Phosphorylation of Tumor Necrosis Factor Receptor 1 (p55) Protects Macrophages from Silica-induced Apoptosis*

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Macrophages play a fundamental role in silicosis in part by removing silica particles and producing inflammatory mediators in response to silica. Tumor necrosis factor α (TNFα) is a prominent mediator in silicosis. Silica induction of apoptosis in macrophages might be mediated by TNFα. However, TNFα also activates signal transduction pathways (NF-κB and AP-1) that rescue cells from apoptosis. Therefore, we studied the TNFα-mediated mechanisms that confer macrophage protection against the pro-apoptotic effects of silica. We will show that exposure to silica induced TNFα production by RAW 264.7 cells, but not by IC-21. Silica-induced activation of NF-κB and AP-1 was only observed in RAW 264.7 macrophages. ERK activation in response to silica exposure was only observed in RAW 264.7 macrophages, whereas activation of p38 phosphorylation was predominantly observed in IC-21 macrophages. No changes in JNK activity were observed in either cell line in response to silica exposure. Silica induced apoptosis in both macrophage cell lines, but the induction of apoptosis was significantly larger in IC-21 cells. Protection against apoptosis in RAW 264.7 cells in response to silica was mediated by enhanced NF-κB activation and ERK-mediation phosphorylation of the p55 TNFα receptor. Inhibition of these two protective mechanisms by specific pharmacological inhibitors or transfection of dominant negative mutants that inhibit IκBα or ERK phosphorylation significantly increased silica-induced apoptosis in RAW 264.7 macrophages. These data suggest that NF-κB activation and ERK-mediated phosphorylation of the p55 TNF receptor are important cell survival mechanisms in the macrophage response to silica exposure.

Environmental exposure to crystalline silica induces pulmonary inflammation that often leads to fibrosis (1). Following phagocytosis of silica particles, macrophages are activated to release cytokines. Among the cytokines secreted by lung macrophages in response to silica, tumor necrosis factor α (TNFα) has been shown to play a critical role in the pathogenesis of silicosis, and procedures that antagonize the biological effects of TNFα ameliorate silica-induced pulmonary fibrosis in mice (2–4).

TNFα transduces its biological activities by binding to two receptors of 55 and 75 kDa (5, 6). Trimeric occupation of TNF receptors by the ligand results in the recruitment of receptor-specific proteins and promotes the activation of transcription factors that induce expression of TNFα-responsive genes (7, 8). Several lines of evidence support the importance of TNF receptors in the pathogenesis of silica-induced lung fibrosis. Following silica exposure an enhanced TNF receptor mRNA expression can be observed in the mouse lung (9). This enhanced TNF receptor expression correlates well with the activation of NF-κB and AP-1 in the lungs of silica-sensitive C57BL/6 mice (10). In contrast to wild-type mice, individual and double TNF receptor-deficient mice (developed on a C57BL/6 genetic background) are protected from the inflammatory and fibrotic effects of silica (9, 10).

Binding of TNFα to its p55 receptor triggers the activation of downstream signaling cascades that lead to apoptosis in a variety of cells (11). However, TNFα interaction with the p55 receptor also induces NF-κB activation that promotes the expression of pro-survival genes (12). Recent studies implicate macrophage apoptosis as an important element in the pathogenesis of silicosis (13, 14). Silica induces apoptosis of human alveolar macrophages in vitro (15). In addition, the number of apoptotic cells in bronchoalveolar lavage fluid from silica-treated rats is significantly higher than in control-treated animals (16). The importance of TNFα-mediated signal transduction and in particular the role of NF-κB and AP-1 activation in silica-induced apoptosis in macrophages is not completely understood.

TNFα induces AP-1 activation via phosphorylation and activation of mitogen activated protein kinases (MAPKs). Stimulation of mouse macrophages with TNFα rapidly promotes ac-

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The phosphorylation of specific members of the ERK, JNK, and p38 subfamilies of MAPKs (17–19). Crystalline silica also induces AP-1 transcription in different cell types (20). Endotracheal instillation of silica in C57BL/6, but not in TNF receptor-deficient mice, is followed by AP-1 activation in the lung (10). These observations suggest that TNF receptors play an important role in regulating AP-1 activation in response to silica. However, the role that specific members of the MAPK family play during silica-induced AP-1 activity is not completely understood.

Engagement of the p55 receptor by the TNFα ligand is followed by ERK activation in murine macrophages (21). Activated ERK2 (p42) is then recruited to the membrane proximal region of the p55 receptor where it induces phosphorylation at the ERK consensus sites (22). Phosphorylated p55 is accumulated in the endoplasmic reticulum where it recruits Bcl-2 and protects against apoptosis (21, 23). Whether or not ERK-mediated phosphorylation of p55 receptor occurs in macrophages in response to silica is unknown.

