Signal Transduction Pathways of Tumor Necrosis Factor-mediated Lung Injury Induced by Ozone in Mice

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Rationale: Increasing evidence suggests that tumor necrosis factor (TNF)- α plays a key role in pulmonary injury caused by environmental ozone (O₃) in animal models and human subjects. We previously determined that mice genetically deficient in TNF response are protected from lung inflammation and epithelial injury after O₃ exposure.

Objectives: The present study was designed to determine the molecular mechanisms of TNF receptor (TNF-R)-mediated lung injury induced by O₃.

Methods: TNF-R knockout (*Tnfr*^{-/-}) and wild-type (*Tnfr*^{+/+}) mice were exposed to 0.3 ppm O₃ or air (for 6, 24, or 48 h), and lung RNA and proteins were prepared. Mice deficient in p50 nuclear factor (NF)- κ B (*Nfkb1*^{-/-}) or c-Jun-NH₂ terminal kinase 1 (*Jnk1*^{-/-}) and wild-type controls (*Nfkb1*^{+/+}, *Jnk1*^{+/+}) were exposed to O₃ (48 h), and the role of NF- κ B and mitogen-activated protein kinase (MAPK) as downstream effectors of lung injury was analyzed by bronchoalveolar lavage analyses.

Results: O₃-induced early activation of TNF-R adaptor complex formation was attenuated in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice. O₃ significantly activated lung NF- κ B in *Tnfr*^{+/+} mice before the development of lung injury. Basal and O₃-induced NF- κ B activity was suppressed in *Tnfr*^{-/-} mice. Compared with *Tnfr*^{+/+} mice, MAPKs and activator protein (AP)-1 were lower in *Tnfr*^{-/-} mice basally and after O₃. Furthermore, inflammatory cytokines, including macrophage inflammatory protein-2, were differentially expressed in *Tnfr*^{-/-} and *Tnfr*^{+/+} mice after O₃. O₃-induced lung injury was significantly reduced in *Nfkb1*^{-/-} and *Jnk1*^{-/-} mice relative to respective control animals.

Conclusions: Results suggest that NF- κ B and MAPK/AP-1 signaling pathways are essential in TNF-R-mediated pulmonary toxicity induced by O₃.

Keywords: tumor necrosis factor receptor; knockout; nuclear factor- κ B; mitogen-activated protein kinase; activator protein-1

Ozone (O₃) is a principal oxidant in air pollution. Elevated ambient O₃ levels have been associated with increased hospital visits and respiratory symptoms in epidemiologic studies (1, 2). Subjects with preexisting allergic/inflammatory airway disorders, such as asthma and rhinitis, are known to be particularly vulnerable to O₃ and at risk of exacerbations (3). Recent evidence also suggested that O₃ enhances the effect of inhaled allergen in patients with asthma (4). Acute O₃ toxicity in rodent airways

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Nuclear factor- κ B and MAPK/AP-1 signaling pathways are essential in tumor necrosis factor receptor-mediated pulmonary toxicity induced by ozone.

What This Study Adds to the Field

NF- κ B and MAPK/AP-1 signaling pathways are essential in TNF receptor-mediated lung injury induced by ozone.

includes predominant neutrophilic inflammation accompanied by airway hyperresponsiveness, chemokine/cytokine production, mucus overproduction and hypersecretion, and cell death and proliferation. However, the mechanisms of O₃-induced effects on the lung are not completely understood.

A number of investigations have focused on the potential roles of inflammatory mediators, including tumor necrosis factor (TNF)- α , in the pathogenesis of O₃-induced lung inflammation and injury. TNF- α is a member of the trimeric cytokine family (5), which has diverse bioregulatory activities engaged in inflammation/immunity responses, cell proliferation/differentiation, and apoptosis. TNF- α has a critical role in many acute and chronic inflammatory diseases, and anti-TNF strategies have proven to be clinically effective (6). TNF- α binds to two distinct cellular membrane receptors (TNF-R1p55, TNF-R2p75). Ligand binding to TNF-R1 induces sequential recruitment of intracellular adaptor proteins, including TNF-R1-associated death domain protein (TRADD) and TNF-R-associated factor 2 (TRAF2) to the membrane. TRAF2 is also a well-defined intracellular adaptor for TNF-R2. The interaction of TRAF2 in the TNF-R complex with the inhibitor of κ B (I κ B) kinase (IKK) and subsequent phosphorylation of IKK and I κ B eventually activates the transcription factor, nuclear factor (NF)- κ B (7). Another pathway that becomes activated by the TRADD/TRAF2 complex is the mitogen-activated protein kinase (MAPK) cascade, which induces nuclear transactivation of activator protein (AP)-1 transcription factors (7). TNF- α signaling directs transcriptional regulation of inflammatory mediator genes, including early-response cytokines (e.g., interleukin [IL]-1 β), chemokines (e.g., macrophage inflammatory protein [MIP]-2), and adhesion molecules (e.g., intercellular adhesion molecule [ICAM]-1) in various airway cells (8, 9).

An essential role for TNF- α has been recently documented in animal models of pulmonary inflammation and oxidative injury responses caused by bleomycin and several environmental toxicants, including hyperoxia, endotoxin, and cigarette smoke (10–14). Inhaled O₃ also enhances TNF- α release and TNF-R expression in the airway cells or tissues (15, 16). Our positional cloning studies in inbred mice identified *Tnf* as a candidate

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susceptibility gene for lung inflammation induced by subacute exposures to 0.3 ppm O₃ (17). In support of this hypothesis, we and others have demonstrated that lack of TNF response provided significant protection from O₃-induced inflammation and airway hyperreactivity in rodent lungs (16–20). Moreover, recent studies (21, 22) demonstrated in human subjects an association of O₃-induced lung functional changes with a *TNF* polymorphism haplotype including –308A, which is also known to be involved in increased risk of asthma (23). In the present study, we elucidated molecular mechanisms underlying TNF-R-mediated pulmonary pathogenesis of subacute O₃ toxicity. Some of the results of this study have been previously reported in abstracts (24, 25).

