Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-α receptors

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Cho, Hye-Youn, Liu-Yi Zhang, and Steven R. Klee-berger. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-α receptors. *Am J Physiol Lung Cell Mol Physiol* 280: L537–L546, 2001.—This study was designed to investigate the mechanisms through which tumor necrosis factor (*Tnf*) modulates ozone (O_3)-induced pulmonary injury in susceptible C57BL/6J (B6) mice. B6 [wild-type (*wt*)] mice and B6 mice with targeted disruption (knockout) of the genes for the p55 TNF receptor [*TNFR1*(−/−)], the p75 TNF receptor [*TNFR2*(−/−)], or both receptors [*TNFR1/TNFR2*(−/−)] were exposed to 0.3 parts/million O_3 for 48 h (subacute), and lung responses were determined by bronchoalveolar lavage. All TNFR(−/−) mice had significantly less O_3-induced inflammation and epithelial damage but not lung hyperpermeability than *wt* mice. Compared with air-exposed control mice, O_3 elicited upregulation of lung TNFR1 and TNFR2 mRNAs in *wt* mice and downregulated TNFR1 and TNFR2 mRNAs in *TNFR2*(−/−) and *TNFR1*(−/−) mice, respectively. Airway hyperreactivity induced by acute O_3 exposure (2 parts/million for 3 h) was diminished in knockout mice compared with that in *wt* mice, although lung inflammation and permeability remained elevated. Results suggested a critical role for TNFR signaling in subacute O_3-induced pulmonary epithelial injury and inflammation and in acute O_3-induced airway hyperreactivity.

susceptibility; tumor necrosis factor receptor knockout; pulmonary injury; gene targeting

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OZONE (O_3) is a highly toxic principal oxidant found in urban environments and workplaces throughout the world. Although airway toxicity to O_3 has been studied extensively in laboratory animals and humans (4), the precise mechanisms underlying pathogenesis of the pulmonary airways after exposure to O_3 are not clear.

Recent studies have raised concern about the potentially susceptible subpopulations that are at increased risk to the effects of environmental pollutants including O_3. Smoking status, preexisting pulmonary disease, age, gender, and race may affect the variability of airway responses to O_3 (4, 28, 37, 39). Interindividual variation in the pulmonary function responses to O_3 has also been observed in healthy nonsmokers (28, 41, 43). Similarly, a wide range of neutrophilic inflammation has been reported from bronchial biopsies or bron-choalveolar lavage (BAL) from healthy humans after acute O_3 exposure (2, 9, 12). These observations have led investigators to suggest that genetic background is a host risk factor in humans that contributes to the differential susceptibility to the adverse health effects of this air pollutant.

Significant differences in O_3 sensitivity have also been reported in animal models (8, 11, 33). Kleeberger and colleagues (16, 18) previously demonstrated that C57BL/6J (B6) inbred mice were more sensitive to pulmonary injury with subacute [0.3 parts/million (ppm) for 72 h] and acute (2 ppm for 3 h) O_3 exposures than C3H/HeJ (C3) mice. To investigate the genetic factors that determine differential susceptibility, a genomewide linkage analysis was done with a B6C3F$_2$ cohort phenotyped for inflammatory responses to 0.3 ppm O_3 (19). In that study, significant and suggestive quantitative trait loci (QTLs) were identified on chromosomes 17 and 11, respectively. The chromosome 17 QTL included a potential candidate gene [tumor necrosis factor (*Tnf*)] that encodes TNF-α. TNF-α is known to be a key proinflammatory cytokine released after O_3 exposure from lung cells including alveolar macrophages and epithelial cells (1). It has also been proposed as a central mediator in airway hyperresponsiveness and inflammation in rodent airways after O_3 exposure (22). Pretreatment of susceptible B6 mice with anti-TNF-α antibody significantly attenuated O_3-induced pulmonary injury and provided strong evidence for *Tnf* as an O_3 susceptibility gene (19). However, the role of TNF-α in acute lung injury and inflammation has not been thoroughly studied.

The cellular effects of TNF-α are mediated by two structurally related but functionally distinct receptors: TNF receptor type 1 (TNFR1; 55 kDa) and TNF receptor type 2 (TNFR2; 75 kDa). TNFR1 and TNFR2 are members of the nerve growth factor/TNFR superfamily of proteins that are characterized by their conserved extracellular cysteine-rich repeat (5). Both receptors are coexpressed on the surface of most cells and may be proteolytically released as soluble molecules to inhibit TNF-α action in the inflammatory response (42, 44). TNF-α binds to the two receptors with similar affinity (24) and

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induces the transcription of genes that regulate acute inflammation, including early-response cytokines (e.g., interleukin-1β), chemokines (e.g., macrophage inflammatory protein-2), and adhesion molecules (e.g., intercellular adhesion molecule-1) (21, 34, 44).