TNFα production by mouse macrophage cell lines in response to silica exposure is not universal (24). RAW 264.7 cells constitute a macrophage cell line derived from BALB/c mice that react to silica exposure with enhanced TNFα production (24). In contrast, macrophages from the IC-21 mouse cell line (derived from C57BL/6 mice) do not stimulate TNFα production upon exposure to silica (24). These differences are important as the expression of TNFα correlates with the mouse strain sensitivity to silica, and we reported that mice from both C57BL/6 and BALB/c strains enhance TNFα production and undergo silica-induced lung injury (9). Therefore, we took advantage of the difference in TNFα production by these macrophage cell lines to study the role that TNFα-mediated activation of NF-κB and MAPKs plays on the protection of macrophages against silica-induced apoptosis. In particular, we investigated the importance of ERK-mediated phosphorylation of p55 TNF receptor in protecting macrophages from silica-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials—**Crystalline silica (α-quartz, average size, 1.7 μm) was obtained from U.S. Silica Co. (Berkeley Springs, WV). These particles were sterilized at 350 °C for 16 h to inactivate endotoxin contamination. Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Goat polyclonal anti-TNFFR1 (p55) (sc-1069), anti-TNFFR2 (p75) (sc-1074), anti-TRADD (sc-1164), anti-p-Bcl-2 (sc-16323), rabbit polyclonal anti-ERK1 (sc-94), anti-TRAF1 (sc-674), anti-TRAF2 (sc-676), anti-Bcl-2 (sc-783), goat polyclonal anti-hamster IgG peroxidase-conjugated, anti-p65 (sc-372x) NFκB subunits, and anti-c-jun/AP-1 (sc-44x) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

PhosphoPlus SAPT/JNK (Thr183/Tyr185) and PhosphoPlus p38 MAPK (Thr180/Tyr182) antibody kits for detection of total and phosphorylated SAPT/JNK and p38 MAPKs, and rabbit polyclonal anti-phospho-ERK1/2 antibody were purchased from New England Biolabs Inc. (Beverly, MA). Hamster monoclonal anti-mouse TNFFR1 (p55) antibody (clone 55R-286), propidium iodide staining and Annexin V-FITC were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). The compounds BAY 11-7085, SB 203580, PD 98059, and 5-iotubercid were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). All compounds were dissolved in Me2SO and stored in aliquots at −70 °C.

**Cell Lines and Cultures—**RAW 264.7 (ATCC TIB 71) and IC-21 (ATCC TIB 186) murine macrophage cell lines were obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 and IC-21 cells were maintained in Dulbecco’s modified Eagle’s medium and RPMI 1640 (Invitrogen, Rockville, MD), respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin and grown at 37 °C in 5% CO2. In all experiments cells were grown to 80% confluence in the media described above.

**Assay of TNFα Production by Macrophages—**Cells were plated in 6-well plates and grown for 3 days. Cells (∼2 × 105 cells/well) were exposed to crystalline silica (10 μg/cm2) for 6 h in triplicate. Untreated cells and cells stimulated with LPS (5 μg/ml) were used as negative and positive control, respectively. Following silica exposure, TNFα was assessed in cell-free supernatants with the use of an ELISA kit (Mouse ELISA TNF, Endogen, Woburn, MA) following manufacturer’s recommendations. 10 μg/ml was identified as the lower concentration of TNFα detectable with the use of this kit.

**Northern Analysis—**Total RNA was isolated from silica (10, 20, or 50 μg/cm2) for 6 h or control-treated macrophages using the Ultraspec II RNA Isolation System (Biometra, Goettingen, Germany). Total RNA was quantified according to the manufacturer’s instructions. Total cell RNA (20 μg/lane) was separated by electrophoresis on a 1.2% formaldehyde agarose g. RNA was transferred to a polyvinylidene difluoride membrane (Immobilon-NT, Millipore Corp., Bedford, MA), and hybridized overnight at 62 °C to the following [α-32P]dCTP-labeled cDNA probes: 1.104 kb murine TNFα (pMUTNF, ATCC) (25) and murine 36B4 cDNA were provided by Dr. J. A. Lasky (Tulane University Health Sciences Center, New Orleans, LA).

Membranes were probed first for TNFα, then stripped and reprobed for 36B4, as a loading control. Blots were developed after 72 h using Biomax films and intensifying screens (Kodak). To quantitate mRNA analysis, membranes were exposed to a Fuji phosphorimager (Fuji BAS 1000, Fuji USA, Stamford, CT) plate overnight and scanned. Quantitative analysis was determined with the use of MCAS 2.5 software (Fuji USA). For each mRNA band the results were normalized to the internal control (36B4) and expressed as a fold increase between the bands for the control and the bands for the silica-treated cells.