METHODS

Animals and Inhalation Exposure

Male *Tnfr^{-/-}* (B6;129S-*Tnfrsfla^{tm1Imx}Tnfrsflb^{tm1Imx/J}*), *Nfkb1^{-/-}* (B6;129P2-*Nfkb1^{tm1Bal/J}*), and *Jnk1^{-/-}* (B6.129-*Mapk8^{tm1Flv/J}*) mice and their respective wild-type mice (6–8 wk) were purchased from Jackson Laboratories (Bar Harbor, ME). After acclimation, mice were placed in individual stainless-steel wire cages within a Hazelton 1000 chamber (Lab Products, Maywood, NJ) equipped with a charcoal and high-efficiency particulate air-filtered air supply. Mice had free access to water and pelleted open-formula rodent diet NIH-07 (Zeigler Brothers, Gardners, PA.). Mice were exposed continuously (for 6, 24, or 48 h) to 0.3 ppm O₃. On the basis of National Ambient Air Quality Standards for ambient O₃ (0.12 ppm for 1 h and 0.08 ppm for 8 h) (26) and dosimetry studies in which rodents require four- to fivefold higher doses of O₃ than humans to create an equal deposition and pulmonary inflammatory response (27), the O₃ concentration used in the current study is a reasonable exposure level from which to make comparisons with humans. O₃ was generated from ultra-high-purity air (< 1 ppm total hydrocarbons; National Welders, Inc., Raleigh, NC) using a silent arc discharge O₃ generator (model L-11; Pacific Ozone Technology, Benicia, CA). Constant chamber air temperature (72 ± 3° F) and relative humidity (50 ± 15%) were maintained. O₃ concentration was continually monitored (Dasibi model 1008-PC; Dasibi Environmental Corp., Glendale, CA). Parallel exposure to filtered air was done in a separate chamber for the same duration. Immediately after each exposure, mice were killed by sodium pentobarbital overdose (104 mg/kg). All animal use was approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

Lung Histopathology

Left lung tissues were fixed by 10% neutral buffered formalin under constant pressure (25 cm H₂O) and sections were processed for histopathology. Immunohistologic staining was done using an anti-TRAF2 antibody (sc-877; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a proliferating cell nuclear antigen (PCNA) staining kit (Zymed Laboratories, Inc., South San Francisco, CA).

Bronchoalveolar Lavage Analyses

Whole lungs from mice exposed to O₃ or air (48 h) were lavaged, and lung cellular inflammation and hyperpermeability were assessed as described previously (16).

Lung Nuclear Protein Isolation for Electrophoretic Mobility Shift Assay

Nuclear proteins were prepared from pulverized pieces of right lung. Briefly, lung tissues were pulverized in liquid nitrogen using a mortar and pestle, and were homogenized in a hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane [HEPES], pH 7.9; 0.5 M sucrose; 1.5 mM MgCl₂; 10 mM KCl; 10% glycerol; 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0; 1 mM dithiothreitol [DTT]; 1 mM phenylmethylsulfonyl fluoride [PMSF]) using a dounce homogenizer. Homogenates were treated with Nonidet P-40 (0.25%), incubated on ice for 15 minutes, and centrifuged (14,000 g, 20 min, 4°C). After collecting supernatants containing soluble cytoplasmic fraction, pellets were resuspended in a hypertonic lysis buffer (20 mM HEPES, pH 7.9; 420 mM

NaCl; 1.5 mM MgCl₂; 10% glycerol; 0.2 mM EDTA, pH 8.0; 0.5 mM DTT; 0.5 mM PMSF; protease inhibitor cocktail), incubated in ice on a rocking platform (150 rpm, 30 min), and centrifuged (14,000 g, 15 min, 4°C). Supernatants including nuclear proteins were collected and stored at –80°C. DNA binding activity of NF-κB or AP-1 was determined by electrophoretic mobility (gel) shift analysis of nuclear proteins (5–10 μg) as described previously (28). Specific binding activity was determined by preincubation of nuclear proteins with anti-p65NF-κB (sc-372X), anti-p50NF-κB (sc-1190X), or anti-pan Jun AP-1 (sc-44X) antibody followed by electrophoretic mobility shift analysis. The gel was autoradiographed using an intensifying screen at –70°C, and autoradiograph images were scanned and quantified by a Bio-Rad Gel Doc 2000 System (Hercules, PA).

Western Blot Analyses

Total lung proteins from right lung tissues were prepared in radioimmunoprecipitation (RIPA) buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 μg PMSF per ml, 1 mM sodium orthovanadate, protease inhibitor cocktail). Lung cytosolic soluble fractions were acquired during nuclear protein extraction as described above. Proteins (20–100 μg) were analyzed by Western blotting using specific antibodies against TRAF2 (sc-877), TRADD (sc-7868), IκB-α (sc-371), phosphorylated IκB-α (*p*-IκB; sc-8404), IKK (sc-7607), phosphorylated IKK (*p*-IKK; sc-21661-R), c-Jun-NH₂ terminal kinase (JNK1; sc-571), *p*-JNK (sc-6254), extracellular signal-regulated kinase (ERK; sc-154), *p*-ERK (sc-7383), and actin (sc-1615). PCNA was detected in nuclear protein (20 μg) using an anti-PCNA antibody (sc-56). To determine TRADD-bound TRAF2 (an indicator of TNF-R1 signaling complex formation) or TNF-R2-bound TRAF2 (an indicator of TNF-R2 signaling complex formation), 200 μg of total lung protein were immunoprecipitated with anti-TRADD antibody (sc-7868) or anti-TNF-R2 antibody (sc-1074), respectively, and each immunoprecipitate was processed for Western blot analysis with the anti-TRAF2 antibody. Bands were scanned and quantified using the Bio-Rad Gel Doc 2000 System.