The present study was designed to investigate the roles of TNFR1 and TNFR2 signaling pathways in the development of O_3-induced lung injury in mice. Wild-type (*wt*) B6 mice and gene-knockout mice deficient in either TNFR1 [*TNFR1*(−/−)], TNFR2 [*TNFR2*(−/−)], or both TNFR1 and TNFR2 [*TNFR1/TNFR2*(−/−)] were exposed to 0.3 ppm O_3 for 48 h (subacute) or 2 ppm O_3 for 3 h (acute), and lung inflammatory and injury responses were compared. Deficiency of TNFR1 and TNFR2 provided significant protection from O_3-induced inflammation and epithelial injury (at 0.3 ppm) and airway hyperreactivity (at 2 ppm) in murine lungs. These studies demonstrated the importance of TNFR-mediated responses in the detrimental pulmonary effects of O_3.

METHODS

General

Male (6–8 wk old) inbred B6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Breeding pairs of *TNFR1*(−/−) [C57BL/6J-*p55*(−/−)], *TNFR2*(−/−) [C57BL/6J-*p75*(−/−)], and *TNFR1/TNFR2*(−/−) [C57BL/6J-*p55/p75*(−/−)] mice were a generous gift from Dr. Jacques Peschon (Immunex, Seattle, WA) (32). Breeding colonies for each mutant were established in our facilities at the Johns Hopkins School of Hygiene and Public Health (Baltimore, MD). The original mutant strains have been backcrossed to a B6 background through a minimum of seven generations. All animals were housed in a virus- and antigen-free room. Water and mouse chow were provided ad libitum. Cages were placed in laminar flow hoods with high-efficiency particulate-filtered air. Sentinel animals were examined periodically (titers and necropsy) to ensure that the animals had remained free of infection. All experimental protocols conducted in the mice were carried out in accordance with the standards established by the US Animal Welfare Acts and set forth in National Institutes of Health guidelines and the Policy and Procedures Manual (Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee).

O₃ Exposure

Mice were placed individually in stainless steel wire cages with free access to food and water during the exposure. The cages were set inside one of two separate 700-liter laminar flow inhalation chambers (Baker, Sanford, ME) that were equipped with a charcoal-filtered air and a high-efficiency particulate-filtered air supply. Chamber air was renewed at the rate of ~20 changes/h, with 50–65% relative humidity and a temperature of 20–25°C. O_3 was generated by directing air (2 l/min) through an ultraviolet light O_3 generator (Orec, Phoenix, AZ) that was upstream from one of the exposure chambers. The O_3-air mixture was metered into the inlet airstream with computer-operated stainless steel mass flow controllers. O_3 concentrations were monitored regularly at different levels within the chamber with an O_3 ultraviolet light photometer (Dasibi model 1003 AH, Dasibi Environmental, Glendale, CA). The Dasibi model 1003 AH was calibrated regularly against a standard source (Dasibi model 1008-PC, Dasibi Environmental). Mice from each genotype were exposed to 0.3 ppm O_3 for 3, 24, or 48 h to assess the subacute effect of O_3. Other mice from each genotype were exposed to 2 ppm O_3 for 3 h and then put in room air for 6 or 24 h for recovery to assess the acute effect of O_3. Mice assigned to corresponding control groups were exposed to filtered air in the inhalation chambers for the same duration.

Necropsy and BAL

Mice were anesthetized with pentobarbital sodium (104 mg/kg), and the lungs were lavaged in situ four times with Hanks' balanced salt solution (35 ml/kg, pH 7.2–7.4). Recovered BAL fluid was immediately cooled to 4°C and centrifuged. The supernatant from the first lavage return was assayed for total protein (a marker of lung permeability) with the Bradford assay. The four cell pellets were resuspended and pooled in 1 ml of Hanks' balanced salt solution, and the cells were counted with a hemacytometer. An aliquot (10 µl) of BAL cell suspension was cytocentrifuged and stained with Wright-Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL) for differential cell analysis. Differential counts for epithelial cells (a marker of epithelial damage), neutrophils, lymphocytes, and macrophages (markers of inflammation) were done by identifying 300 cells according to standard cytological technique (36).