**Electrophoretic Mobility Shift Assay—**Cells were cultured in 6-well plates as described above. Following exposure to silica (10, 20, or 50 μg/cm2) for 6 h, cells were scraped in 1 ml of ice-cold PBS, pH 7.4, and collected by centrifugation at 500 × g for 10 min at 4 °C. Cell pellets were resuspended in 150 μl of lysis buffer (20 mM HEPES, 125 mM KCl, 1 mM EGTA, 5 mM magnesium chloride, 0.2% (v/v) glycerol, 0.1% (v/v) Nonidet-P40, 5 mM dithiorthreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), sonicated, and centrifuged at 13,000 × g for 10 min at 4 °C. Supernatants were aliquoted and stored at −70 °C until use. For retardation assays, NFκB consensus 5′-oligonucleotides 5′-AGTGGAGGACCTTC-CCAGGC-3′ and 5′-GGGCAAGAGATCCCGGAAA-3′ were end-labeled with γ-[32P]ATP and T4 polynucleotide kinase (Invitrogen, Gaithersburg, MD) and purified using Sephadex G-25 columns (Miniquick Spin Columns, Roche Applied Science, Indianapolis, IN). 5 μg of protein from whole cell lysate was mixed with the labeled probe and bound to the matrix Heps, 7.5% (v/v) glycerol, 3 mM magnesium chloride, 1 mM dithiothreitol, 0.06% (v/v) Nonidet P-40, 1.5 μg of poly(dI-dC) (Amersham Biosciences) in a 20-μl volume and incubated 50 min at room temperature to allow the binding of nuclear transcription factor to the probe. DNA-protein complexes were separated on 6% polyacrylamide gel (Invitrogen, Carlsbad, CA). The gels were vacuum-dried, and labeled complexes were detected by autoradiography. Competition assays were performed using 100-fold excess of unlabeled probe. For supershift experiments, samples were preincubated with antibody to p65, p55 NFκB subunits, or anti-c-jun before adding the labeled probe to the mixture.

**Western Blot Analysis—**Cells were seeded in 6-well plates and stimulated with silica or LPS (positive control) at the doses indicated for 6 h. In experiments where MAPK inhibitors were used cells were pre-treated with p38 MAPK inhibitor SB 203580 (1 μM), ERK inhibitor 5-iodotubercid (5 μM), or PD 098059 (50 μM) for 1 h and 30 min before silica exposure. At the end of the treatment, cells were lysed in buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 10 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, and 1 mM Na3VO4, incubated on ice for 30 min and centrifuged at 16,000 × g for 10 min at 4 °C. Protein concentration of the cell lysates was determined by the Lowry method using the Bio-Rad DC Protein assay kit (Bio-Rad). An equal volume of 2× Laemmli sample buffer was added, and the samples were boiled for 5 min. 50 μg of protein were separated on a SDS-PAGE gel and transferred to nitrocellulose membranes (Schleicher & Schuell, Inc.). Membranes were blocked in blocking buffer (5% nonfat dry milk in Tris-buffered saline, pH 7.6, 0.05% (v/v) Tween 20 (TBST)) for 1 h at room temperature and incubated with the relevant antibodies in blocking buffer overnight at
4 °C. After washing with TBST, blots were incubated with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature and developed using an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences).

**Analysis of Macrophage Apoptosis by Flow Cytometry—Apoptosis** was analyzed by evaluating the fluorescein-labeled annexin V binding and the exclusion of propidium iodide by macrophages, as previously described (66). Cells were seeded in 12-well plates and exposed to silica (20 and 50 μg/cm²), or medium as negative control for 6 h. In some experiments cells were pre-treated with MAPK inhibitors or NFκB inhibitor BAY 11-7085 at the concentrations indicated, for 1 h and 30 min. After treatment cells were washed with PBS, pH 7.4, and incubated for 15 min at room temperature in the presence of FITC-labeled annexin V, propidium iodide, or both, at the same time. Cells were washed twice with binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂), scraped, and subjected to flow cytometry analysis. Flow cytometry analysis was performed on a fluorescence-activated cell sorter (Vantage, BD Biosciences) with argon ion laser tuned to 488 nm. Fluorescence emission was measured through 530/30 nm band-pass (FITC) and 575/26 nm band-pass (propidium iodide) filters. Data were collected as 5000 mode event files and analyzed using CellQuest software (BD Biosciences).

**Immunoprecipitation of TNF Receptors—Cells** were plated in 100-mm dishes and grown to 80% confluence. Following exposure to silica (50 μg/cm²) for 6 h, 2 × 10⁷ cells were lysed in lysis buffer (50 mM Tris/HCl, pH 8.0, 137 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μM leupeptin, 5 μg/ml aprotinin). Lysates were incubated on ice for 20 min, sonicated, and centrifuged at 16,000 × g for 10 min at 4 °C to remove cellular debris. 1 mg of protein from total cell lysate was pre-cleared by i-1 incubation with 20 μl of protein G-agarose (for p55) or protein-A-Sepharose beads (for p75) and then incubated with rotation overnight at 4 °C with 3 μg of goat polyclonal anti-TNFRI (p55) or 10 μg of monoclonal anti-TNFFR2 (p75). 50 μl of protein A-Sepharose (p75) or protein G-agarose (p55) beads were added to each sample and incubated for 1 h followed by washing three times with lysis buffer and then with 50 mM Tris/HCl, pH 8.0. Beads were resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue), boiled for 5 min, and centrifuged.

Immunoprecipitates were separated by SDS-PAGE on 10% polyacrylamide gels under nonreducing conditions and analyzed by Western blotting as described above. Blots were incubated with hamster monoclonal anti-TNFRI (p55) or goat polyclonal anti-TNFFR2 (p75) overnight at 4 °C, followed by incubation with goat polyclonal anti-hamster IgG-peroxidase or rabbit polyclonal anti-goat IgG-peroxidase conjugated, respectively.