Reverse Transcriptase–Polymerase Chain Reaction Analyses

Total RNA was isolated from right lung homogenate, and reverse transcriptase–polymerase chain reaction was performed with mouse-specific primers for TNF-α, lymphotoxin (LT)-β, MIP-2, and IL-1β (29). Forward and reverse primers used for ICAM-1 (GI:194077) amplification were 5'-atgcttcaaccgtgccaa-3' and 5'-gttacttgctcccttcga-3', respectively. Forward and reverse primers used for IL-6 (GI:198367) amplification were 5'-aagaacgatagtcattcca-3' and 5'-gatctcaaatgacttttag-3', respectively. Each mRNA abundance was quantified by the Bio-Rad Gel Doc 2000 System as described previously (28) using 18S ribosomal RNA as an internal control.

Statistics

Data were expressed as the group mean ± SEM. Two-way analysis of variance (ANOVA) was used to evaluate the effects of exposure and genotype in all experiments except p50 NF-κB binding activity in *Nfkb1^{+/+}* mice in which one-way ANOVA was used. The Student-Newman-Keuls test was used for *a posteriori* comparisons of means (*p* < 0.05). All of the statistical analyses were performed using Sigma-Stat 3.0 software program (SPSS, Inc., Chicago, IL).

RESULTS

Differential Activation of TNF-R Signal Pathways by O₃ in *Tnfr^{+/+}* and *Tnfr^{-/-}* Mice

Intracellular TNF-R complex formation. Intracellular TNF-R signal protein complex was measured as an indicator of TNF-R activation after exposure to O₃. TRAF2 is a common intracellular signal transducer that mediates TNF-R1 and TNF-R2 responses, and it has recently been found to be essential for early recruitment of downstream kinases for NF-κB and AP-1 activation (30–32). Immunoprecipitation/Western blotting of total lung protein indicated that TRADD-bound TRAF2 (an indicator of intracellular TNF-R1 signal transducer complex formation) was

elevated after 6 hours of exposure, before the onset of inflammation (Figure 1A). Complex formation was significantly attenuated in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice after O₃ exposure (Figure 1A, Table 1). TRAF2 bound to TNF-R2 (an indicator of TNF-R2 signaling complex formation) was significantly increased by O₃ in *Tnfr*^{+/+} mice, but not in *Tnfr*^{-/-} mice (Figure 1A, Table 1). Soluble TRAF2 was also relatively higher in *Tnfr*^{+/+} mice than in *Tnfr*^{-/-} mice basally and after O₃ expo-

sure, and O₃ reduced soluble TRAF2 levels in both genotypes in a time-dependent manner (Figure 1A, Table 1). O₃-induced early increases in TRAF2-TRADD and TRAF2-TNF-R2 complexes were concurrent with depletion of soluble TRAF2 before lung pathology developed in the wild-type mice, and suggested the recruitment of "free" cytoplasmic TRAF2 to form membrane complex in response to O₃.

Lung TRAF2 was detected constitutively by immunohistochemical staining in cytoplasm and membranes of ciliated and basal bronchial epithelial cells, endothelium, and smooth muscle, and in alveolar macrophages of *Tnfr*^{+/+} mice and *Tnfr*^{-/-} mice (Figure 1B). TRAF2 was also detected in infiltrating inflammatory cells and in terminal bronchiolar cells of the centriacinar region, which was undergoing significant proliferation and reconstitution in O₃-exposed mice (Figure 1B) as demonstrated previously (16, 17). Consistent with immunoprecipitation/Western blot data (Figure 1A), relatively fewer TRAF2-positive cells were found in these pathologic regions of *Tnfr*^{-/-} mice, compared with *Tnfr*^{+/+} mice (Figure 1B).

NF- κ B pathway. As a dimeric transcription factor, the activity of NF- κ B is regulated by its interaction with I κ B, a family of cytoplasmic NF- κ B inhibitors. Activation of the NF- κ B pathway requires sequential phosphorylation of the upstream kinase complex IKK and its substrate I κ B, which leads to phosphorylation degradation of I κ B and nuclear translocation of NF- κ B after having been liberated from NF- κ B-I κ B complexes. After O₃ exposure, lung IKK(α/β) and I κ B- α were enhanced similarly in both genotypes (Figure 2A). However, *p*-IKK(α/β) level normalized by IKK(α/β) was significantly lower in *Tnfr*^{-/-} mice than in *Tnfr*^{+/+} mice basally and after O₃ (Table 1). A time-dependent increase of phosphorylated I κ B- α (*p*-I κ B- α /I κ B- α) by O₃ was evident in *Tnfr*^{+/+} mice but marginal and significantly lower in *Tnfr*^{-/-} mice (Figure 2A, Table 1). Baseline DNA binding activities of total NF- κ B and p50 κ B subunits were significantly suppressed in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice (Figure 2B, Table 1). O₃ significantly enhanced the binding activity of total NF- κ B and specific p50 κ B over the constitutive level in both genotypes (Figure 2B, Table 1). However, O₃-induced total (6 and 24 h) and specific p65 (24 h) and p50 (24 h) NF- κ B activity was significantly lower in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice (Figure 2B, Table 1).