Measurement of Airway Pressure-Time Index

The airway pressure-time index (APTI) procedure for assessment of airway reactivity in mice has been previously described in detail (23). Briefly, each animal was initially anesthetized with an inspired concentration of 1.5% halothane. After the trachea was cannulated, each mouse was given ketamine (50 mg/kg iv in sterile saline), paralyzed with decamethonium (25 mg/kg iv in sterile saline; Sigma, St. Louis, MO), and ventilated at 120 breaths/min with a constant tidal volume (0.2 ml). These drugs do not have apparent strain-specific effects on airway caliber that may influence interpretation of airway responses to agonists. Airway pressure was measured at a distal port of the tracheal cannula and recorded on a strip chart. Acetylcholine (ACh; 50 µg/kg in sterile saline; Sigma) was injected into the inferior vena cava, and airway reactivity was estimated as the integrated change in airway pressure from the initial change until the return to baseline airway pressure (APTI; in $cmH_2O \cdot s$). Airway reactivity was measured 24 h after a 3-h exposure because Zhang et al. (46) had previously demonstrated the greatest difference in ACh responsiveness between air- and O_3-exposed B6 mice occurred at this time. We did not measure airway responsiveness in mice exposed to 0.3 ppm O_3 because we found that this exposure does not significantly increase responsiveness to ACh in C57BL/6J mice (Kleeberger, unpublished observations).

Analysis of TNFR1 (p55) and TNFR2 (p75) mRNA Expression

Total RNA was isolated from lung homogenates according to the method of Chomczynski and Sacchi (7) as indicated in the TRIzol reagent (Life Technologies, Gaithersburg, MD) specifications. Five hundred nanograms of pooled RNA from mice of each group were reverse transcribed into cDNA in a volume of 50 µl containing 1× PCR buffer (50 mM KCl and 10 mM Tris, pH 8.3), 5 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate, 125 ng of oligo(dT)$_{15}$, and 50 U of Moloney

murine leukemia virus (MMLV) RT (Life Technologies) at 45°C for 15 min and 95°C for 5 min with a Gene Amp PCR system 9700 (PerkinElmer Applied Biosystems, Foster City, CA). PCR amplification was performed with an aliquot of cDNA (10 μl) with a final concentration of 1× PCR buffer, 4 (TNFR1) or 2 (TNFR2 and β-actin) mM MgCl$_2$, 400 μM each deoxynucleotide triphosphate, 120 nM each forward and reverse primer, and 1.25 U of *Taq* polymerase in a total volume of 25 μl, with forward and reverse primers specific for mouse TNFR1 (5′-GCA CCA AGT GCC ACA AAG-3′ and 5′-ACG GTG TTC TGA GTC TCC-3′, respectively), TNFR2 (5′-AGC TGC AGT TCG AAG ACC A-3′ and 5′-CTT CCT GTA CCA CTG ACC A-3′, respectively), and β-actin (5′-GTG GGC CGC TCT AGG CAC CA-3′ and 5′-CGG TTG GCC TTA GGG TTC AGG GGG G-3′, respectively) as internal controls. PCR was started with a 5-min incubation at 94°C followed by a three-step temperature cycle: denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min for 25~30 cycles. A final extension step at 72°C for 10 min was included after the final cycle to complete polymerization. The number of cycles was chosen to ensure that the amplification product did not reach a plateau level. The predicted amplified sizes of the cDNA product for TNFR1, TNFR2, and β-actin were 319, 286, and 221 bp, respectively. Reactions were electrophoresed in 2% agarose gels containing ethidium bromide, and the volume of each cDNA band was quantitated with a Bio-Rad Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA), and the ratios of TNFR1 and TNFR2 cDNA to β-actin cDNA were determined.

Lung Tissue Preparation for Histopathology

Left lung lobes excised from mice exposed to either 0.3 ppm O$_3$ or filtered air for 48 h were fixed with zinc formalin, embedded in paraffin, and sectioned 5 μm thick. Tissue sections were histochemically stained with hematoxylin and eosin for morphological comparison of pulmonary injury between genotypes. The terminal bronchioles and alveoli were the primary focus of study because a subacute exposure to 0.3 ppm O$_3$ causes histologically evident inflammation and epithelial lesions in these regions of the mouse lung (see Ref. 19).

