously examined in our previous study (1). TNF activates certain important trans-acting factors including AP-1 represented by cognate cis elements in the 5'-flanking region of some genes. In this paper, macrophage cell lines and other cells were initially tested for suitability in transfection of MSR gene promoter and responses to TNF. However, no significant MSR gene promoter-driven luciferase reporter gene activity was detected in most transfected samples, indicating low transfection efficiency in the tested cells (data not shown). Therefore, we examined the response of MSR gene promoter-driven luciferase activity in human embryonic kidney 293 T cells to TNF-mediated signaling pathways. Specifically, to explore the concept that components in TNF-mediated pathways of PKC/MEK1/ERK, of PI-3 kinase/Rac1/PAK/JNK, and of PI-3 kinase/Rac1/PAK/p38 are necessary in the regulation of MSR gene, we investigated how certain signaling molecules including NAC, MEK1, PKC, JNK, and p38 influence luciferase activity driven by MSR gene promoter in 293 T cells. As shown in Fig. 10, TNF increases MSR gene promoter-driven luciferase activity; in the absence of TNF, the stimulation of relative luciferase activity by PMA demonstrates PKC involves transcriptional regulation of MSR gene as indicated in our previous report (1). Preincubation of NAC, an effective antioxidant, in MSR gene promoter-transfected 293 T cells followed by TNF treatment resulted in marked reduction of relative luciferase activity (Fig. 10). This result shows that the important role of TNF-initiated ROS in the transcriptional regulation of MSR gene, although the species of ROS needs to be further identified, is comparable with the finding of H₂O₂-relevant oxidative stress in MSR gene expression (54) (see below). Both PD098059 and calphostin C significantly reduce TNF-induced MSR gene promoter-driven luciferase activity, indicating TNF-mediated PKC/MEK1/ERK pathway involves MSR gene regulation at the transcriptional level in the early stage of TNF treatment. Moreover, a ternary complex consisting of transcription factors c-Jun and JunB binding to the region IV has been reported (37, 74). TRAF2 has been demonstrated to activate the JNK pathway, which in turn stimulates transcription factor AP-1 (75) mainly via phosphorylation of the c-Jun component (68). At the early stage of TNF treatment, blockade of JNK activity via co-transfection of DN-JNK construct in 293 T cells significantly decreased the TNF-induced MSR gene promoter-driven luciferase activity, demonstrating TNF-mediated JNK activity plays a positive role in the transcription regulation of MSR gene. Similarly, it has been reported that both AP-1 binding and JNK activation induced by PMA and oxidative stress (e.g. H₂O₂), respectively, associated with MSR gene expression (54) also provide the direct link between JNK and MSR regulation. Interestingly, our data showed in the presence of TNF that there was a slight reduction of relative luciferase activity in cells pretreated with SB203580, indicating p38 less involved in TNF-mediated transcriptional regulation of the MSR gene. Alternatively, a C/EBP element located between the −44- and −21-bp site of MSR gene promoter has been identified (54); mutation of the C/EBP site results in reduction of the inducible activity of the promoter in SMCs by 50%. However, this specific element exhibited no significant role in either basal or inducible MSR gene expression in macrophage (74). The role of C/EBP element in induction of MSR gene expression between macrophages and SMC probably reflects a fact of the constitutive expression of MSR on macrophages, whereas SMC express little or no MSR mRNA and protein unless induced by cytokines, growth factors, or PMA. Taken together, these findings reveal interesting information that at a certain point, transcriptional regulation of MSR gene is cell-specific, but the biological significance is unclear.

By stimulating J774A.1 cells with TNF, up-regulation of NF-xB-mediated gene MnSOD expression was observed (Fig. 9B) as described (44); this is also consistent with the increased NF-xB gene promoter-driven luciferase activity indicating that TNF stimulates NF-xB activity in cells. Indeed, exposure of cells to TNF leads to rapid activation of an important transcription factor, NF-xB, via TNF-R1 signaling downstream of TRAF2 (76). The induction of MnSOD would help the cell resist against TNF-induced ROS cytotoxicity (Fig. 9A). Our preliminary data showed that the NF-xB-like cis element probably exists proximal to the transcriptional start site of MSR gene promoter, although NF-xB has not been reported in MSR regulation. The exact location and functional property of the specific cis element in TNF regulation of MSR expression are needed to investigate further.