MAPK/AP-1 pathway. Phosphorylation activation of the MAPK regulates nuclear AP-1 transactivation. Total JNK and ERK MAPK levels were not significantly changed by O₃ in either genotype (Figure 3A). Total activated levels of ERK and JNK (determined by ratio of phosphorylated level to nonphosphorylated level) were significantly attenuated basally and after O₃ in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice, although O₃ also significantly enhanced activation of these MAPKs in *Tnfr*^{-/-} mice (Figure 3A, Table 1). Basal and O₃-induced (6 h) DNA binding activity of nuclear total AP-1 was significantly suppressed in *Tnfr*^{-/-} mice, compared with wild-type mice (Figure 3B, Table 1). Specific DNA-binding activity of AP-1 Jun proteins was constitutively lower in *Tnfr*^{-/-} than in *Tnfr*^{+/+} mice, and was not significantly enhanced by 24 hours after O₃ in *Tnfr*^{-/-} mice (Figure 3B, Table 1).

Downstream inflammatory gene induction. Transcriptional induction of several O₃-inducible genes containing *cis*-acting elements for NF- κ B and/or AP-1 binding in their promoters (33–35) was compared in *Tnfr*^{+/+} and *Tnfr*^{-/-} mice. Constitutive expression of TNF- α and IL-1 β mRNA was significantly lower in *Tnfr*^{-/-} compared with *Tnfr*^{+/+} mice (Figure 4). O₃ caused a significant increase of IL-1 β mRNA expression at 24 and 48 hours over the control level, whereas TNF- α , LT- β , MIP-2, and ICAM-1 mRNA was significantly elevated above respective baseline expression only at 48 hours when inflammation and

Figure 1. Intracellular tumor necrosis factor receptor (TNF-R) signal transducers were suppressed in *Tnfr*-deficient mice. (A) Differential activation of TNF-R proximal signaling complex in *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after exposure to air and 0.3 ppm O₃ (6 or 24 h). Aliquots of total lung homogenates were immunoprecipitated (IP) using anti-TNF-R1-associated death domain protein (TRADD) or anti-TNF-R2 antibody followed by Western blot (WB) with anti-TNF-R-associated factor 2 (TRAF2) antibody to determine TRADD-bound TRAF2 or TNF-R2-bound TRAF2 levels, respectively. Soluble TRAF2 was determined by Western blot of nonparticulate fractions from lung homogenates. Representative images from multiple analyses (*n* = 3–4/group) are presented. Data are normalized to air-exposed *Tnfr*^{+/+} mice, and normalized group mean $\pm$ SEM and results from statistical analyses (two-way analysis of variance [ANOVA], *p* < 0.05) are shown in Table 1. (B) Differential levels of TRAF2 localized in terminal bronchioles of *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after 48 hours of exposure to air and 0.3 ppm O₃. TRAF2-positive lung cells were immunohistologically stained with anti-TRAF2 antibody. High magnification shows TRAF2 localized on the membrane and in the cytoplasm. Bars indicate 100 μ m. Representative light photomicrographs are presented.

TABLE 1. QUANTIFIED RESULTS OF WESTERN BLOT ANALYSES AND ELECTROPHORETIC MOBILITY SHIFT ASSAYS IN *Tnfr*^{+/+} AND *Tnfr*^{-/-} MICE