Statistics

All data are expressed as group means ± SE. The data were natural log (ln) transformed, if necessary, to normalize the distribution and make the variances approximately equal. Two-way ANOVA was used to evaluate the effects of the subacute O$_3$ exposure on pulmonary toxicity in *wt* and TNFR-deficient mice. The factors in the analysis were exposure (air or O$_3$) and genotype [*wt, TNFR1*(−/−), *TNFR2* (−/−), or *TNFR1/TNFR2*(−/−)]. The dependant variables were BAL fluid protein concentration and cell number. The data from the acute O$_3$ experiment were analyzed by three-way ANOVA to determine the effect of exposure (air or O$_3$), genotype [*wt, TNFR1*(−/−), or *TNFR2*(−/−)] and postexposure time (6 or 24 h) on APTI and BAL fluid protein and cells. Because there was no postexposure time effect on the BAL fluid protein and cells in air-exposed mice, the data from two air control groups were pooled and are expressed as pooled means ± SE. The Student-Newman-Keuls a posteriori test was used for comparisons of the group means. All statistical analyses were performed with a commercial statistical analysis package (SuperANOVA, Abacus Concepts, Berkeley, CA). Significance was accepted at P ≤ 0.05.

RESULTS

Role of TNFR in O$_3$-Induced Pulmonary Inflammation and Injury

To determine the role of TNFR-mediated responses in O$_3$-induced lung inflammation, epithelial injury, and hyperpermeability, *wt* and TNFR-knockout mice were exposed to 0.3 ppm O$_3$ for 48 h (subacute) or 2.0 ppm O$_3$ for 3 h (acute). The number of neutrophils, macrophages, lymphocytes (markers of inflammation), and epithelial cells (a marker of epithelial injury) and the concentration of total protein (a marker of the permeability change) in BAL returns were determined as indexes of response.

Subacute O$_3$ exposure. In mice exposed to air, there were no significant differences in the number of inflammatory and epithelial cells or in the amount of protein between genotypes [i.e., *wt, TNFR1*(−/−), *TNFR2* (−/−), and *TNFR1/TNFR2*(−/−); Figs. 1 and 2]. Compared with air exposure, O$_3$ caused significant increases in the number of neutrophils, macrophages, lymphocytes, and epithelial cells in *wt* mice (24-, 2.5-, 6-, and 4-fold, respectively). In the three genotypes of TNFR-knockout mice exposed to O$_3$, there was a marked decrease in macrophages (30%), neutrophils (80–90%), and epithelial cells (40–50%) compared with those in *wt* mice exposed to O$_3$. No significant effects of O$_3$ were detected on lymphocyte infiltration in *TNFR1*(−/−), *TNFR2*(−/−), and *TNFR1/TNFR2*(−/−) mice. There were no significant differences in the number of inflammatory and epithelial cells among *TNFR1*(−/−), *TNFR2*(−/−), and *TNFR1/TNFR2*(−/−) mice exposed to O$_3$.

Compared with air exposure, O$_3$ caused a fivefold increase in total BAL fluid protein in *wt* mice (Fig. 2). All O$_3$-exposed TNFR-deficient mice had significantly higher BAL fluid protein concentrations than genotype-matched air-exposed control mice, and the magnitude of the O$_3$-induced hyperpermeability change in those mice was not significantly different from that in *wt* mice.

Acute O$_3$ exposure. Neutrophil infiltration and hyperpermeability were assessed 6 and 24 h, respectively, after acute O$_3$ exposure because Kleeberger et al. (16) previously determined that the responses peak at these times. As opposed to the subacute O$_3$ exposure model, we have not found that the number of BAL fluid macrophages, lymphocytes, or epithelial cells are significantly elevated in B6 mice (i.e., *wt*) after acute O$_3$ exposure (16). Compared with air-exposed *wt* control mice, however, there was an ~20-fold greater BAL fluid neutrophilic inflammation in *wt* mice exposed to O$_3$ (Fig. 3*A*). In contrast to subacute exposure, acute O$_3$ exposure induced a significant elevation in neutrophil number in both *TNFR1*(−/−) and *TNFR2*(−/−) mice, and the O$_3$-induced neutrophilic inflammation in *TNFR1*(−/−) and *TNFR2*(−/−) mice was not significantly different from that in *wt* mice.

