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Tumor Necrosis Factor Receptor (TNFR) 1, but Not TNFR2, Mediates Tumor Necrosis Factor-α–Induced Interleukin-6 and RANTES in Human Airway Smooth Muscle Cells: Role of p38 and p42/44 Mitogen-Activated Protein Kinases

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ABSTRACT

Little information is available regarding the mechanisms involved in cytokine-induced synthetic function of human airway smooth muscle (ASM) cells. Here, we report that tumor necrosis factor receptor (TNFR) 1-induced p38 and p42/44 mitogen-activated protein kinase (MAPK) activation modulates tumor necrosis factor-α (TNFα)-mediated synthetic responses: expression of intercellular adhesion molecule-1 (ICAM-1) and secretion of interleukin (IL)-6 and the regulated-on-activation, normal T-cell expressed and secreted (RANTES) chemokine in human ASM cells. Pretreatment of ASM cells with SB203580, a p38 MAPK inhibitor, slightly enhanced TNFα-induced ICAM-1 expression in a dose-dependent manner but partially inhibited secretion of RANTES and IL-6. In contrast, PD98059, a p42/44 inhibitor, reduced ICAM-1 expression by 50% but had no effect on TNFα-induced RANTES or IL-6 secretion. SB203580 and PD98059 had little effect on TNFα-induced nuclear factor-κB (NF-κB) activation as determined in cells transfected with a NF-κB–luciferase reporter construct. We also found that agonistic antibodies specific for either TNFR1 or TNFR2 stimulated IL-6 and RANTES secretion and activated p38 and p42/44 MAPKs. In addition, both antibodies induced NF-κB-mediated gene transcription. Using receptor-specific blocking antibodies, we found that TNFR1 primarily regulates TNFα-induced IL-6 and RANTES secretion and activation of p38 and p42/44 MAPK pathways. Interestingly, we found that TNFR1 and TNFR2 are expressed differently on the cell surface of ASM cells. Our data suggest that despite the presence of functional TNFR2, TNFR1 associated with MAPK-dependent and -independent pathways is the primary signaling pathway involved in TNFα-induced synthetic functions in ASM cells.

Airway smooth muscle (ASM) is an important effector cell in asthma. Recent evidence suggests that cytokine-induced changes in the airway smooth muscle phenotype may modulate bronchial hyperresponsiveness and airway inflammation (Amrani et al., 2000a; Chung, 2000). Therefore, characterizing the cellular and molecular mechanisms that regulate ASM function will probably lead to new therapeutic approaches in the management of asthma.

Using cultured ASM cells that retain their physiological responsiveness to agonist (Panettieri et al., 1989), investigators have shown that TNFα, a cytokine present in high levels in the bronchoalveolar lavage fluid of asthmatic patients, stimulates ASM to express and/or secrete many proinflammatory mediators such as cytokines, chemokines, growth factors, and adhesion molecules known to be involved in asthma (Amrani et al., 2000a; Chung, 2000). The mechanisms underlying TNFα-induced synthetic responses of ASM have not been fully elucidated. TNFα initiates its pleiotropic action by binding to two receptors designated as p55 (TNFR1) and p75 (TNFR2) according to their apparent molecular mass. These receptors are coexpressed on the surface of most cells (for review, see Tartaglia and Goeddel, 1992). Although both TNFR1 and TNFR2 were found to be coexpressed on ASM cells and in native tissues (Amrani et al., 1996, 2000b), the majority of TNFα effects on ASM are mediated by TNFR1 (for review, see Amrani et al., 2000a). TNFR1 was shown to regulate TNFα-induced potentiation of agonist-evoked calcium signals, ASM cell proliferation (Amrani et al., 1996), and expression of adhesion molecules (Amrani et al., 2000b). Whether TNFR1 and/or TNFR2 activation

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ABBREVIATIONS: ASM, airway smooth muscle; TNFα, tumor necrosis factor-α; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; IL, interleukin; PDGF, platelet-derived growth factor; ICAM-1, intercellular adhesion molecule-1; RANTES, regulated on activation, normal T-cell expressed and secreted; NF-κB, nuclear factor-κB; FBS, fetal bovine serum; BSA, bovine serum albumin; COX, cyclooxygenase.

coordinately regulates ASM synthetic function remains unclear. Furthermore, the downstream signaling that mediates TNFα-induced ASM synthetic function is not clearly understood.

Mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases, consist of at least three distinct members: extracellular signal-regulated kinase (ERK, also called p42/p44 MAPK), p38 MAPK, and c-Jun NH_2-terminal kinase (Davis, 1994). MAPKs regulate a variety of cellular responses, including inflammation, cell cycle progression, proliferation, and differentiation (for review, see Cowley et al., 1994). Recently, we and others have shown that p38 and p42/44 MAPKs are activated by a variety of proinflammatory agents such as cytokines (TNFα and IL-1β), growth factors (epidermal growth factor and PDGF), or contractile agonists (histamine and thrombin) (Orsini et al., 1999; Page et al., 1999). Collectively, these studies support the notion that MAPKs play an essential role in modulating contractile, proliferative, or synthetic responses in ASM cells. To better define the role of MAPK activation in modulating ASM-induced synthetic responses, we examined the role of p38 and p42/44 in TNFα-induced ICAM-1 expression and IL-6 and RANTES secretion. In separate experiments, we also asked whether p38 and p42/44 modulate TNFα-induced NF-κB activation because NF-κB seems to be critically important in regulating cytokine-induced ICAM-1 and IL-6 secretion in ASM cells and other cell types (Roebuck et al., 1995; Sanceau et al., 1995; Amrani et al., 1999). In addition, we investigated the contribution of TNFα receptor subtypes in these responses.

We report that TNFR1 and TNFR2 initiate similar cellular responses when activated individually using specific agonistic antibodies. However, we found that TNFα-induced ICAM-1 expression, RANTES, and IL-6 secretion are mediated primarily via TNFR1. P38 and p42/44 pathways differentially regulate these cellular responses. In addition, although TNFα activated p38 and p42/44, inhibition of p42/44 had little effect on TNFα-induced IL-6 and RANTES secretion. Inhibition of p38 only partially affected these responses. Our data support the concept that TNFα-induced synthetic responses are mediated primarily via the actions of TNFR1 and occur via MAPK-independent and -dependent pathways.

Materials and Methods

Human Airway Smooth Muscle Cell Culture. Human trachea was obtained from lung transplant donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was removed under sterile conditions and the trachealis muscle isolated. The muscle was then centrifuged and resuspended in 10 ml of buffer containing 0.2 mM $CaCl_2$, 640 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 10 U/ml elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 105-μm Nytex mesh, and the filtrate was washed with equal volumes of cold Ham's F-12 medium supplemented with 10% FBS (Hyclone Laboratories, Logan, UT). Aliquots of the cell suspension were plated at a density of 1.0×10^4 cells/cm^2. The cells were cultured in Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml amphotericin B, and this was replaced every 72 h. Human ASM cells in subculture during the second through fifth cell passages were used because, during these cell passages, the cells retain native contractile protein expression, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin.

Flow Cytometry. Flow cytometric analysis was performed as described previously (Amrani et al., 1999). Human ASM cells were stained using either a fluorescein isothiocyanate-conjugated monoclonal antibody specific for ICAM-1 or an isotype-matched control (R & D Systems, Minneapolis, MN). Samples were then analyzed using an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). ICAM-1 expression was expressed as the increase in mean fluorescence intensity over background.