**Metabolic Labeling of Macrophages with [32P]Orthophosphate—Following pre-treatment with MEK1 (PD 98059 (30 μM), pSB (SB 203580 (1 μM)), or ERK (5-iodotubercidin (5 μM)), or ERK inhibitor BAY 11-7085 at the concentrations indicated, for 1 h and 30 min, RAW 264.7 and IC-21 cells were exposed to silica (20, or 50 μg/cm²) or control in the presence of inhibitors and metabolically labeled as previously described (21). Cells were vacuum-dried, and radiolabeled receptors were detected by autoradiography.

**Transfection of RAW 264.7 Macrophages with Dominant Negative IκB and ERK Mutants—**To determine the biological specificity of the results observed in RAW 264.7 macrophages with the use of the kinase inhibitors on the silica-induced apoptosis, we transfected these cells with constructs expressing dominant negative IκBα and ERK mutants.

The adenoval vector directing the expression of dnIkBa, in which serines 32 and 36 have been mutated to alanine and a triple influenza hemagglutinin tag added at its 5’-end, has been described previously (27, 28). The CDNA plasmid of pLPCMV-IκBαS32A/S36A, which contains a hemagglutinin-tagged human super-repressor of NF-κB, was subcloned into the XbaI site of the pACCMV MLPAIrS10 plasmid to construct the plasmid pACCMV-IκBaS10, in which IκBa is driven by the cytomegalovirus (CMV) promoter/enhancer. The adenoval encoding the enhanced green fluorescent protein gene under a CMV promoter (AdEGFP) was used as a control (29).

Dominant negative ERK1 (Lys71Arg) and ERK2 (Lys89Arg) mutants each cloned in pCEPA vector were obtained from Dr. Melanie Cobb and have been previously described (30). Extracellular signal-regulated protein kinases (ERK) 1 and 2 and the respective mutants were expressed in bacteria with a hexahistidine tag and purified using nickel-chelate chromatography. Basal activity of wild type ERK2 was ~2 nmol/min/mg. Self-catalyzed phosphorylation occurred in vitro on the major physiological site of tyrosine phosphorylation in an intramolecular reaction. Rabbit muscle ERK activator activated ERK2 500-1000-fold up to a specific activity (~2 μmol/min/mg) approximating that of ERK1 purified from stimulated cells (31). The ERK activator to the same extent could also activate ERK1. These ERK mutants lack the major site of tyrosine phosphorylation, are autophosphorylated at a greatly reduced rate, and are no longer activated by the ERK kinase.

1.5 × 10⁶ cells were plated in 12-well plates and grown for 24 h as described above. Cells were infected with adenoviral vectors (10^9 viral particles/well) or transfected with 1 μg of DNA using GeneJuice transfection reagent following manufacturer’s recommendations (EMD Biosciences, Inc., Novagen brand, Madison, WI). 48 h later, cells were exposed to silica or medium alone for 6 h as described above. After 6 h, cells were harvested for Western blot or flow cytometry studies as described above.

**Statistical Analysis—**Experiments were performed in triplicates. Results are reported as means ± S.E. Statistical comparisons were made using one-way analysis of variance (Statview 4; Abacus Concepts, Inc., Berkeley, CA) of variance followed by a Student-Neuman-Keuls test for post-hoc pairwise comparisons. A p value of <0.05 was considered significant.

**RESULTS**

Silica Induces TNFα Production in RAW 264.7, but Not in IC-21, Macrophages—Previous studies have established that the phagocytosis of silica particles by macrophages induces TNFα production (32). However, the ability of macrophages to secrete TNFα in response to silica is not universally observed and Claudia and associates (24) reported that different macrophage cell lines respond to silica with variable TNFα production. Therefore, we studied the differences in TNFα production in two macrophage cell lines in response to in vitro silica exposure.

TNFα mRNA expression was studied by Northern analysis in RAW 264.7 and IC-21 macrophages after 6 h exposure to silica (10 μg/cm²), LPS (4 μg/ml), or control media. As illustrated in Fig. 1A, exposure of RAW 264.7 cells to LPS or silica resulted in significant (p < 0.001) increases in TNFα mRNA expression compared with unstimulated controls. In contrast, IC-21 macrophages did not significantly increase (p > 0.2) TNFα mRNA expression in response to silica, although they demonstrated significantly increased (p < 0.02) TNFα mRNA expression in response to LPS stimulation.

The secretion of TNFα (measured by ELISA in picograms/ml) by the RAW 264.7 and IC-21 macrophage cell lines paralleled the results of the TNFα mRNA levels following silica, LPS, or control media exposure (Fig. 1B). RAW 264.7 cells secreted significant (p < 0.001) amounts of TNFα in response to LPS (6660 ± 206 pg/ml) or silica (528 ± 77 pg/ml) when compared with unstimulated cells (44 ± 12 pg/ml). In contrast to RAW 264.7 cells, IC-21 macrophages did not significantly increase (p > 0.5) their TNFα secretion following silica exposure (28 ± 5 pg/ml). However, when compared with unstimulated cells (41 ± 32 pg/ml) IC-21 macrophages demonstrated significant (p < 0.001) TNFα secretion in response to LPS stimulation (2223 ± 206 pg/ml).