Mean BAL fluid protein concentrations were not significantly different among *wt, TNFR1*(−/−), and

Fig. 1. Number of neutrophils (A), macrophages (B), lymphocytes (C), and epithelial cells (D) in bronchoalveolar lavage (BAL) fluid recovered from wild-type (*wt*) and tumor necrosis factor receptor type 1 [*TNFR1*(−/−)], type 2 [*TNFR2*(−/−)], and type 1 and 2 [*TNFR1/TNFR2*(−/−)] knockout mice after a 48-h exposure to 0.3 parts/million (ppm) ozone (O$_3$) or filtered air. Values are means ± SE; $n = 4$–12 mice/group. *Significantly different from genotype-matched air-exposed control mice, $P < 0.05$. +Significantly different from O$_3$-exposed TNFR knockout mice, $P < 0.05$.
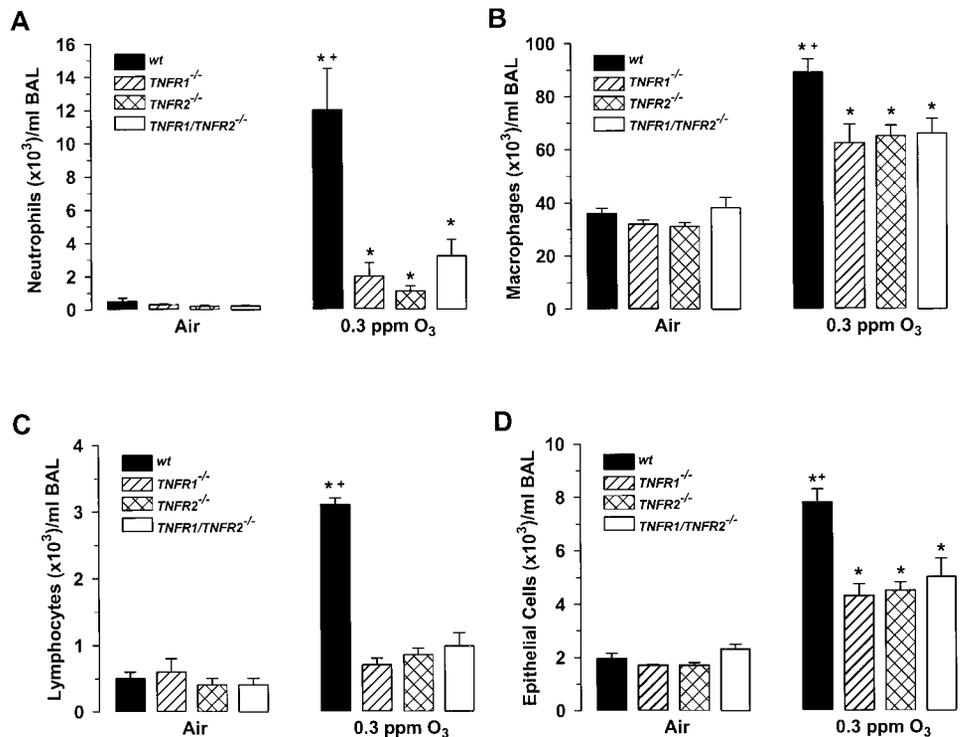
TNFR2(−/−) mice after air exposure (Fig. 3B). At 24 h after exposure, there were significant increases (two-fold) in the amount of protein in the lungs of *wt*, *TNFR1*(−/−), and *TNFR2*(−/−) mice compared with genotype-matched air-exposed control mice. However, there were no significant differences in the O$_3$-induced lung hyperpermeability among *wt*, *TNFR1*(−/−), and *TNFR2*(−/−) mice. Because there were no significant differences in the acute exposure-induced inflammation, hyperpermeability, or reactivity phenotypes between single-TNFR knockout mice and *wt* mice (see below), double-knockout mice were not investigated.

Role of TNFR in Acute O$_3$-Induced Airway Hyperreactivity

The role of TNFR in the acute O$_3$-induced pulmonary functional response was assessed by measuring airway
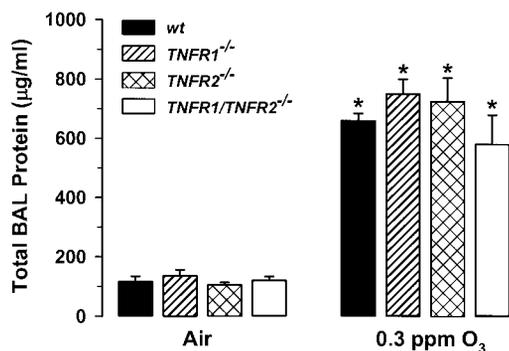