In summary, we dissected the role of TNF-mediated signal transduction pathways in the regulation of MSR gene expression and function related to foam cell formation. We demonstrate for the first time that TNF could trigger three MAPK signaling pathways and other related protein kinases in macrophage J774A.1 cells (Fig. 11). Furthermore, we evaluated the role of individual protein kinase in TNF regulation of MSR expression and in OxLDL accumulation of foam cell formation. The existing two time-dependent phases of MSR expression and activity in TNF-treated cells indicate TNF-mediated specific signal transduction pathways play different mechanisms in regulation of MSR at different stages. Specifically, our current findings indicate that TNF activation of ERK, JNK, and p38 in the context of TNF-mediated other functional transducing signal molecules such as PAK, Rac1, and PI-3 kinase, resulting in the regulation of MSR expression to occur at more than one level. In the early stage, TNF-induced PKC/MEK1/ERK pathway and JNK activity mediate interactions of activated transcription factors mostly in transcriptional regulation of the MSR gene, leading to an increase of MSR gene activity. Extending TNF incubation time, the diverse roles of JNK and p38 in PI 3-kinase/Rac1/PAK/JNK and PI 3-kinase/Rac1/PAK/p38 pathways also participate in the post-transcriptional regulation of MSR such as destabilization of MSR mRNA (1) to decrease MSR expression and function. TNF reduction of MSR
expression and activity likely results in decreasing foam cell number. By using a specific anti-TNF-R1 agonist antibody, we demonstrate TNF-R1-mediated signal transduction most similar to those of TNF induction. Our results expose and demonstrate the differential roles of TNF-mediated specific protein kinase pathways at different stages mediate multiple regulatory functions in MSR expression. Future studies are needed to investigate the biological role of TNF-mediated specific signal-regulating molecule regulation of MSR expression in the early and late stage of atherogenesis at animal model.

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REFERENCES

1. Hsu, H. Y., Nicholson, A. C., and Hajjar, D. P. (1996) J. Biol. Chem. 271, 7769–7777.
2. Moss, M. L., Jin, S. L., Milla, M. E., Bickett, D. M., Burkhart, W., Carter, H. L., Chou, M. T., Chou, W. J., Chou, C. J., and Chou, C. Y. (1996) J. Biol. Chem. 271, 367–375.
3. Jovinge, S., Ares, M. P., Kallin, B., and Nilsson, J. (1996) J. Biol. Chem. 271, 17176–17179.
4. Liu, R. Y., Fan, C., Olashaw, N. E., Wang, X., and Zuckerman, K. S. (1999) J. Biol. Chem. 274, 13877–13885.
5. Adam-Rälges, S., Adam, D., Wiegmann, K., Struve, S., Kolanus, W., Schneider-Mergener, J., and Kronke, M. (1996) Cell 86, 973–987.
6. Kull, F. C., Jacobs, S., and Cuatrecasas, P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5760–5765.
7. Ross, R. (1999) N. Engl. J. Med. 340, 115–126.
8. Schreyer, S. A., Peschon, J. J., and LeBeouf, R. C. (1996) J. Biol. Chem. 271, 26174–26178.
9. Steinberg, D., and Goto, A. M. J. (1999) J. Am. Med. Assoc. 282, 2043–2050.
10. Steinberg, D., Carew, T. E., Fielding, C., Fogelman, A. M., and Williams, L. T. (1998) J. Clin. Invest. 101, 122–133.
11. Basu, S. K., Anderson, R. G., Goldstein, J. L., and Brown, M. S. (1977) J. Biol. Chem. 252, 7728–7735.
12. Hsu, H. Y., Nicholson, A. C., and Hajjar, D. P. (1994) J. Biol. Chem. 269, 9213–9220.
13. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. (1995) J. Biol. Chem. 270, 27995–27998.
14. Shio, M., Kono, Y., and Kato, T. (1999) Biochem. Biophys. Acta 1467, 303–306.
15. Persons, D. A., Wilkinson, W. O., Bell, R. M., and Finn, G. O. (1998) Cell 32, 447–458.
16. Hsu, H. Y., Hajjar, D. P., Khan, K. M., and Falcone, D. J. (1998) J. Biol. Chem. 273, 1240–1246.