**Silica Alters Expression of TNF Receptor and TNF Receptor-associated Proteins (TRADD, TRAF1, and TRAF2) in RAW 264.7, but Not in IC-21, Macrophages—**We have previously reported that exposure of mice to silica is followed by an up-regulated expression of the p75, but not the p55, TNFα receptor mRNA in the mouse lung (9). Therefore, we characterized the expression of TNFα receptors in RAW 264.7 and IC-21 macrophages following silica exposure. To study TNFα receptor expression, we performed immunoprecipitation against each individual (p55 or p75) receptor. As shown in Fig. 1C, untreated RAW 264.7 and IC-21 expressed both p55 and p75 TNF receptors. Silica exposure (20 μg/cm², for 6 h), of RAW 264.7, but not IC-21, macrophages was associated with enhanced expression of the p75 TNFα receptor. In contrast, no effect on total p55
expression was observed in either macrophage cell line following silica exposure (Fig. 1C).

To determine whether or not silica induced changes in TNF receptor-associated proteins in the macrophage cell lines, we studied the protein expression for the p55 TNF receptor-associated death domain (TRADD), and the p75 TNF receptor factor-associated (TRAF1 and TRAF2) proteins, by immunoblotting (Fig. 1D). TRADD is the p55 receptor death domain-associated protein and is involved in TNF-α-mediated apoptosis and NF-κB activation (33, 34). TRAF1 and TRAF2 contribute in a direct manner to NF-κB and AP-1 activation (8, 35, 36). In addition, TRAF1 and 2 recruit inhibitor of apoptosis proteins (cIAP) to the p55 receptor complex. The recruitment of IAP inhibits the activation of caspase 3 (37, 38) and caspase 8 (39), preventing TNF-α-induced apoptosis. As shown in Fig. 1D, RAW 264.7 macrophages demonstrate low levels of TRADD expression. This low level of TRADD expression was not greatly modified in these cells by exposure to either silica (5–50 μg/cm²) or LPS (4 μg/ml). In contrast to RAW 264.7 macrophages, IC-21 cells did not express detectable levels of TRADD protein in control-treated cells. TRADD expression was not enhanced in IC-21 cells following LPS (4 μg/ml) or silica (5–50 μg/cm²) exposure.

Similar to the data regarding TRADD, differences in the baseline expression of TRAF1 were observed between the macrophage cell lines. RAW 264.7, but not IC-21, cells showed baseline TRAF1 expression. This baseline expression was clearly enhanced in both cell lines following LPS (4 μg/ml), but not silica (5–50 μg/cm²), exposure (Fig. 1D).

Basal expression of TRAF2 protein was observed in both RAW 264.7 and IC-21 macrophage cell lines. However, the TRAF2 signal observed in RAW 264.7 cells was greater than that of IC-21 cells. TRAF2 expression was not significantly
Therefore, we studied NF-κB and AP-1 activation in RAW 264.7, but not in IC-21, macrophages in response to silica. AP-1 activation as AP-1 binding in crude nuclear extracts isolated from RAW 264.7 (A) and IC-21 (B) cells 6 h after incubation with silica (10 and 20 μg/ml), LPS (4 μg/ml), or media as control. Gel is representative of four experiments.

Silica Promotes NF-κB and AP-1 Activation in RAW 264.7, but Not in IC-21, Macrophages—Expression of TNF-α-responsive genes is mediated via the activation of specific transcription factors (40). The most prominent transcription factors activated by the TNF-α signal pathway are NF-κB and AP-1 (40, 41). Therefore, we studied NF-κB and AP-1 activation in silica-stimulated RAW 264.7 and IC-21 cells (by electrophoretic mobility shift assay). Silica treatment induced NF-κB (Fig. 2A) and AP-1 (Fig. 3A) activation in RAW 264.7, but not in IC-21 cell line. However, IC-21 cells activated NF-κB and AP-1 in response to LPS (Figs. 2A and 3A). The specificity of the observed NF-κB binding was demonstrated by the fact that adding a non-labeled NF-κB oligonucleotide inhibited the binding. Also the use of an antibody specific to the p50, but not the p65, subunit of NF-κB caused a shift of the NF-κB complexes (Fig. 2A).

Silica Induces Differential Activation of MAPKs in RAW 264.7 and IC-21 Cells—Phosphorylation of MAPKs, including p38, ERK1/2, and SAPK/JNK kinases, lead to the formation of homo- or heterodimers of c-Fos/c-Jun and AP-1 activation (42, 43). Therefore, we studied the effect of silica exposure on the activation (phosphorylation) measured by Western blotting, of MAPKs in RAW 264.7 and IC-21 macrophages.

Silica exposure induced ERK1/2 activation in RAW 264.7, but not in IC-21 cells (Fig. 4A). Baseline p38 expression was similar in both macrophage cell lines. However, silica exposure enhanced p38 MAPK phosphorylation in IC-21, but not in RAW 264.7, macrophages (Fig. 4B). No differences in total or phosphorylated SAPK/JNK MAPKs were observed in either of two cell lines (Fig. 4C).

Maximal silica-induced ERK phosphorylation was evident at 10 μg/cm² and appeared to plateau at 20 μg/cm² with no further changes observed when these cells were exposed to higher concentrations of particles (Fig. 5A).