Measurement of IL-6 and RANTES Secretion by ASM Cells. Near-confluent, growth-arrested human ASM cells were pretreated with SB203580, SB202474 (negative congener), or with diluent for 30 min, before stimulation with TNFα. In parallel experiments, cells were exposed to TNFR1, TNFR2 agonistic antibody (R & D Systems and Cell Sciences, Inc., Norwood, MA, respectively), or isotype-matched goat or mouse IgG (R & D Systems). In experiments with receptor-blocking antibodies, cells were first preincubated with either anti-TNFR1 or anti-TNFR2 (R & D Systems) for 30 min before addition of TNFα. After 24 h, cell supernatants were harvested and IL-6 or RANTES measured by an enzyme-linked immunosorbent assay according to the manufacturer's instructions (R & D Systems).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Immunoblot analysis for p38 and p42/44 was performed as described previously (Amrani et al., 1999): Briefly, ASM cells were washed with cold phosphate-buffered saline and resuspended in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 10 μg/ml aprotinin and leupeptin. Proteins were analyzed on a 12.5% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The membranes were blocked in 3% BSA in Tris-buffered saline then incubated with a rabbit monoclonal IgG against the phosphorylated form of p38 or p42/44 (Cell Signaling, Beverly, MA). After incubation with the appropriate peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals, Minneapolis, MN), the bands were visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) and autoradiographed.

Transfection of Human ASM Cells. Transfection of human ASM cells was performed as described previously (Amrani et al., 1999). Briefly, 4×10^6 cells were harvested and resuspended in 5 ml of Dulbecco's modified Eagle's medium [containing 200 μg of DEAE-dextran, 3×10^8 plaque-forming units of Ad5-GPT, and 10 μg of pNF-κB-Luc designed for monitoring activation of NF-κB (CLONTECH, Palo Alto, CA)] and 2 μg of pSV-b-galactosidase control vector to normalize transfection efficiencies (Promega, Madison, WI). The mixture was added to cells grown on 10-cm tissue culture plates and incubated for 2 h at 37°C. The media were then removed and the cells were washed for 1 min with 10% dimethyl sulfoxide in calcium- and magnesium-free phosphate-buffered saline and incubated with Ham's F-12 medium for 48 h. Cells were then rendered quiescent in medium containing 0.2% FBS for 16 h and exposed to TNFα for 4 h in the absence or the presence of inhibitors or receptor-blocking antibodies. Cells were then harvested, and luciferase and β-galactosidase activities were assessed using a Promega kit according to the manufacturer's instructions.

Immunostaining of TNFα Receptors on Human ASM Cells. ASM cells were washed with HEPES buffer containing 137.5 mM NaCl, 1.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 6 mM KCl, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA. The cells were fixed with 4% paraformaldehyde solution for 30 min at 4°C and then blocked in HEPES buffer (supplemented with 0.1% BSA) for 30 min at room temperature. ASM cells were then incubated with either anti-TNFR1 (htr-9) or anti-TNFR2 (utr-1) antibodies (Bachem Biosciences, King of Prussia, PA) for 120 min at 37°C. Negative controls included cells incubated with a mouse isotype IgG1 control (R & D Systems). After three washings, cells were incubated with a goat anti-mouse Alexa 594 (Molecular Probes, Eugene, OR). To stain the

nucleus, cells were then exposed to 1/5000 dilution of 4,6-diamidino-2-phenylindole (2 mg/ml). After washing, the glass coverslips were mounted onto glass slides, examined under epifluorescence microscopy (Nikon, Tokyo, Japan), and photographed.

Statistical Analysis. Statistical analysis was calculated using the Student's *t* test for paired values (two-tailed test). Values were considered statistically significant if the probability (*P*) of chance alone causing the effect was less than 5%.

Results

Effect of SB203580 and PD98059 on TNFα-Induced ICAM-1 Expression. TNFα is known to be a potent stimulus for activation of p38 and p42/44 MAPK (for review, see Ono and Han, 2000). To address the role of TNFα-induced p38 and p42/44 activation in modulating cytokine-induced ICAM-1 expression, cell monolayers were stimulated with TNFα in the presence and absence of SB203580, a p38 MAPK inhibitor; SB202474, an inactive congener; or with PD98059, a p42/44 inhibitor. As shown in Fig. 1A, TNFα markedly induced ICAM-1 expression, and in a dose-dependent manner, SB203580 augmented TNFα-induced ICAM-1 expression. TNFα alone induced a 5.3 ± 0.5-fold increase in ICAM-1 expression at 4 h, and in the presence of 1 and 10 μM SB203580, there was a 7.8 ± 0.4- and 9.1 ± 0.7-fold increase in ICAM-1 expression compared with cells that were diluent-treated ($P < 0.05$, $n = 3$; Fig. 1A). Treatment of human ASM cells with SB202474, however, had little effect on TNFα-induced ICAM-1 expression as shown in Fig. 1A. Similar effects were also seen at 24-h incubation where SB203580 also increased TNFα-induced ICAM-1 expression (treated 18 ± 2.3 versus control 12 ± 1.2, $n = 4$). In contrast, PD98059, an inhibitor of $p42/44^{MAPK}$, induced a 52 ± 5% decrease in TNFα-induced ICAM-1 expression (Fig. 1B). The effect of PD98059 on TNFα-induced ICAM-1 expression at 24 h was unchanged (treated 5 ± 1.5 versus control 11 ± 2.7, $n = 3$, $P < 0.05$, $n = 4$). This suggests that the effect of both inhibitors on ICAM-1 expression in ASM cells is unaffected by the exposure time to TNFα. Together, these data suggest that p38 MAPK may act as a negative regulator of TNFα-induced ICAM-1 expression, whereas activation of p42/44 partially mediates TNFα-induced ICAM-1 expression.

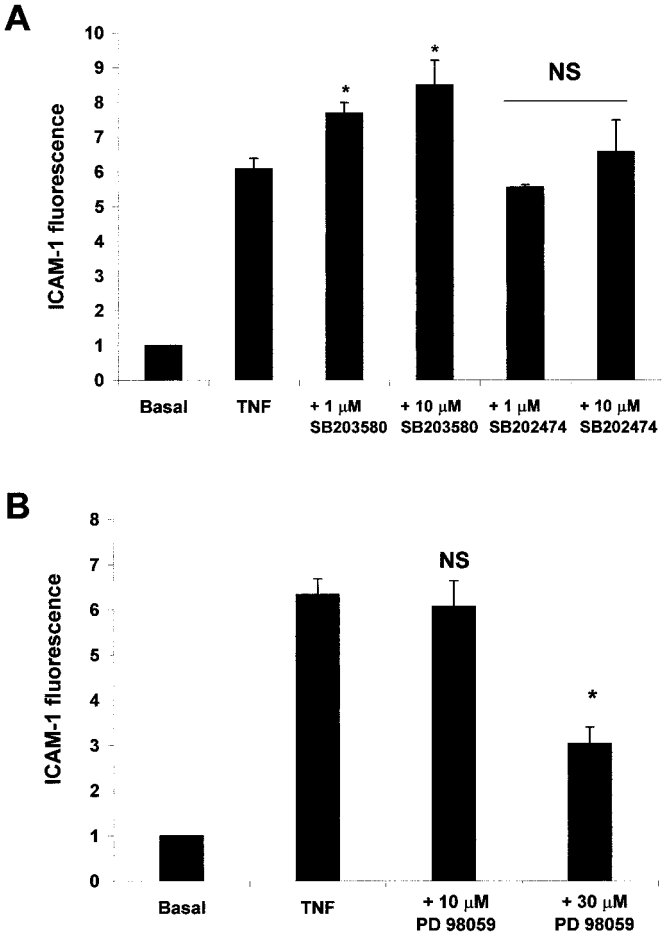

Fig. 1. Effect of SB203580 and PD98059 on TNFα-induced ICAM-1 expression. ASM cells were incubated with the indicated concentrations of SB203580, SB202474, or PD98059 for 30 min and then stimulated with 10 ng/ml TNFα for 4 h. ICAM-1 expression was assessed as described under *Materials and Methods*. Values shown are mean ± S.E.M. and are significantly different from basal, $n = 3$ different experiments. *$P < 0.05$ significant from cells treated with cytokine alone. N.S., nonsignificant compared with cells treated with cytokine alone.