Proteins Detected	Genotype	Air	6 h O ₃	24 h O ₃	Figure (sample/group)
TRADD(IP)/ TRAF2(WB)	<i>Tnfr</i> ^{+/+}	1 ± 0.01	3.37 ± 0.24*	1.91 ± 0.37*	1A
	<i>Tnfr</i> ^{-/-}	0.59 ± 0.01	2.07 ± 0.05*	0.57 ± 0.23†	(n = 3)
TNF-R2(IP)/ TRAF2(WB)	<i>Tnfr</i> ^{+/+}	1 ± 0.01	1.63 ± 0.10*	1.87 ± 0.19*	1A
	<i>Tnfr</i> ^{-/-}	0.03 ± 0.03†	0.07 ± 0.00†	0.03 ± 0.03†	(n = 3)
Soluble TRAF2	<i>Tnfr</i> ^{+/+}	1 ± 0.25	0.65 ± 0.25	0.46 ± 0.16*	1A
	<i>Tnfr</i> ^{-/-}	0.65 ± 0.09	0.32 ± 0.11†	0.21 ± 0.13*	(n = 4)
<i>p</i> -IKK/IKK(α/β)	<i>Tnfr</i> ^{+/+}	1 ± 0.12	1.38 ± 0.14*	1.45 ± 0.07*	2A
	<i>Tnfr</i> ^{-/-}	0.00 ± 0.00†	0.00 ± 0.00†	0.81 ± 0.17*	(n = 3)
<i>p</i> -IκB/IκB(α)	<i>Tnfr</i> ^{+/+}	1 ± 0.21	2.02 ± 0.50	5.31 ± 1.22*	2A
	<i>Tnfr</i> ^{-/-}	0.29 ± 0.15	0.98 ± 0.23	1.28 ± 0.64†	(n = 3)
Total NF-κB (EMSA)	<i>Tnfr</i> ^{+/+}	1 ± 0.19	1.73 ± 0.38*	3.59 ± 0.67*	2B
	<i>Tnfr</i> ^{-/-}	0.68 ± 0.14†	0.96 ± 0.26†	1.88 ± 0.32*	(n = 3)
p65 NF-κB (EMSA)	<i>Tnfr</i> ^{+/+}	1 ± 0.08	2.31 ± 0.52*	2.45 ± 0.46*	2B
	<i>Tnfr</i> ^{-/-}	0.77 ± 0.07	1.24 ± 0.14	1.06 ± 0.33†	(n = 3)
p50 NF-κB (EMSA)	<i>Tnfr</i> ^{+/+}	1 ± 0.06	1.10 ± 0.01	2.21 ± 0.19*	2B
	<i>Tnfr</i> ^{-/-}	0.47 ± 0.11†	0.68 ± 0.22	1.33 ± 0.07*	(n = 3)
<i>p</i> -ERK/ERK	<i>Tnfr</i> ^{+/+}	1 ± 0.02	1.28 ± 0.06	3.32 ± 0.15*	3A
	<i>Tnfr</i> ^{-/-}	0.36 ± 0.02†	1.25 ± 0.15*	2.01 ± 0.04*	(n = 3)
<i>p</i> -JNK/JNK	<i>Tnfr</i> ^{+/+}	1 ± 0.10	2.07 ± 0.04*	2.92 ± 0.41*	3A
	<i>Tnfr</i> ^{-/-}	0.75 ± 0.22	1.34 ± 0.30†	1.82 ± 0.11*	(n = 3)
Total AP-1‡ (EMSA)	<i>Tnfr</i> ^{+/+}	1 (0.98, 1.02)	1.37 (1.48, 1.26)	1.57 (1.8, 1.34)*	3B
	<i>Tnfr</i> ^{-/-}	0.62 (0.65, 0.58)†	0.93 (0.95, 0.91)†	1.15 (1.38, 0.93)*	(n = 2)
Jun AP-1 (EMSA)	<i>Tnfr</i> ^{+/+}	1 ± 0.07	1.43 ± 0.01*	1.45 ± 0.01*	3B
	<i>Tnfr</i> ^{-/-}	0.63 ± 0.02†	0.65 ± 0.13†	0.86 ± 0.07†	(n = 3)

Definition of abbreviations: AP-1 = activator protein-1; EMSA = electrophoretic mobility shift assay; ERK = extracellular signal-regulated kinase; IκB = inhibitor of κB; IKK = IκB kinase; IP = immunoprecipitation; JNK = c-Jun-NH₂ terminal kinase; NF-κB = nuclear factor-κB; *p*- = phosphorylated; TNF-R2 = tumor necrosis factor receptor-2; TRAF2 = TNF-R-associated factor 2; TRADD = TNF-R1-associated death domain protein; WB = Western blot.

Data are presented as mean ± SEM of relative ratio to air-exposed *Tnfr*^{+/+} mice.

* Significantly different from genotype-matched air controls (*p* < 0.05).

† Significantly different from exposure-matched *Tnfr*^{+/+} mice (*p* < 0.05).

‡ Data presented as mean and individual normalized ratio to air-exposed *Tnfr*^{+/+} mice.

injury by O₃ had reached a peak (Figure 4). Induced levels of these genes were significantly lower in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice (Figure 4). In contrast, IL-6 transcript level was significantly greater in *Tnfr*^{-/-} mice than in *Tnfr*^{+/+} mice after 24- and 48-hour exposure (Figure 4).

Functional Role of NF-κB in O₃-induced Pulmonary Toxicity

Lung inflammation, injury, and proliferation. Because NF-κB binding activity was significantly lower in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice after O₃ exposure, we hypothesized that mice deficient in a functional subunit of NF-κB (*Nfkb1*^{-/-}) would be less responsive to inflammatory effects of O₃ compared with wild-type (*Nfkb1*^{+/+}) control animals. No significant differences in mean total protein concentration or cell differentials were found in bronchoalveolar lavage (BAL) fluid between *Nfkb1*^{+/+} and *Nfkb1*^{-/-} mice after air exposure (Figure 5A). O₃-induced increases in the mean total protein concentration and numbers of neutrophils and epithelial cells were significantly attenuated (70–50%) in *Nfkb1*^{-/-} mice relative to the *Nfkb1*^{+/+} control animals (Figure 5A). However, no significant O₃ or genotype effects on the numbers of BAL macrophages were found (Figure 5A).

Immunohistologic localization of PCNA indicated a few proliferating cells throughout the lung sections in both genotypes of mice exposed to air (Figure 5B). Cellular proliferation in the injured regions (mainly in terminal bronchioles) caused by O₃ was markedly reduced in *Nfkb1*^{-/-} mice compared with *Nfkb1*^{+/+} mice (peak at 48 h, Figure 5B). Western blot analysis (Figure 5B) determined significant differences (twofold) in nuclear PCNA (36 kD) between the two genotypes basally and after O₃ (48 h).

Nuclear NF-κB–DNA binding activity. O₃ significantly stimulated total NF-κB and specific p50 and p65 binding in the lungs of *Nfkb1*^{+/+} mice at 6 and/or 24 hours (Figure 6). In *Nfkb1*^{-/-} mice, total κB activity (SB) was slightly enhanced by O₃ at 24 hours (Figures 6A–6D), whereas specific binding activity of p50 κB (SSB) was not detected (Figure 6B). Compared with *Nfkb1*^{+/+} mice, total (SB in Figure 6A) and p65 (SSB in Figure 6C) κB binding activity was significantly depressed in *Nfkb1*^{-/-} mice, basally and after O₃ exposure (Figure 6D). This suggested that absence of p50 subunit inhibited heterodimerization of p65–p50 κB for DNA binding.