To further determine the specificity of the ERK activation, we investigated the effects of specific MEK1 (PD 98059) (44), an upstream activator of ERKs, ERK (5-iidotubercidin), or p38 (SB 203580) (45), inhibitors on silica-induced ERK phosphorylation in RAW 264.7 macrophages (Fig. 5B).

No change in silica-induced ERK phosphorylation was observed when RAW 264.7 cells were pre-treated with 1 μM SB 203580 (Fig. 5B). In contrast, silica-induced ERK activation was prevented when RAW 264.7 cells were exposed to silica in the presence of the PD 98059 (50 μM) or 5-iidotubercidin (5 μM) (Fig. 5B).

Macrophage Apoptosis in Response to Silica is Enhanced in IC-21 Macrophages—It has been suggested that macrophage apoptosis may be important in initiating the inflammatory response to silica (14, 15, 46). Therefore, we evaluated, by flow cytometry, the induction of apoptosis by silica in RAW 264.7 and IC-21 cells.

Fig. 6 illustrates the binding of fluorescein-labeled annexin V and the exclusion of propidium iodide by the RAW 264.7 and IC-21 macrophage cell lines, following exposure to silica 20–50 μg/cm² for 6 h. Control-treated RAW 264.7 cells demonstrated minimal (~3% of the cells stained positively) binding of annexin V and universally excluded propidium iodide (~1% of the cells demonstrated propidium iodide uptake) suggesting that...
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When RAW 264.7 cells were exposed to silica at a concentration of 20 μg/cm², for 6 h. Following silica exposure p55 was immunoprecipitated, and its degree of phosphorylation was determined by autoradiography. As illustrated in Fig. 7, p55 phosphorylation was only observed in silica-induced apoptosis of RAW 264.7 macrophages. Therefore, we examined the effect of inhibiting the ERK-mediated phosphorylation of p55 on the silica-induced apoptosis of RAW 264.7 macrophages. As shown in Fig. 8, ERK inhibition by PD98059 (50 μM) or 5-iodotubercidin (5 μM) significantly (p < 0.001) increased silica-induced apoptosis in RAW 264.7 macrophages. To determine the biological specificity of the results observed with the pharmacological inhibitors of ERK, we transfected RAW 264.7 macrophages with genetically manipulated mutant ERK1/2 that can not be phosphorylated and therefore, behave as dominant negative proteins (30) to measure the effect of overexpressing these proteins on the silica-induced apoptosis.

As discussed above, silica exposure induced NF-κB activation in RAW 264.7 macrophages. Because NF-κB activation is an important mechanism against apoptosis, we studied whether or not the enhanced apoptosis observed in RAW 264.7 cells following the exposure to the ERK inhibitors could be attributed to inhibitory effects of these compounds on NF-κB activation. Therefore, we studied the effects of PD98059 or 5-iodotubercidin treatments on the silica-induced activation of NF-κB in RAW 264.7 macrophages (Fig. 2B). To clarify the role of NF-κB activation on the silica induction of apoptosis we incubated RAW 264.7 macrophages with BAY 11-7085, which is a powerful inhibitor of IκB phosphorylation, or infected these cells with adenovirus containing a mutant IκB protein that cannot be phosphorylated and therefore prevents NF-κB activation (48). As can be observed in Figs. 2 and 6, the addition of BAY (40 μM) to cell cultures eliminated silica-induced phosphorylation of ERK proteins (Fig. 5C) and significantly (p < 0.001) increased the silica induction of apoptosis in these cells (Fig. 9), thus confirming the results observed with the pharmacological inhibitors of ERK.

As discussed above, silica exposure induced NF-κB activation in RAW 264.7 macrophages. Because NF-κB activation is an important mechanism against apoptosis, we studied whether or not the enhanced apoptosis observed in RAW 264.7 cells following the exposure to the ERK inhibitors could be attributed to inhibitory effects of these compounds on NF-κB activation. Therefore, we studied the effects of PD98059 or 5-iodotubercidin treatments on the silica-induced activation of NF-κB in RAW 264.7 macrophages (Fig. 2B). To clarify the role of NF-κB activation on the silica induction of apoptosis we incubated RAW 264.7 macrophages with BAY 11-7085, which is a powerful inhibitor of IκB phosphorylation, or infected these cells with adenovirus containing a mutant IκB protein that cannot be phosphorylated and therefore prevents NF-κB activation (48). As can be observed in Figs. 2 and 6, the addition of BAY (40 μM) to cell cultures eliminated silica-induced phosphorylation of ERK proteins (Fig. 5C) and significantly (p < 0.001) increased the silica induction of apoptosis in these cells (Fig. 9), thus confirming the results observed with the pharmacological inhibitors of ERK.

Macrophages play an important role in the pathogenesis of silica-induced lung fibrosis in part through their ability to clear silica particles and apoptotic cells from the lung (1, 49). In addition, macrophages are the initial source of inflammatory mediators produced in response to silica exposure that include oxygen radicals, leukotrienes, cytokines, and growth factors (1). Therefore, the concept has been advanced that the excessive loss of lung macrophages in response to silica could compromise the ability of the host to clear silica particles and remove apoptotic cells while promoting excessive inflammation. Thus, understanding the mechanisms regulating macro-
Phage cell death in response to silica constitute a first step in promoting adequate resolution of this type of lung injury. A large body of experimental evidence implicates TNFα as a fundamental cytokine in the pathogenesis of silicosis. TNFα expression is up-regulated in the lungs of silica-treated mice in a manner that precedes the development of lung fibrosis (2). Furthermore, enhanced TNFα expression in the lung correlates with the murine strain sensitivity to silica and C57BL/6 mice.