Effect of SB203580 and PD98059 on TNFα-Induced IL-6 and RANTES Secretion. Human airway smooth muscle is a rich source of cytokines and chemokines that modulate airway inflammation. We and others previously reported that TNFα stimulates IL-6 and RANTES secretion in human ASM cells (John et al., 1997; Ammit et al., 2000; McKay et al., 2000). To further dissect cellular and molecular signaling mechanisms that regulate cytokine secretion by ASM, studies were performed to address whether p38 and p42/44 MAPK activation plays a role in regulating TNFα-induced IL-6 and RANTES secretion. Cells were treated with TNFα in the presence and absence of SB203580 or PD98059, and after 24 h, IL-6 and RANTES levels were measured by enzyme-linked immunosorbent assay. Maruoka et al. (2000) showed that in human ASM cells, SB203580 and PD98059 abrogated p38 and ERK activity, respectively. Similar observations were also obtained in our laboratory (data not shown). At baseline, ASM cells secrete little IL-6, but after stimulation with TNFα, there is an approximately 37-fold increase in IL-6 secretion (Fig. 2A). TNFα-induced IL-6 levels were reduced by 40% in cells treated with SB203580 from 3705 ± 222 to 1878 ± 112 pg/ml ($P < 0.01$, $n = 5$; Fig. 2A), whereas the inactive congener SB202474 had no effect. TNFα-induced RANTES was also partially reduced by SB203580 (25%) with levels from 13,362 ± 682 to 10,092 ± 1,398 pg/ml, respectively ($P < 0.05$, $n = 5$; Fig. 2A). In contrast, TNFα-induced IL-6 (Fig. 2A) and RANTES (Fig. 2B) secretion were unaffected by pretreating cells with PD98059 (N.S., $n = 5$). Thus, although TNFα-induced ICAM-1 expression seems to be ERK-dependent and p38-independent, other synthetic responses such as IL-6 and RANTES secretion are p38 MAPK-dependent and p42/44-independent.

Effect of SB203580 and PD98059 on TNFα-Induced NF-κB Activation. We have previously shown that NF-κB activation is critically important in TNFα-induced ICAM-1 expression and that both TNFα and IL-1β activate NF-κB in human ASM cells (Amrani et al., 1999). Because p38 MAPK inhibition seemed to modulate TNFα-induced synthetic functions, we postulated that p38 MAPK may affect cytokine-induced NF-κB activation. Cells were transfected with a NF-κB reporter construct then stimulated with TNFα in the presence or absence of SB203580 or PD98059. As shown in

Fig. 3, TNFα induced a 7.2 ± 0.7-fold increase in NF-κB reporter activity at 4 h compared with cells treated with diluent alone. SB203580 used at 1 or 10 μM had no significant effect on TNFα-induced NF-κB reporter activation (N.S., $n = 3$; Fig. 3A). In contrast, PD98059 modestly inhibited TNFα-induced NF-κB reporter activation by 16 ± 4% compared with cells treated with TNFα and diluent alone ($P < 0.01$, $n = 3$; Fig. 3B). Collectively, these data suggest that cytokine-induced effects that are MAPK-dependent are not mediated by NF-κB activation in ASM cells.

TNFR1 and TNFR2 Engagement Stimulates IL-6 and RANTES Secretion. Recent evidence suggests that TNFR1 plays an important role in mediating TNFα effects on ICAM-1 expression and calcium signaling (for review, see Amrani et al., 2000a). To further dissect the potential role of TNFR1 in TNFα-induced synthetic function, we studied the effect of an activating antibody against TNFR1 on IL-6 and RANTES secretion. We found that treatment of cells with the TNFR1-activating antibody at 2 and 5 μg/ml induced a dose-dependent increase in IL-6 levels of 3654 ± 245 and 4395 ± 223 pg/ml, respectively ($P < 0.01$, $n = 3$; Fig. 4A). The TNFR1 agonistic antibody also stimulated RANTES secretion in a dose-dependent manner, stimulating levels of 3566 ± 347 and 5456 ± 1328 pg/ml in cells treated with 2 and 5 μg/ml antibody, respectively ($P < 0.01$, $n = 3$; Fig. 4B). At the same concentrations, the isotype-matched antibody had no effect on cytokine secretion (Fig. 4, A and B). To test whether TNFR2 has functional properties in human ASM cells, we studied the effect of an agonistic antibody against TNFR2 cells on IL-6 and RANTES secretion. The agonistic properties of this antibody have been well described in several previous studies (Leeuwenberg et al., 1994; De Cesaris et al., 1999). The specificity of the agonistic TNFR2 antibody was tested by immunoblot analysis and showed that this antibody does not cross-react with the 55-kDa band recognized by the anti-TNFR1 antibody (data not shown). We found that treatment of cells with TNFR2-activating antibody (10 μg/ml, a concentration used in those previous studies) stimulated IL-6 secretion to levels of 2994 ± 689 pg/ml ($P < 0.01$, $n = 3$; Fig. 4A). The TNFR2 agonistic antibody also induced RANTES secretion to levels of 3266 ± 558 pg/ml ($P < 0.01$, $n = 3$; Fig. 4B).