Fig. 2. Concentration of total proteins in BAL fluid recovered from *wt* and TNFR knockout mice after a 48-h exposure to 0.3 ppm O$_3$ or filtered air. Values are means ± SE; $n = 4$–12 mice/group. *Significantly different from genotype-matched air-exposed control mice, $P < 0.05$.
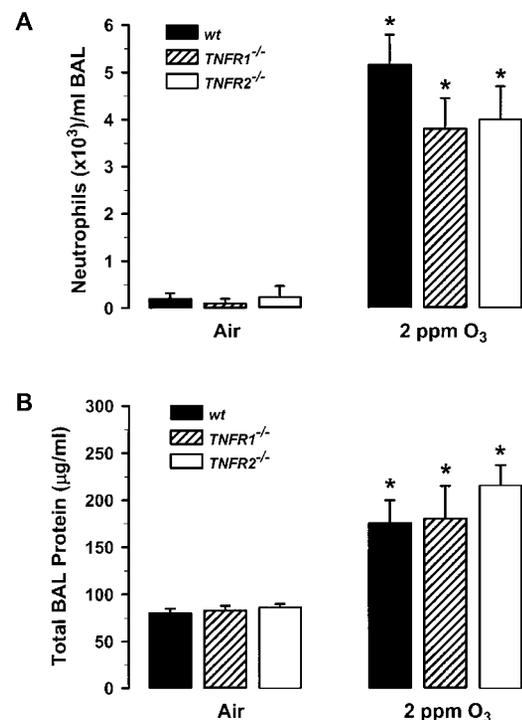

Fig. 3. Number of neutrophils (A) and concentration of total protein (B) in BAL fluid recovered from *wt* and TNFR knockout mice after a 3-h exposure to 2 ppm O$_3$ or filtered air. Neutrophils and proteins were assessed 6 and 24 h, respectively, after O$_3$ exposure because Kleeberger et al. (16) determined previously that the responses peak at these times. Values are means ± SE; $n = 4$–8 mice/group. *Significantly different from genotype-matched air-exposed control mice, $P < 0.05$.
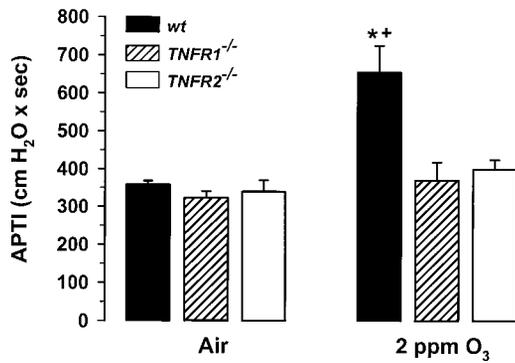
Fig. 4. Airway pressure-time index (APTI) response to ACh (50 μg/kg) in *wt* and TNFR knockout mice 24 h after a 3-h exposure to 2 ppm O$_3$ or filtered air. Values are means ± SE; n = 4–8 mice/group. *Significantly different from genotype-matched air-exposed control mice, $P < 0.05$. +Significantly different from O$_3$-exposed *TNFR-1(−/−)* and *TNFR2 (−/−)* mice, $P < 0.05$.

reactivity as determined by APTI. Mean baseline airway pressures measured 3 min before ACh challenge were not significantly different between *wt* and TNFR-knockout mice after air or O$_3$ exposure (data not shown). O$_3$ significantly increased (twofold) the APTI response to ACh in *wt* mice (Fig. 4). However, the ACh response in O$_3$-exposed *TNFR1(−/−)* and *TNFR2(−/−)* mice was not significantly different from that in genotype-matched air-exposed control mice.