**Fig. 5.** Silica induces dose-dependent ERK1/2 activation in RAW 264.7 macrophages. RAW 264.7 cells were treated with increasing concentrations of silica (5, 10, or 20 μg/cm²), LPS (4 μg/ml), or roridin (RA) as positive controls, or media alone as control for 6 h. A, cell lysates were analyzed by Western blotting for total and phosphorylated ERK1/2 (using an antibody that only detects the TYR204 forms of ERK1/2). B and C, to study specificity of the silica-induced ERK phosphorylation, RAW 264.7 cells were pretreated with PD98059 (30 μM), 5-iodotubercidin (5 μM), or SB203580 (1 μM) for 1 h, or transfected with dominant negative mutants of ERK (dnERK) or empty vector (pCEP40) as control and then exposed to silica (20 μg/cm²) or LPS (4 μg/ml) for 6 h. Cell lysates were analyzed by Western blotting for total and phosphorylated ERK1/2 as described in the text. Gels are representative of four additional experiments.

**Annexin V**

Flow cytometry colocalization of annexin-V binding and propidium iodine uptake for quantitation of apoptotic macrophage populations following silica exposure (20 μg/cm²) as described under “Experimental Procedures.” Each panel depicts data gathered from 5000 individual cells. The horizontal axis depicts fluorescein-labeled annexin V; the vertical axis shows binding of propidium iodide fluorescence. Also illustrated is the effect of treatment of RAW 264.7 cells with BAY 11-7085 compound (40 μM) prior to silica treatment.

**Fig. 6.** Silica induces apoptosis in RAW 264.7 and IC-21 macrophages.
which up-regulate TNF expression in their lungs, are more sensitive to silica-induced lung injury than C3H mice that demonstrate much lower TNF expression in their lungs following silica exposure (4). In humans, polymorphism of the TNFα gene has been associated with worse clinical outcome in populations of silica-exposed miners (50, 51). Antagonism of TNFα, by treating silica-exposed mice with anti-TNFα antibodies or soluble TNF receptors that bind and inactivate TNFα, ameliorates silica-induced lung injury in mice (2, 3). The importance of TNF signaling in the pathogenesis of silicosis has been demonstrated by the fact that transgenic mice, engineered on a silica-sensitive C5BL/6J background, in which both p55 and p75 TNF receptors have been deleted, are protected from silica-induced lung injury despite enhanced TNF expression in their lungs (9).

The enhanced TNFα production observed in macrophages in response to silica has been suggested to be responsible for the induction of macrophage apoptosis. However, TNFα also induces NF-κB activation, which promotes anti-apoptotic effects in different cells (52, 53). Therefore, TNFα activation in macrophages in response to silica could signal for opposite effects at the same time. Confounding the role of the enhanced TNFα expression in macrophages are the facts that macrophage production of TNFα in response to silica is not universal and macrophage cell lines have been described that do not up-regulate their TNFα expression in response to silica exposure (24). In the present work we found that TNFα expression correlates inversely with the silica induction of apoptosis, because IC-21 cells, which do not up-regulate TNFα expression in response to silica, exhibit greater induction of apoptosis than RAW 264.7 macrophages.

To further study the role that TNFα plays in the silica induction of apoptosis, we characterized the TNF-mediated signal transduction pathways in RAW 264.7 and IC-21 cell lines. RAW 264.7 cells react to silica with enhanced TNFα production and activation of TNF receptor-mediated signal transduction leading to NF-κB and AP-1 activation. In contrast to RAW 264.7 cells, IC-21 macrophages do not up-regulate TNFα expression nor activate TNF-mediated signal transduction in response to silica. However, when IC-21 macrophages were treated with LPS, TNFα release, NFκB, and AP-1 DNA binding were observed. These findings suggest that TNFα is an important determinant of the activation of NFκB and AP-1 observed in macrophages in response to silica.

Intratracheal administration of freshly fractured silica induces AP-1 transactivation in the lung of AP-1 luciferase reporter transgenic mice (20). Hubbard et al. (54) reported that the treatment of NF-κB luciferase reporter mice with silica resulted in enhanced NF-κB activation that was traced to bronchiolar epithelial cells and alveolar macrophages in these animals. Consistent with these reports is our previous work suggesting that TNF receptors mediate NF-κB and AP-1 activation in response to silica in mouse lung (10). Thus, silica-sensitive C57Bl/6 mice enhanced NFκB and AP-1 activation in their lungs following treatment with crystalline silica. These responses were absent in double, and significantly reduced in individual, TNF receptor-deficient mice that were protected from the effects of silica (10).