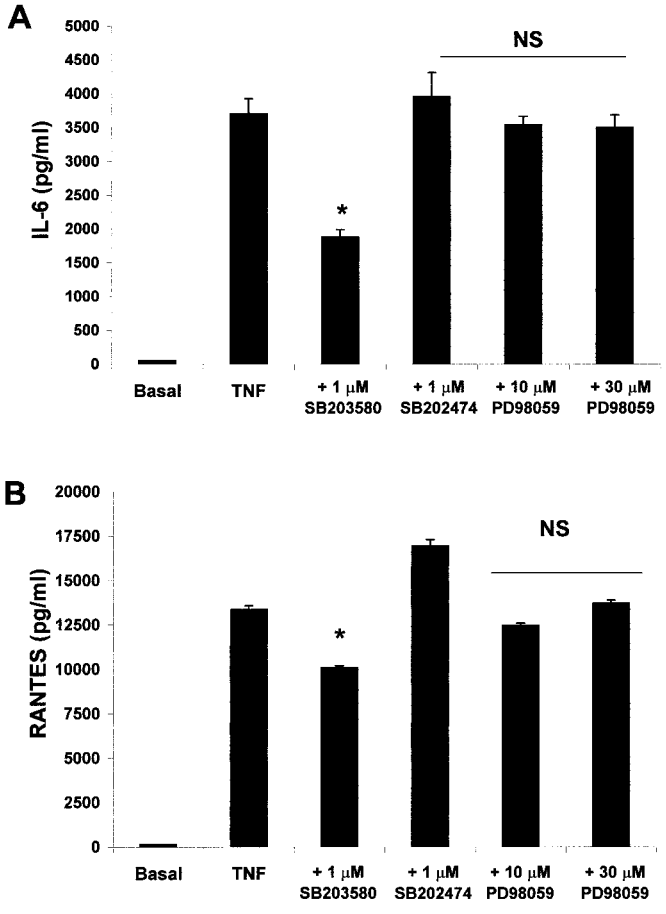

Fig. 2. Effect of SB203580 and PD98059 on TNFα-induced IL-6 and RANTES secretion. ASM cells were incubated with 1 μM SB203580, SB202474, or the indicated concentration of PD98059 for 30 min and then stimulated with 10 ng/ml TNFα for 24 h. IL-6 (A) or RANTES (B) secretion in supernatant was then assessed as described under *Materials and Methods*. Values shown are mean ± S.E.M. and are significantly different from basal, $n = 5$ different experiments. *$P < 0.05$ significant from cells treated with cytokine alone. N.S., nonsignificant compared with cells treated with cytokine alone.

TNFα-Induced IL-6 and RANTES Secretion via TNFR1. To further investigate the relative contribution of TNFR1 and TNFR2 in mediating TNFα-induced synthetic functions, we measured IL-6 and RANTES secretion in ASM cells pretreated with receptor-blocking anti-TNFR1 or anti-TNFR2 antibodies (Murray et al., 1997; Murakami-Mori et al., 1999). As shown in Fig. 5, A and B, neutralizing anti-TNFR1 significantly inhibited TNFα-induced RANTES and IL-6 secretion with 95 and 50% inhibition at 20 μg/ml antibody, respectively ($P < 0.05$, $n = 3$). Neutralizing anti-TNFR2 also partially abrogated TNFα-induced RANTES (50% inhibition) but had little effect on IL-6 secretion. Increasing the concentration of neutralizing anti-TNFR2 to 20 μg/ml did not increase this inhibitory effect (data not shown). Surprisingly, we found that neutralizing anti-TNFR1, but not anti-TNFR2, stimulated IL-6 secretion ($P < 0.05$, $n = 3$; Fig. 5A), suggesting that this blocking antibody also exhibits agonistic activity. No notable agonistic effect, however, was observed on RANTES levels (Fig. 5B). These data suggest that the inability of blocking anti-TNFR1 antibodies to fully prevent the IL-6 secretion induced by TNFα may be due to its partial agonistic effect.

Engagement of TNFR1 or TNFR2 Activates p38, p42/44 MAPKs, and NF-κB Pathways. Although there is growing evidence supporting a role for MAPKs in TNFα-induced biological activities, relatively little is known about the TNFα receptor subtype involved in MAPK regulation in ASM cells. We found that engagement of TNFR1 and TNFR2 alone by using agonistic antibodies stimulates p38 and p42/44 pathways as demonstrated by the phosphorylation of p38 and p42/44 (Fig. 6A). Agonistic antibodies specific for TNFR1 and TNFR2 also activated NF-κB-dependent gene transcription, inducing 6.1 ± 0.9- and 3.6 ± 0.4-fold increases over basal, respectively ($n = 3$; Fig. 6B). Pretreating cells with blocking anti-TNFR1 antibodies, but not with anti-TNFR2, or an isotype control IgG (data not shown), almost completely abrogates TNFα-induced phosphorylation of p38 and p42/44 MAPKs (Fig. 7A). In addition, receptor-blocking anti-TNFR1 antibody but not anti-TNFR2 antibody reduced TNFα-induced NF-κB-dependent gene transcription by 56% (Fig. 7B). Similar to its effects on IL-6 secretion, we found that blocking anti-TNFR1 antibody, but not blocking anti-TNFR2 antibody, stimulates NF-κB–dependent gene transcription (3.86 ± 0.25-fold increase

over basal, $n = 6$; Fig. 7B), explaining the lack of neutralizing anti-TNFR1 antibodies to completely prevent TNFα effects on NF-κB reporter constructs. Collectively, these studies show that TNFα regulates p38, p42/44 MAPKs and NF-κB activation in ASM cells by mainly activating TNFR1.

Expression of TNFR1 or TNFR2 on Human ASM Cells. In a previous report, we showed the expression of both TNFR1 and TNFR2 in cultured ASM cells (Amrani et al., 1996). To extend those studies, we now use a modified immunocytochemical technique to define the cell surface expression of TNFR1 and TNFR2. We found that TNFR1 seems to be distributed evenly over the plasma membrane in a punctuate manner with increasing expression perinuclear (Fig. 8). Interestingly, TNFR2 expression is more located to the edge of the cells with little expression in the perinuclear region. We also noted that 80% of the cultured cells do express the TNFR2 receptor, whereas 90% express positively for TNFR1 (data not shown). Together, these data show that both TNFα receptors are differentially expressed on the plasma membrane of ASM cells.

Discussion

Airway smooth muscle, the primary effector cell that regulates bronchomotor tone in asthma, may modulate airway inflammatory responses by secreting cytokines, chemokines, and growth factors or by expressing adhesion molecules (for review, see Chung, 2000). Although cytokines, such as TNFα, serve as important mediators in modulating ASM synthetic function, the molecular mechanisms regulating cytokine-induced ASM function are not fully understood. We have shown that TNFα stimulates IL-6 and RANTES secretion in ASM cells primarily via TNFR1, despite the ability of TNFR2 to also stimulate similar responses. We also report that TNFR1 stimulation activates p38 and ERK MAPKs, which differentially modulate TNFα-induced synthetic functions in ASM cells.

A number of studies have demonstrated the involvement of ERK MAPKs in modulating ASM cell proliferation. We and others have previously shown that the mitogenic effect induced by epidermal growth fator, PDGF, or the contractile