Histopathology of Lungs From wt and TNFR2(−/−) Mice After Subacute O₃ Exposure

Because the magnitude and characteristics of light microscopically examined pathology of the O$_3$-induced lung injury were similar in the three genotypes of TNFR-knockout mice, we present one [*TNFR2(−/−)*] as a representative of each. Figure 5 shows light photographs of representative hematoxylin and eosin-stained lung tissues from *wt* (*A* and *B*) and *TNFR2(−/−)* (*C* and *D*) mice exposed to either 0.3 ppm O$_3$ or filtered air for 48 h. In the lungs of *wt* (Fig. 5*A*) and *TNFR2(−/−)* (Fig. 5*C*) mice exposed to filtered air, there were no significant changes in the lung pathology as assessed by light microscopy. The lungs appeared normal, with regularly shaped alveoli and 1–2 layer thickness of epithelium lining the bronchioles and ter-
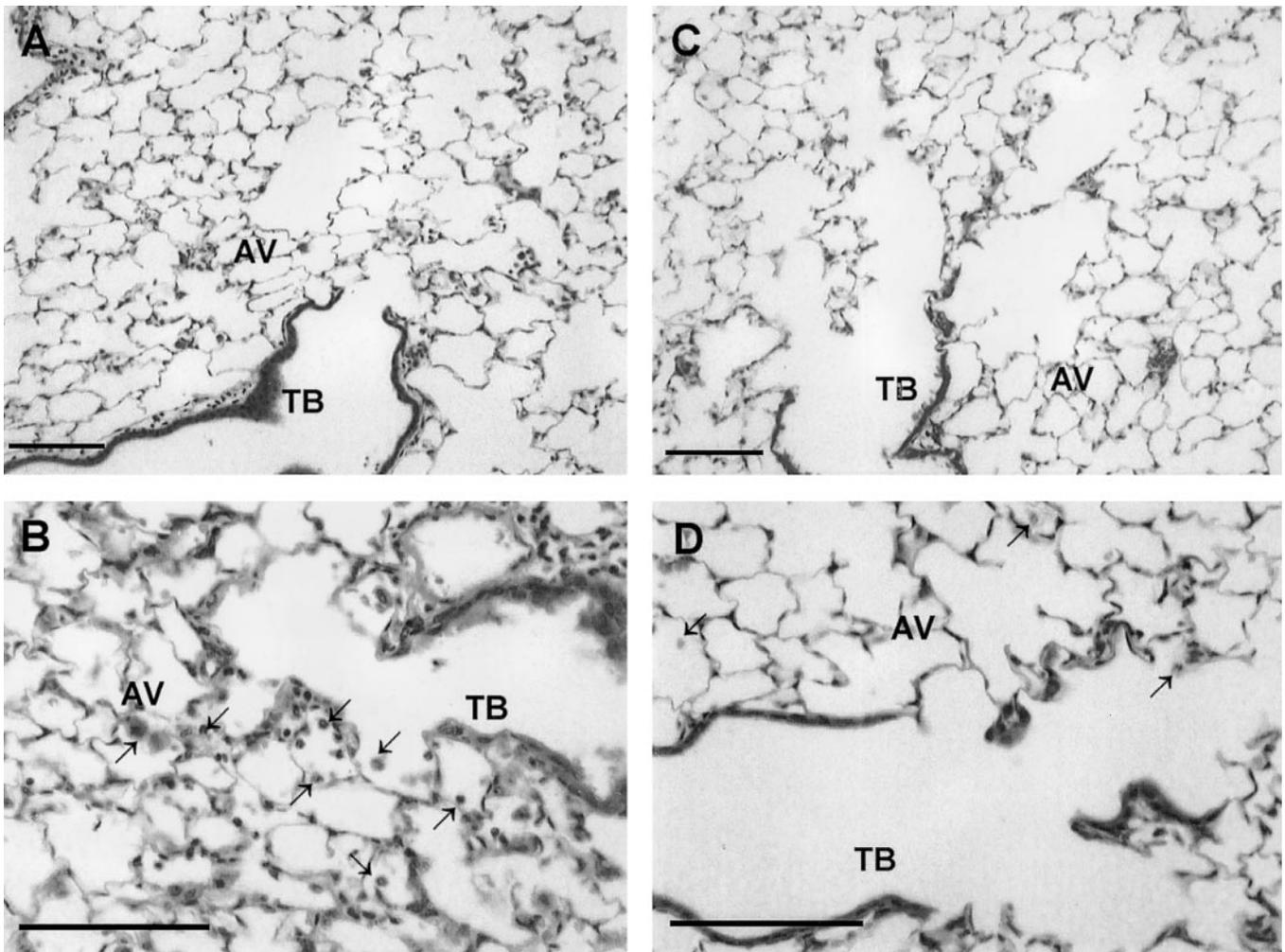