Functional Role of MAPK8 (JNK1) in O₃-induced Pulmonary Toxicity

Because activated JNK (*p*-JNK) was also suppressed in *Tnfr*^{-/-} mice compared with *Tnfr*^{+/+} mice after O₃ exposure, we compared inflammatory responses to O₃ in *Jnk*^{-/-} and *Jnk*^{+/+} mice to address functional relevance of this signaling pathway. No significant differences in mean BAL total protein concentration or numbers of neutrophils and epithelial cells were found between air- and O₃-exposed *Jnk1*^{-/-} mice (Figure 7). These lung injury indices were significantly lower (60–75%) in *Jnk1*^{-/-} mice compared with those in *Jnk1*^{+/+} mice after O₃ (Figure 7). O₃ did not significantly change the mean numbers of BAL macrophages in either genotype (Figure 7).

DISCUSSION

The functional importance of TNF-α as a key modulator of O₃-induced airway toxicity has been documented by multiple animal studies. Anti-TNF-α antibody pretreatment decreased airway

Figure 2. Nuclear factor (NF)- κ B pathway was attenuated in *Tnfr*-deficient mice. (A) Differential activation of IKK and I κ B in the lungs of *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after exposure to air and 0.3 ppm O₃ (6 or 24 h) as assessed by Western blotting with phospho-specific antibodies. Representative images from multiple analyses (n = 3–4/group) are presented. Data are normalized to air-exposed *Tnfr*^{+/+} mice, and normalized group mean $\pm$ SEM and results from statistical analyses (two-way ANOVA, p < 0.05) are shown in Table 1. (B) Differential nuclear NF- κ B-DNA binding activity in the lungs of *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after exposure to air and 0.3 ppm O₃ (6 or 24 h). Aliquots of nuclear protein isolated from pieces of right lung (n = 3 mice/group) were incubated with an end-labeled oligonucleotide probe containing NF- κ B consensus sequence. Total NF- κ B-DNA binding was determined by gel shift analysis (top panel). To detect specific binding activity of each NF- κ B subunit by gel supershift analyses, either anti-p65 (middle panel) or anti-p50 (bottom panel) subunit antibodies were added to the binding reactions. SB indicates shifted bands of total bindings (NF- κ B motif-protein complex); SSB indicates super-shifted bands of specific bindings (NF- κ B motif-protein antibody complex). Representative images from multiple analyses (n = 3/group) are presented. Data are normalized to air-exposed *Tnfr*^{+/+} mice, and normalized group mean $\pm$ SEM and results from statistical analyses (two-way ANOVA, p < 0.05) are shown in Table 1.

Figure 3. Mitogen-activated protein kinase/activator protein-1 (MAPK/AP-1) pathway was attenuated in *Tnfr*-deficient mice. (A) Differential phosphorylation of MAPK (p-JNK1, p-ERK) levels in the lungs of *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after exposure to air and 0.3 ppm O₃ (6 or 24 h) as assessed by Western blotting. Representative images from multiple analyses (n = 3–4/group) are presented, and group mean $\pm$ SEM normalized to air-exposed *Tnfr*^{+/+} mice and statistical analyses are shown in Table 1. (B) Differential AP-1-DNA binding activity in the lungs of *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after air and 0.3 ppm O₃ (6 or 24 h). Aliquots of nuclear protein isolated from pieces of right lung (n = 3 mice/group) were incubated with an end-labeled oligonucleotide probe containing AP-1 consensus sequence. Total AP-1-DNA binding was determined by gel shift analysis (top panel). To detect specific binding activity of AP-1 Jun proteins by gel supershift analyses, anti-Jun antibody was added to the binding reactions. SB indicates shifted bands of total bindings (AP-1 motif-protein complex); SSB indicates super-shifted bands of specific bindings (AP-1 motif-protein-antibody complex). Representative images from multiple analyses are presented. Data are normalized to air-exposed *Tnfr*^{+/+} mice, and normalized group mean $\pm$ SEM (n = 3/group) or mean and individual values (total AP-1 binding, n = 2/group) are shown with results from statistical analyses (two-way ANOVA, p < 0.05) in Table 1.

inflammation, hyperpermeability, and cell proliferation after acute or subacute O₃ exposure in rodents (17, 19, 20, 36). Lung inflammation and epithelial injury were also reduced after subacute O₃ exposure in mice genetically deficient in TNF-R (*Tnfr1*^{-/-}, *Tnfr2*^{-/-}, or *Tnfr1*^{-/-}*Tnfr2*^{-/-}) (16). Furthermore, acute O₃-induced airway hyperreactivity was decreased in these TNF-R knockout mice (16, 18). In the present study, we determined that relative to wild-type mice, activation of NF- κ B and MAPK/AP-1 pathways was significantly reduced in *Tnfr*^{-/-} mice,

Figure 4. *Tnfr* deficiency reduced transcriptional induction of inflammatory mediators. Inflammatory gene expression was detected by reverse transcriptase–polymerase chain reaction using total lung RNA isolated from *Tnfr*^{+/+} and *Tnfr*^{-/-} mice after exposure to air and 0.3 ppm O₃ (n = 3/group). Representative cDNA band images for each gene are shown in (A). Quantitated intensities of digitized cDNA bands were normalized to the intensities of 18S bands, and relative intensity to air-exposed *Tnfr*^{+/+} mice of each gene is shown in (B). Data are presented as group means ± SEM. *Significantly higher than genotype-matched air controls (two-way ANOVA, p < 0.05); +significantly higher than exposure-matched *Tnfr*^{-/-} mice (two-way ANOVA, p < 0.05). ICAM-1 = intercellular adhesion molecule-1; LT-β = lymphotoxin-β; MIP-2 = macrophage inflammatory protein-2; TNF-α = tumor necrosis factor-α.

and significantly lower lung injury and inflammation were found in *Nfkb1*^{-/-} and *Jnk1*^{-/-} mice after O₃ exposure. Our observations are the first to demonstrate that NF-κB and MAPK/AP-1 pathways are key signaling components of TNF-mediated pulmonary pathogenesis by inhaled O₃ (Figure 8).