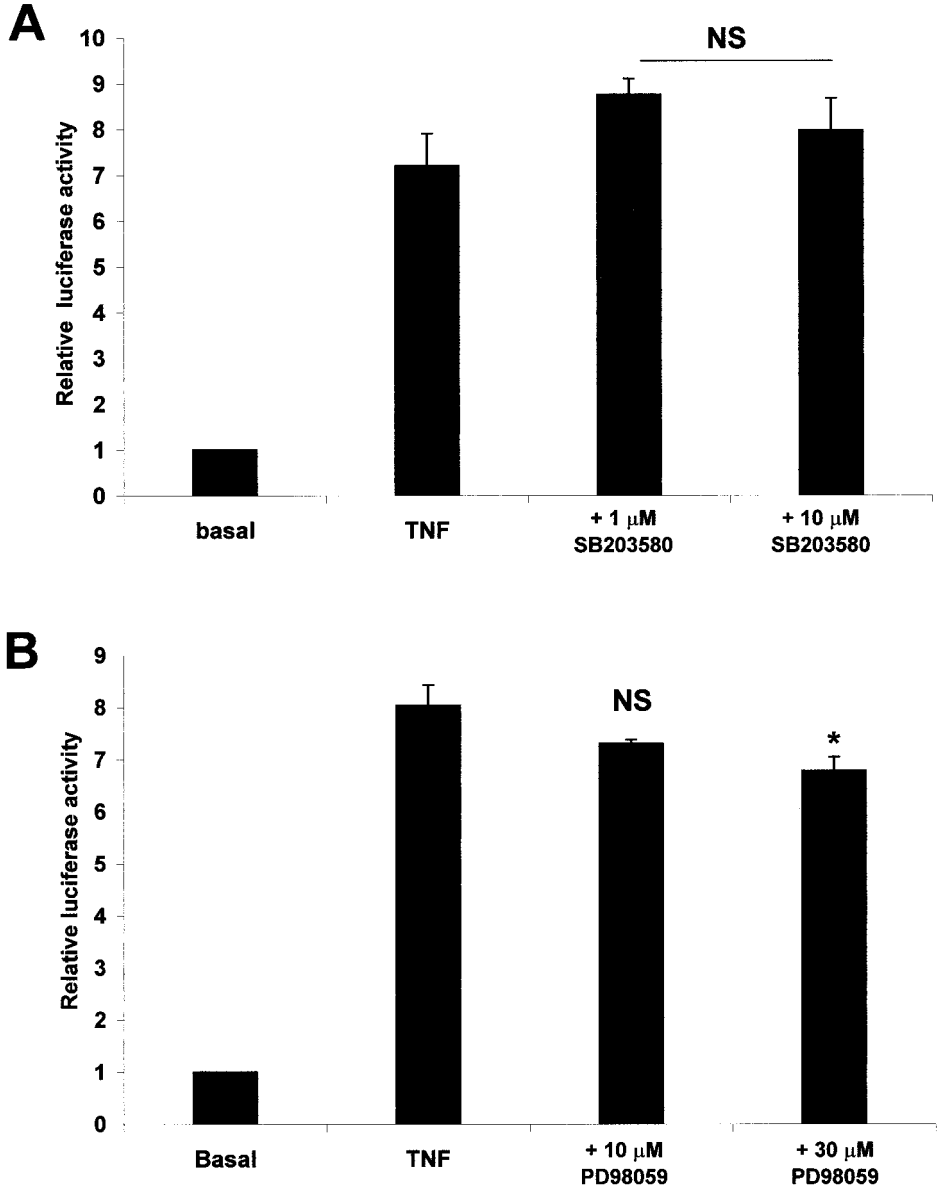

Fig. 3. Effect of SB203580 and PD98059 on TNFα-induced NF-κB activation. ASM cells transfected with NF-κB-luciferase reporter construct were incubated for 30 min with the indicated concentration of SB203580 or PD98059 and then stimulated with 10 ng/ml TNFα for 4 h. Luciferase activity in cell extracts was normalized for β-galactosidase activity as described under *Materials and Methods*. Data represent normalized luciferase activity relative to untreated cells and are representative of three different experiments performed in duplicate. $^*P < 0.05$ significant from cells treated with cytokine alone. N.S., nonsignificant compared with cells treated with cytokine alone.

agonist thrombin correlates with a strong and sustained activation of p42/44 (Orsini et al., 1999; Rakhit et al., 2000; for review, see Page and Hershenson, 2000). TNFα and IL-1β also modulate cell proliferation in human ASM cells (Stewart et al., 1995; Amrani et al., 1996). Recently, Yang and colleagues (Luo et al., 2000) showed that in canine ASM cells, mitogenic responses induced by these cytokines were completely abrogated by the p42/44 inhibitor PD98059. Together, these data suggest that p42/44 MAPK activation represents a common pathway used by a variety of stimuli to regulate ASM cell mitogenesis.

The role of p42/44 MAPK in synthetic functions is not as well delineated. In a recent report, we showed that TNFα is a potent stimulator of IL-6 and RANTES secretion (Ammit et al., 2000). Here, we show that PD98059 did not prevent cytokine-induced IL-6 and RANTES expression. These data are in agreement with those of Maruoka et al. (2000) and suggest that the p42/44 MAPK pathway plays a minor role in synthetic function induced by TNFα in ASM cells. In contrast, PD98059 was found to suppress platelet-activating factor-induced chemokine release in ASM cells and in human lung fibroblasts (Hayashi et al., 2000; Maruoka et al., 2000). These differences may be explained, in part, by the fact that cellular responses are highly stimuli-specific, whereas cytokines, such as TNFα, and G protein-coupled receptor-activating agonists, such as bradykinin, may differentially signal to regulate similar cellular responses in a particular cell type.

We have demonstrated that TNFα activates p38 MAPK in ASM cells and that inhibition of p38 MAPK by SB203580 partially suppresses TNFα-induced IL-6 and RANTES secretion. Other investigators have shown that SB203580 not only partially inhibits RANTES secretion stimulated by platelet-activating factor but also prevented ASM migration in response to PDGF and IL-1β, respectively (Hedges et al., 1999; Maruoka et al., 2000). Recently, Laporte et al. (2000) used SB203580 to demonstrate a possible role for a p38 MAPK pathway in IL-1β-induced COX-2 protein expression. A p38 MAPK pathway also seems to regulate the expression of proinflammatory cytokines such as IL-6 and IL-8 in response to Cytomix (Hedges et al., 2000). Together, these data support a role for p38 MAPK in regulating ASM synthetic functions induced by proinflammatory cytokines and by G protein-coupled receptor-activating agonists. Similar observations were made in bronchial epithelial cells or synovial fibroblasts where p38 MAPK seems to play

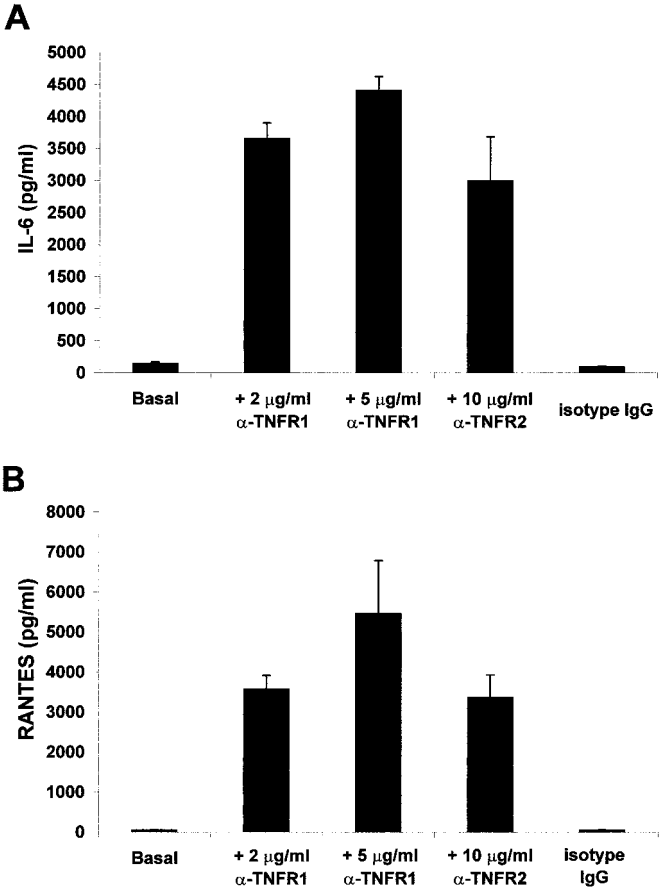

Fig. 4. Selective activation of TNFR1 or TNFR2 stimulates IL-6 and RANTES secretion. ASM cells were incubated with the indicated concentrations of anti-TNFR1, anti-TNFR2 agonistic antibodies, or isotype control IgG for 24 h. IL-6 (A) or RANTES (B) levels in supernatant were then assessed as described under *Materials and Methods*. Values shown are mean ± S.E.M. and are significant from basal (n = 3 different experiments, $P < 0.05$).