Differences in expression of TNF receptors and TNF receptor-associated proteins (TRADD, TRAF1, and TRAF2) in response to silica were also observed in RAW 264.7 and IC-21 macrophages. Basal levels of p55 and p75 TNF receptor protein expression were similar in both cell lines. Silica exposure enhanced p75, but not p55, TNF receptor protein expression in RAW 264.7, but not in IC-21 macrophages. These results are consistent with our previous observation that silica exposure induced up-regulation for p75, but not p55, mRNA in the lung of silica-sensitive mice (9). Low level of TRADD protein expression was only observed in RAW 264.7 macrophages. Basal expression of both TRAF1 and 2 was almost undetectable in IC-21 cells but was obvious in RAW 264.7 cells. Silica treatment did not alter the baseline levels of expression of TRAF proteins. In contrast, the levels of expression for TRAF proteins were greatly enhanced in both macrophage cell lines by treatment with LPS. These data indicate that there are differences in the way in which silica and LPS induce NF-κB activation.

In the present work we found that NF-κB activation inversely correlated with the silica induction of apoptosis in both macrophage cell lines. To further study the role of NF-κB activation in the silica induction of apoptosis we inhibited, in RAW 264.7 macrophages, the phosphorylation of the 1κBα repressor with the use of a pharmacological inhibitor (BAY compound) or transfection of these cells with a mutated protein in which serine 32 (Ser32) and 36 (Ser36) residues have been substituted by alanines and cannot be phosphorylated (dnIkB) by the kinase (29). BAY treatment or transduction with dnIkB of RAW 264.7 macrophage prior to or during silica exposure significantly enhanced silica-induced apoptosis in these macrophages suggesting that NF-κB activation mediates a protective effect against apoptosis in response to silica. The mechanisms by which activated NF-κB protects from apoptosis have not been directly studied in silica-treated cells. However, in other systems NF-κB enhances the regulation of c-IAP genes and inhibits the activation of caspase-8, therefore blocking the apoptotic cascade (39). In addition, it has been recently reported that suppression of constitutive NF-κB activation in RAW 264.7 macrophages induces caspase-independent apoptosis by generating loss of mitochondrial transmembrane potential and mitochondrial dysfunction (55).

TNFα induces AP-1 activation by promoting phosphorylation of the MAPKs (40). Little information is available regarding the activation of MAPKs in response to silica (20, 56, 57). Ding and associates (20) showed that exposure of mouse and rat epidermal cell lines to silica is followed by ERK and p38 phosphorylation that subsequently induce AP-1 activation (20). In our studies we observed consistent differences in the MAPK activation in response to silica in RAW 264.7 and IC-21 cells. Silica exposure predominantly induced ERK1/2, with minimal effect on p38 and no effect upon SAPK/JNK, phosphorylation in RAW 264.7 macrophages. In contrast, minor p38 phosphorylation was the only response observed in silica-exposed IC-21. Pharmacological (by treatment with the MEK1-specific inhibitor PD98059 or the ERK2 phosphorylation inhibitor 5'-iodotubercidin) or genetic (by overexpression of dominant negative ERK mutants) inhibition of ERK activation abrogated AP-1 activation in response to silica. Furthermore, silica exposure of RAW 264.7 cells in the presence of PD 98059 inhibitor was
associated with increased NF-κB transactivation compared with cells exposed to silica only. Thus, this observation suggests that the MEK-ERK pathway exerts a negative feedback on NF-κB activation. These data confirm the report by Janssen-Heininger et al. (58) demonstrating that overexpression of MEK in rat lung epithelial cells resulted in decreased ability of these cells to activate NF-κB in response to TNF or oxidants.

But what is the role of ERK activation in silica-induced macrophage apoptosis? It is clear from our data that ERK phosphorylation inversely correlates with the induction of macrophage apoptosis by silica. Furthermore, inhibition of ERK activation in RAW 264.7 cells significantly enhanced silica-induced apoptosis. Recent data indicate that the mechanism by which ERK mediates protection from apoptosis involves the phosphorylation of the p55 TNF receptor (22). Cottin et al. (22) demonstrated that TNFα treatment of bone marrow-derived macrophages is followed by rapid ERK2 activation, which in turn targets the cytoplasmic domain of the p55 receptor and induces its phosphorylation at ERK binding sites (22). ERK2-mediated phosphorylation of p55 cytoplasmic domain promotes receptor translocation to the endoplasmic reticulum where it recruits the anti-apoptotic protein Bcl-2 (21, 23). In our work we found that silica induced p55 phosphorylation only in RAW 264.7 macrophages that activated TNF expression. Furthermore, we also found that the use of specific inhibitors of ERK, but not of p38, activation prevented p55 phosphorylation and significantly enhanced silica-induced apoptosis in RAW 264.7 macrophages.

In summary, in the present work we find that expression of TNFα and activation of TNF receptor mediated signal transduction, leading to NF-κB and AP-1 activation, protect macrophages from silica-induced apoptosis. AP-1 activation observed in macrophages in response to silica appears to be mediated by activation of the ERK family of MAPKs. Activated ERK induces phosphorylation of p55 TNF receptor and protects macrophages from apoptosis. This mechanism of protection appears...
to be independent of the protection conferred by NF-κB activation, because ERK inhibition enhances silica-induced apoptosis while promoting NF-κB activation. Further studies looking at downstream effects of phosphorylated p55 are necessary to further clarify the role of TNF receptor phosphorylation in silica-induced apoptosis.

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