NF-κB and MAPK/AP-1 pathways are critical in developmental processes and immune responses by orchestrating expression of multiple genes involved in inflammation and immunity, development, lymphoid differentiation, oncogenesis, and apoptosis. Use of NF-κB subunit- or *Jnk*-deficient mice has supplied direct evidence for the role of NF-κB and MAPK in pulmonary

inflammation and allergy models. For example, *Nfkb1*^{-/-} mice were resistant to allergic airway eosinophilic inflammation and mycobacterial infection (37, 38). c-Rel κB deficiency also reduced airway hyperresponsiveness and chemokine induction after allergen challenge (39). Lack of JNK (*Jnk1* or *Jnk2*) inhibited neutrophilic influx and chemokine expression after mechanical ventilation (40). These two redox-sensitive transcription factor signaling pathways have also been shown to be induced by O₃ in airway cells and tissues (41–44). More recently, Fakhrazadeh and colleagues (45) determined a functional role of pulmonary NF-κB in the increase of inducible nitric oxide synthase and

Figure 5. Nuclear factor (NF)- κ B was essential in O_3 -induced pulmonary pathogenesis. (A) Effect of targeted disruption of *Nfkb1* was determined by bronchoalveolar lavage (BAL) phenotypes after 48 hours of exposure to air and 0.3 ppm O_3 . Data are presented as means $\pm$ SEM ($n = 5$ mice/group). *Significantly different from genotype-matched air control mice (two-way ANOVA, $p < 0.05$); ⁺significantly different from O_3 -exposed *Nfkb1*^{+/+} mice (two-way ANOVA, $p < 0.05$). (B) Differential proliferation of pulmonary cells in *Nfkb1*^{+/+} and *Nfkb1*^{-/-} mice after 48 hours of exposure to air or 0.3 ppm O_3 . S-phase cells undergoing proliferation were detected by proliferating cell nuclear antigen (PCNA) immunostaining. Representative light photomicrographs are shown. Bars indicate 100 μ m. Representative Western blot image demonstrates NF- κ B p50-dependent increase of nuclear PCNA in mouse lungs. Graph depicts mean $\pm$ SEM from duplicates normalized to air-exposed *Nfkb1*^{+/+}. *Significantly different from genotype-matched air control mice (two-way ANOVA, $p < 0.05$); ⁺significantly different from exposure-matched *Nfkb1*^{+/+} mice (two-way ANOVA, $p < 0.05$).

TNF- α levels by inhaled O_3 . Our current observations support NF- κ B and MAPK as key mediators of TNF-R responses.

The present study, however, indicated that TNF signaling does not account for all O_3 -induced NF- κ B and MAPK/AP-1 activities. As depicted in Figures 2 and 3 (also see Table 1), O_3 exposure significantly activated signal transducers of NF- κ B and MAPK/AP-1 pathways even in the absence of TNF-R. This suggests that receptor-mediated signals other than TNF-R activate these pathways in response to O_3 . It is possible that greater fold increases of certain NF- κ B and MAPK signal proteins in *Tnfr*^{-/-} mice than in *Tnfr*^{+/+} mice compared with genotype-matched air-exposed control animals may be associated with compensatory activation of these non-TNF-R signals in the absence of TNF-R. In support of this concept, Alcamo and associates (46) determined that TNF-R1/NF- κ B p65-double deficient

mice were significantly more resistant to lung neutrophilic inflammation and chemokine/cytokine expression (e.g., ICAM-1, MIP-2) than TNF-R1 single knockout mice during acute lung injury induced by endotoxin. These studies thus indicated that activation of pulmonary NF- κ B may also occur independently of TNF-R signaling after stimulation with exogenous stimuli. It has become clear that proinflammatory responses by the pulmonary innate immune system are partially mediated through pattern recognition receptors including the Toll-like receptor (TLR) family of proteins (47–49). We previously determined that TLR4 contributes significantly to the pulmonary hyperpermeability response to subacute O_3 exposure (50), and that mechanisms underlying hyperpermeability are dissociated from those for TNF-R-mediated cellular inflammation (16). Accumulating evidence shows that MAPK and NF- κ B signaling pathways are essential

Figure 6. p50 Nuclear factor (NF)-κB deficiency abolished total NF-κB activity in the lung. Nuclear NF-κB-DNA binding activity in the lungs of *Nfkb1*^{+/+} and *Nfkb1*^{-/-} mice after exposure to air and 0.3 ppm O₃ (6 or 24 h). Aliquots of nuclear protein isolated from pieces of right lung (n = 3 mice/group) were incubated with an end-labeled oligonucleotide probe containing NF-κB consensus sequence. Total NF-κB-DNA binding was determined by gel shift analysis (A). To detect specific binding activity of each NF-κB subunit by gel supershift analyses, either anti-p50 (B) or anti-p65 (C) subunit antibody was added to the binding reactions. SB indicates shifted bands of total bindings (NF-κB motif-protein complex); SSB indicates super-shifted bands of specific bindings (NF-κB motif-protein-antibody complex); FP indicates free probes. The total (indicated as SB in A) and specific (indicated as SSB) p50 (B) or p65 (C) NF-κB-DNA binding activity was quantified using a Bio-Rad Gel Doc 2000 System, and mean ± SEM (n = 3/group) or mean and individual values (n = 2/group) normalized to air-exposed *Nfkb1*^{+/+} mice were presented (D). *Significantly different from genotype-matched air control mice (two-way ANOVA for total and p65 κB, one-way ANOVA for p50 κB, p < 0.05); ⁺significantly different from exposure-matched *Nfkb1*^{+/+} mice (two-way ANOVA, p < 0.05).