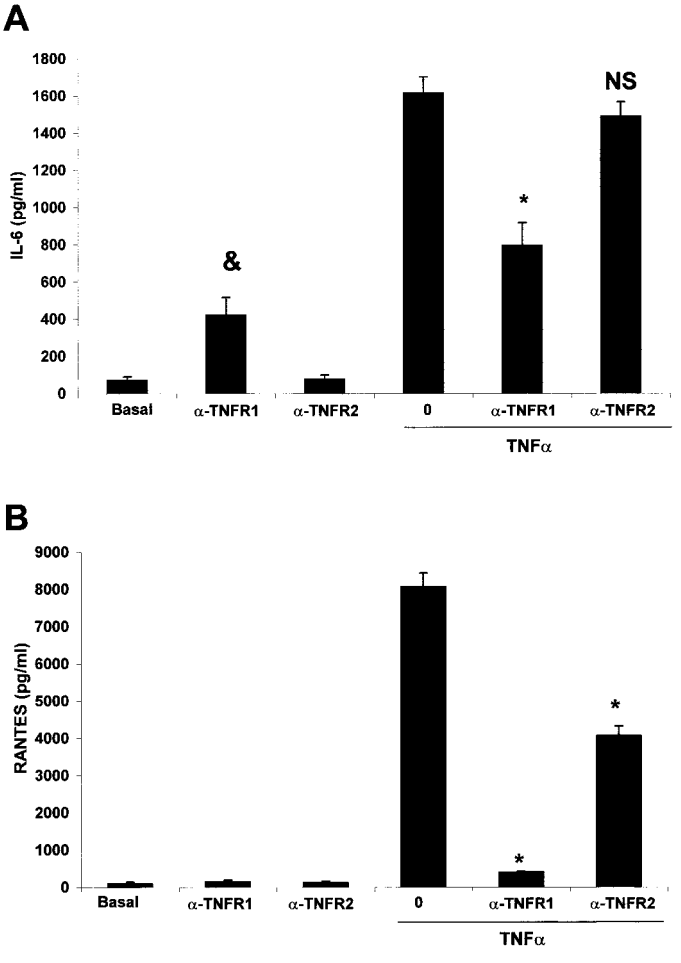

Fig. 5. TNFR1 and TNFR2 are involved in mediating TNFα-induced IL-6 and RANTES secretion. ASM cells were incubated for 24 h with TNFα (1 ng/ml) or in the presence or the absence of neutralizing anti-TNFR1 (20 μg/ml) or anti-TNFR2 (10 μg/ml) antibodies at the indicated concentrations added 1 h before TNFα. IL-6 (A) or RANTES (B) secretion in supernatant was then assessed as described under *Materials and Methods*. Values shown are mean ± S.E.M. of three independent experiments. Values are significantly different from basal, $^{*}P < 0.05$ and $^{\&}P < 0.05$ compared with cells treated with TNFα or with diluent, respectively. N.S., nonsignificant compared with cells treated with cytokine alone.

a role in TNFα- and IL-1β-induced secretion of IL-6, IL-8, and RANTES (Hashimoto et al., 2000; Suzuki et al., 2000).

The effect of p38 MAPK on TNFα-induced ICAM-1 expression is likely to be more complex. In the present study, SB203580 partially increased cytokine-induced ICAM-1 expression, suggesting that p38 MAPK may act as a negative regulator of ICAM-1 expression. In endothelial cells, as well as in epithelial cells, SB203580 was found to have no effect on ICAM-1 expression induced by TNFα or IL-1β (Pietersma et al., 1997; Chen et al., 2000). The reasons underlying the differential action of p38 MAPK on cytokine-induced synthetic functions (inhibiting IL-6 and RANTES and slightly enhancing ICAM-1 response) are yet not known. The involvement of COX-2 is unlikely because 1) indomethacin was found to have no effect on cytokine-induced IL-6 or RANTES secretion (Ammit et al., 2000), and 2) TNFα does not induce COX-2 expression in ASM cells (Amrani et al., 1999). In cardiac myocytes, it has been shown that p38 MAPK regulates NF-κB activation to mediate TNFα-induced IL-6 gene expression (Craig et al., 2000). In human synovial fibroblasts, SB203580 suppressed IL-6 secretion in response to TNFα or IL-1β without affecting NF-κB activation (Suzuki et al., 2000). ASM cells transfected with a κB luciferase reporter construct allowed us to demonstrate that TNFα-induced NF-κB activation was insensitive to SB203580. This is important because we showed that activation of NF-κB is required for cytokine-induced ICAM-1 expression (Amrani et al., 1999). Together, these data suggest that p38 MAPK modulates ICAM-1 gene expression in human ASM cells by involving NF-κB-independent pathways. Because the transcriptional regulation of ICAM-1 and other genes, such as IL-6, involves the cooperation of multiple transcription factors (Roebuck et al., 1995; Sanceau et al., 1995), it is plausible that the p38 MAPK pathway may use other transcription factors to regulate cytokine-induced ICAM-1 expression in human ASM cells.

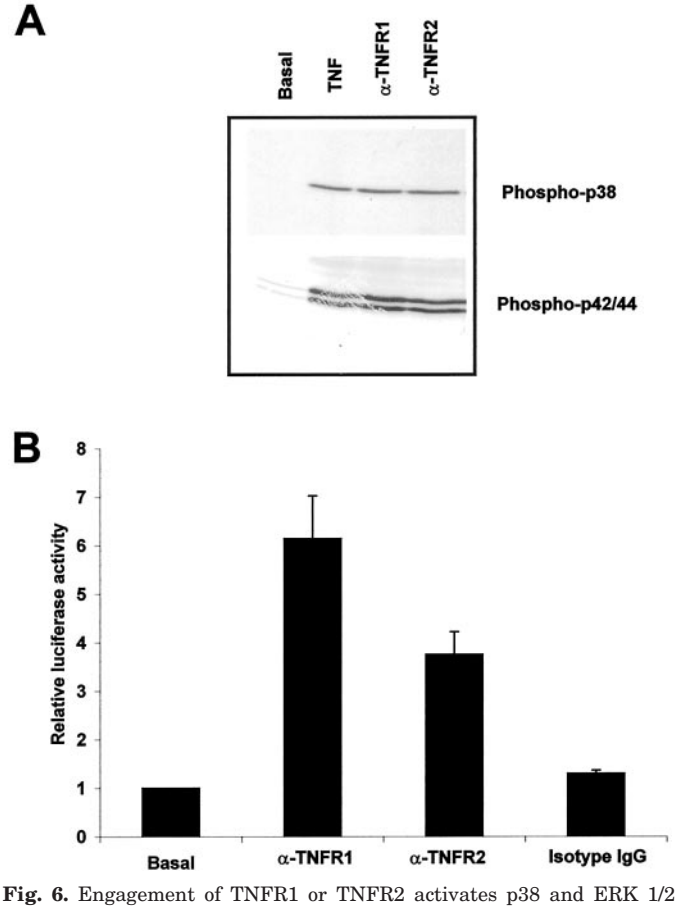

Fig. 6. Engagement of TNFR1 or TNFR2 activates p38 and ERK 1/2 MAPKs and NF-κB pathways. A, ASM cells were incubated for 20 min with agonistic antibodies anti-TNFR1 (2 μg/ml), anti-TNFR2 (10 μg/ml), or TNFα (1 ng/ml). Cells were lysed and cytoplasmic extracts were prepared and assayed for the phosphorylated form of p38 and p42/44 MAPK by immunoblot analysis as described under *Materials and Methods*. Results are representative of three separate blots. B, in separate experiments, ASM cells were also preincubated with both agonistic antibodies for 4 h before assessing NF-κB activation. Luciferase activity in cell extracts was normalized for β-galactosidase activity as described under *Materials and Methods*. Data represent normalized luciferase activity relative to untreated cells and are representative of three different experiments performed in duplicate.

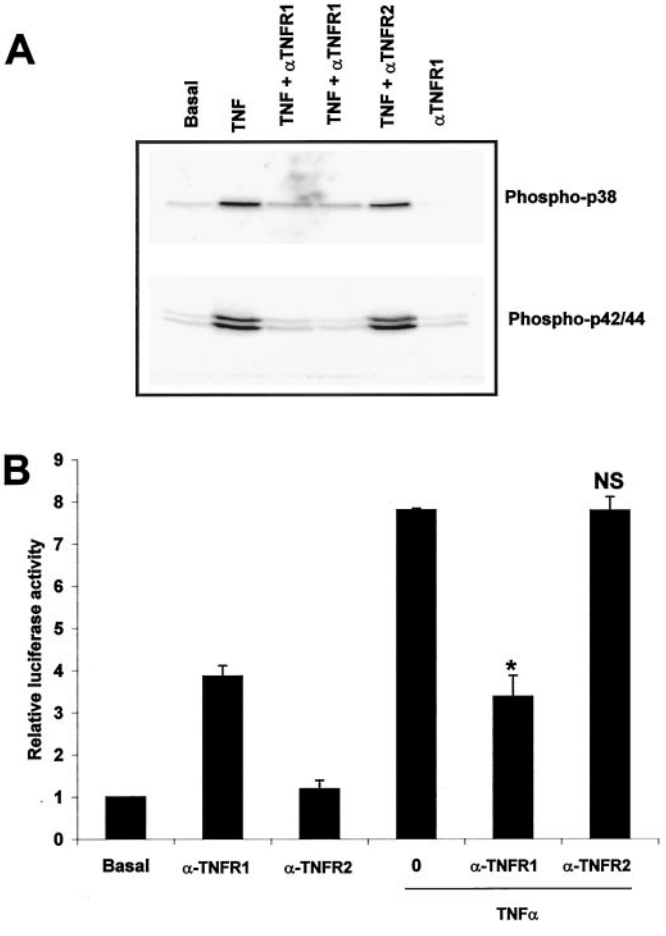

Fig. 7. TNFα regulates p38, ERK 1/2, and NF-κB activation via activation of the TNFR1 receptor. A, ASM cells were incubated for 1 h with neutralizing antibodies to anti-TNFR1 (10 or 20 μg/ml) or anti-TNFR2 (10 μg/ml) before exposing cells to TNFα (1 ng/ml) for 20 min. Cells were lysed and cytoplasmic extracts were prepared and assayed for the phosphorylated form of p38 and p42/44 by immunoblot analysis as described under *Materials and Methods*. Results are representative of three separate blots. B, ASM cells transfected with NF-κB-luciferase reporter construct were incubated for 4 h with 1 ng/ml TNFα in the presence or absence of neutralizing anti-TNFR1 (20 μg/ml) or anti-TNFR2 (10 μg/ml) antibodies added 1 h before. Luciferase activity in cell extracts was normalized for β-galactosidase activity as described under *Materials and Methods*. Data represent normalized luciferase activity relative to untreated cells and are representative of three separate experiments. $P <$ 0.05 significant from cells treated with TNFα alone. N.S., nonsignificant compared with cells treated with cytokine alone.

The observation that TNFα induces synthetic responses that are differentially regulated by p38 and ERK inhibitors suggests that these synthetic responses may be initiated by different TNFα receptor subtypes. To date, two TNFα receptors have been identified with molecular masses of p55 and p75 (for review, see Tartaglia and Goeddel, 1992). We have previously shown that in human ASM cells, TNFα exerts its biological activities mainly via the TNFR1 receptor, despite the presence of TNFR2 on the cell surface of human ASM in vivo (for review, see Amrani et al., 2000a). To elucidate the TNFα receptor subtype involved in cytokine-induced cellular responses, we used agonistic and antagonistic antibodies (Leeuwenberg et al., 1994; Murray et al., 1997; De Cesaris et al., 1999; Murakami-Mori et al., 1999). Although activation of TNFR1 or TNFR2 alone stimulates NF-κB, p38, and p42/44 MAPK pathways, we demonstrated that TNFR1 was the predominant receptor stimulating those signaling events. The role of TNFR1 in regulating p38 and p42/44 MAPK has been described previously in other cell types such as human Kaposi's sarcoma cells (Murakami-Mori et al., 1999) and mouse L929 sA (Brinkman et al., 1999).

The ability of each TNFα receptor subtype to mimic TNFα-induced cellular responses is surprising because both receptors, which have distinct signaling cascades, regulate separate functions in various cell types (for review, see Wallach et al., 1999). In Sertoli cells, the use of similar agonistic antibodies showed that TNFα-induced p38 MAPK, p42/44 MAPK, and c-Jun NH_2-terminal kinase/stress-activated protein kinase predominately involves TNFR1 but not TNFR2 (De Cesaris et al., 1999). Several other studies, however, showed that both receptors activated alone have the potential to regulate similar biological activities. In human fibroblasts, agonistic antibodies that activate either TNFR1 or TNFR2 initiate similar cellular responses such as IL-6 and IL-8 expression (Butler et al., 1994). Baxter et al. (1999) also demonstrate that receptor-specific mutants induce comparable cytotoxic effects in human erythroleukemic cells, despite the major role played by TNFR2 in TNFα effects in this cell