in TLR4/MyD88-dependent cell signaling (51, 52). In addition, lung injury induced by a particle (residual oil fly ash) was significantly attenuated in mice with dominant mutant *Tlr4* (C3H/HeJ) compared with *Tlr4* normal mice (C3H/HeOuJ), and this resistance was shown to be mediated through suppressed

activation of downstream MAPK/AP-1 and NF-κB pathways (29). Collectively, these investigations suggest that interaction exists between TNF and TLR4 signaling mechanisms through NF-κB and MAPK pathways during the pathogenesis of pulmonary oxidative injury. In the current study, abolishment of

Figure 7. c-Jun-NH₂ terminal kinase (JNK) was essential in O₃-induced pulmonary pathogenesis. Effect of targeted disruption of *Jnk1* was determined by bronchoalveolar lavage (BAL) phenotypes after 48 hours of exposure to 0.3 ppm O₃. Data are presented as means ± SEM (n = 3–5 mice/group). *Significantly different from genotype-matched air control mice (two-way ANOVA, p < 0.05); ⁺significantly different from O₃-exposed *Jnk1*^{+/+} mice (two-way ANOVA, p < 0.05).

Figure 8. A hypothetical molecular mechanism underlying inhaled O_3 -induced pulmonary inflammation and injury. O_3 may cause ligand binding to tumor necrosis factor receptor (TNF-R) on pulmonary cells to elicit trimerization of TNF-R and receptor complex formation by recruitment of accessory proteins, including TNF-R1-associated death domain protein (TRADD) and TNF-R-associated factor 2 (TRAF2). This event will trigger phosphorylation of downstream signal transducers, including mitogen-activated protein kinase (MAPK) kinase (MEK) and inhibitor of κ B ($I\kappa$ B) kinase (IKK), which in turn would induce phosphorylation of MAPK, including c-Jun-NH₂ terminal kinase (JNK) and phosphorylation and degradation of $I\kappa$ B, respectively. Activator protein (AP)-1 proteins activated by phosphorylated MAPK and nuclear factor (NF)- κ B subunits (e.g., p50, p65) liberated from $I\kappa$ B-NF- κ B complex would be subsequently translocated into nuclei for DNA binding to modulate inflammatory effector gene expression. These signaling pathways and possibly feedback regulation by TNF- α (dashed arrows) and/or by other cytokines and receptors (dotted arrow) may be essential to propagate airway inflammation and injury caused by O_3 , and exacerbate symptoms in subjects with preexisting respiratory disease (e.g., asthma).

O_3 -induced hyperpermeability in *Jnk1*^{-/-} mice (see Figure 7) and *Nfkb1*^{-/-} mice (see Figure 5A) supports this possibility.

The current study also identified multiple proinflammatory genes that were differentially regulated in *Tnfr*^{-/-} and *Tnfr*^{+/+} mice during O_3 -induced lung inflammation. Included among these is the potent neutrophil chemoattractant MIP-2, which is also TNF dependent in murine pulmonary models of silica and cigarette smoke toxicity (10, 13). We also observed TNF-R-mediated induction of TNF- α (autoregulation) in O_3 -exposed lungs. A similar observation was reported in the lungs after cigarette smoke exposure, and mice deficient in TNF-R had decreased expression of TNF- α , whereas TNF- α was induced in wild-type mice after exposure (13). Presence of functional AP-1 and NF- κ B binding sites in mouse MIP-2 (34, 53) and TNF- α (54, 55) gene promoters further supports TNF-mediated MIP-2 and TNF- α regulation via these transcription factors. The injurious effects of TNF-dependent IL-1 β have also recently been determined after acute O_3 exposure (56). Interestingly, in the present study, IL-6 mRNA was overexpressed in O_3 -resistant *Tnfr*^{-/-} mice, which may suggest a protective role for this cytokine. IL-6 has been shown to have antiinflammatory properties. For example, IL-6 deficiency augmented hydrogen peroxide-induced murine alveolar epithelial cell death (57), and anti-IL-6 antibody treatment significantly increased neutrophilic inflammation caused by O_3 exposure in rats (58). However, converse effects have also been reported, and IL-6 was determined to be proinflammatory during the early phase of O_3 exposure in mice (59, 60).

Figure 8 depicts a schematic representation of the molecular mechanisms that we have investigated and identified as putative signal transduction pathways leading to pulmonary toxicity caused by inhaled O_3 . In summary, we uncovered that NF- κ B and MAPK/AP-1 play key roles in subacute O_3 -induced lung inflammation and injury mediated through TNF-R. Although further investigation is required to clarify the complex link between these two pathways and downstream inflammatory mediator networks, the current study provided details of molecular events underlying pulmonary O_3 toxicity. Our observations may have important implications for understanding the pathogenesis of inflammatory sequelae after environmental O_3 exposure in normal subjects and individuals with preexisting lung disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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