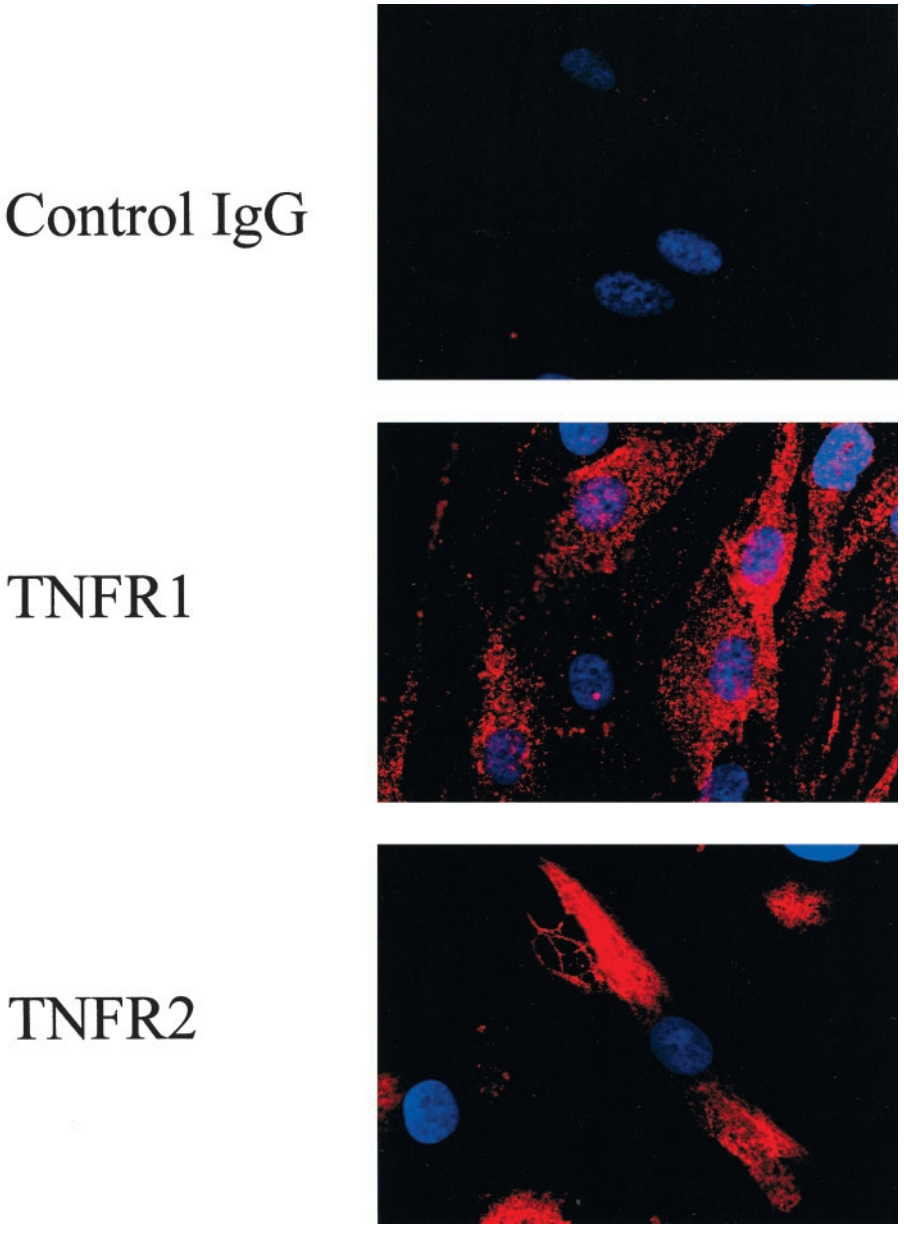

Fig. 8. Cellular distribution of TNFR1 and TNFR2 by immunofluorescence on human ASM cells. Cells were incubated with the optimal dilution (10 μg/ml) of the isotype-matched mouse control IgG1, anti-TNFR1, and anti-TNFR2. Cell surface expression of TNFα receptors and nucleus staining were then determined using a secondary Alexa 594-coupled antibody and 4,6-diamidino-2-phenylindole, respectively.

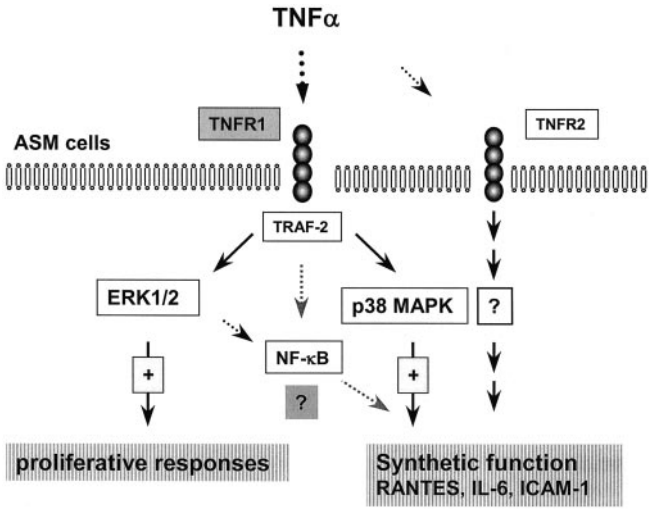

Fig. 9. Putative signaling pathways mediating TNFα-induced synthetic functions in human ASM. TNFα, via the TNFR1 receptor coupled to TRAF-2, activates both p38 MAPK and ERK 1/2 pathways. p38 MAPK activation by TNFα modulates either positively or negatively cytokine-induced synthetic responses. In contrast, ERK 1/2 activation plays a minor role in cytokine-induced cellular responses, an effect that is probably due to the modulation of the NF-κB-dependent pathway. Based on current evidence, ERK 1/2 pathway seems to play a more important role in regulating cell mitogenesis induced by TNFα. The physiological role of TNFR2-mediated activation of p38, ERK 1/2, and NF-κB pathways in TNFα effects on ASM cells remains to be defined.

line. Therefore, it remains possible that both TNFR1 and TNFR2 participate in the synthetic functions induced by TNFα in human ASM cells.

This latter hypothesis was further confirmed using receptor-blocking antibodies to both TNFα receptors to determine the relative contribution of TNFR1 and TNFR2 in TNFα-induced RANTES and IL-6 secretion. In human fibroblasts, both receptors were shown to be involved in TNFα-induced IL-6 and IL-8 secretion (Butler et al., 1994). Here, we found that TNFR1 seems to play a critical role in regulating RANTES and IL-6 secretion, the latter being insensitive to receptor-blocking anti-TNFR2. One possible explanation for the diminished sensitivity of cytokine-induced IL-6 secretion to receptor-blocking anti-TNFR1 antibodies is simply because those antibodies also have partial agonistic properties in our cells. This neutralizing anti-TNFR1 antibody was found to activate NF-κB pathways, suggesting that NF-κB may play a role in mediating TNFα-induced IL-6 gene expression as described recently in cardiac myocytes (Craig et al., 2000). Using neutralizing anti-TNFR2 antibodies, we found that TNFR2 is also involved in TNFα-induced RANTES secretion but not in IL-6 secretion. The mechanisms by which both TNFR1 and TNFR2 regulate TNFα-induced RANTES secretion in human ASM cells are yet unclear. In other cell types, investigators show that TNFR2 enhances cellular responses by activating common downstream signaling events triggered by TNFR1 (Murray et al., 1997) or by facilitating TNFα binding to TNFR1, a phenomenon known as ligand passing (Tartaglia et al., 1993). However, in ASM cells this phenomenon seems unlikely because most of the signaling pathways (p38, ERK MAPK, and NF-κB) and cellular responses (IL-6 and ICAM-1) activated by TNFα in ASM cells are mediated predominately by TNFR1 receptor activation. It is plausible that TNFR2 is involved in TNFα-induced RANTES secretion by involving a TNFR1-independent signaling pathway.

Although the MAPK inhibitors have been widely used to study the contribution of MAPK in ASM and other cell types, our present study does not exclude the possibility that inhibition of TNFα responses in ASM cells by SB203580 may result from "nonspecific effects". The use of p38 dominant negative or MKK6, an upstream activator of p38, may be necessary to confirm the role of p38 MAPK in TNF-induced synthetic functions. Studying the effect of such proteins on the activity of luciferase-tagged RANTES or IL-6 promoter (with their deletion constructs) will provide critical information about the transcriptional regulation of IL-6 and RANTES by p38 MAPK.

Together, our data show that TNFα regulates the intracellular signaling pathways such as NF-κB, p38, and ERK 1/2 MAPKs, mainly via TNFR1, despite the fact that TNFR2 also activates similar pathways when activated individually. A simplified diagram is provided to summarize the respective role of each MAPK in the regulation of ASM function induced by TNFα (Fig. 9). Further studies exploring the precise role of each TNFα receptor isotypes in the modulation of gene expression may offer now therapeutic approaches to inhibit the synthetic responses of ASM cells.

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