Fig. 5. Light photomicrographs of lungs from filtered air-exposed *wt* (*A*) and *TNFR2(−/−)* (*C*) mice and from 0.3 ppm O$_3$-exposed *wt* (*B*) and *TNFR2(−/−)* (*D*) mice. All exposures were for 48 h. Tissue sections were stained with hematoxylin and eosin. AV, alveoli; TB, terminal bronchiole. Arrows, inflammatory cells. Bars, 100 μm.
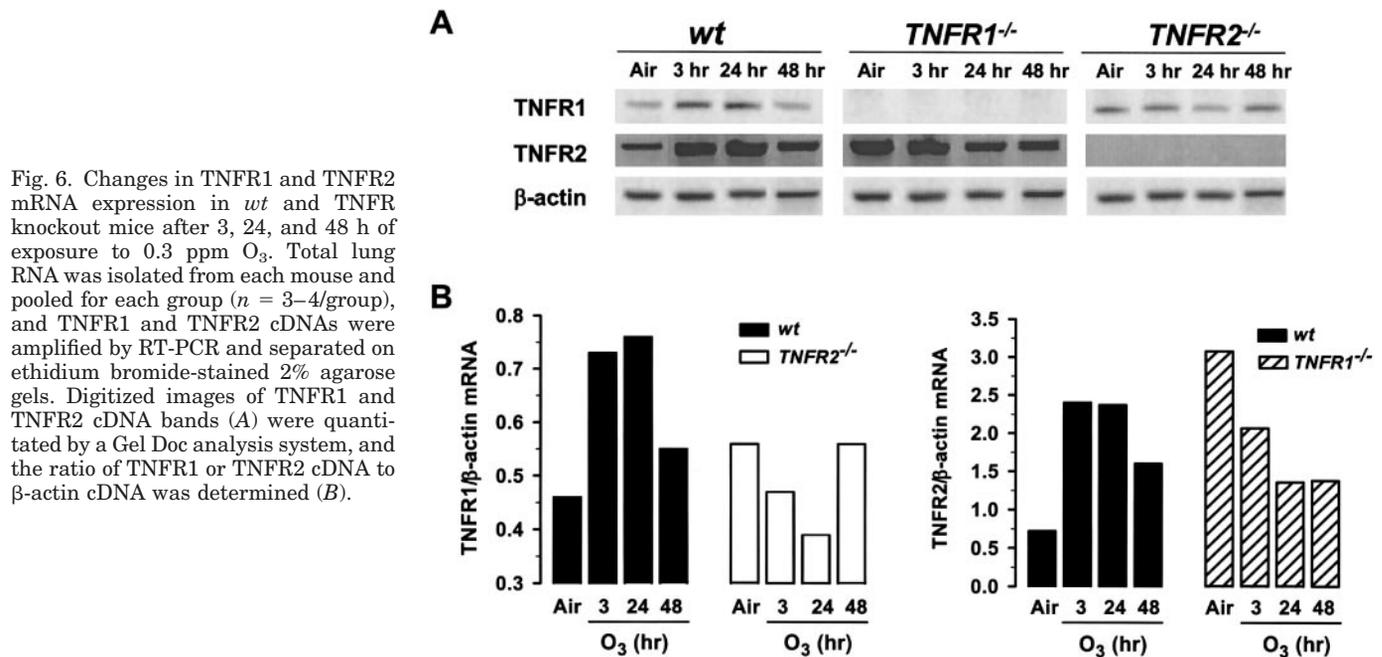
Fig. 6. Changes in TNFR1 and TNFR2 mRNA expression in *wt* and TNFR knockout mice after 3, 24, and 48 h of exposure to 0.3 ppm O_3. Total lung RNA was isolated from each mouse and pooled for each group ($n = 3$–4/group), and TNFR1 and TNFR2 cDNAs were amplified by RT-PCR and separated on ethidium bromide-stained 2% agarose gels. Digitized images of TNFR1 and TNFR2 cDNA bands (*A*) were quantitated by a Gel Doc analysis system, and the ratio of TNFR1 or TNFR2 cDNA to β-actin cDNA was determined (*B*).

minal bronchioles in those animals. There were few infiltrating neutrophils and alveolar macrophages in the air spaces and lung tissues in these air-exposed animals. At 48 h, marked pathological changes by inhaled O_3 were evident in the centriacinar pulmonary regions (i.e., terminal bronchiole and alveolar duct) and throughout the alveoli. The *wt* mice exposed to O_3 had severe inflammation characterized by numerous neutrophils and macrophages in the alveolar air spaces, lung parenchyma, and blood vessels (Fig. 5*B*).

Concurrent with the inflammation was extensive exudation throughout the alveoli, which resulted in the loss of alveolar structures in these mice. In addition, thickening of terminal bronchial epithelium and alveolar septa was evident in *wt* mice exposed to O_3. This is likely due to the epithelial cell proliferation (hyperplasia) and hypertrophy that Kleeberger et al. (19) have previously demonstrated in B6 mice. In *TNFR2*(−/−) mice exposed to O_3, there was focal exudation and mild inflammatory cell infiltration mainly in the blood ves-
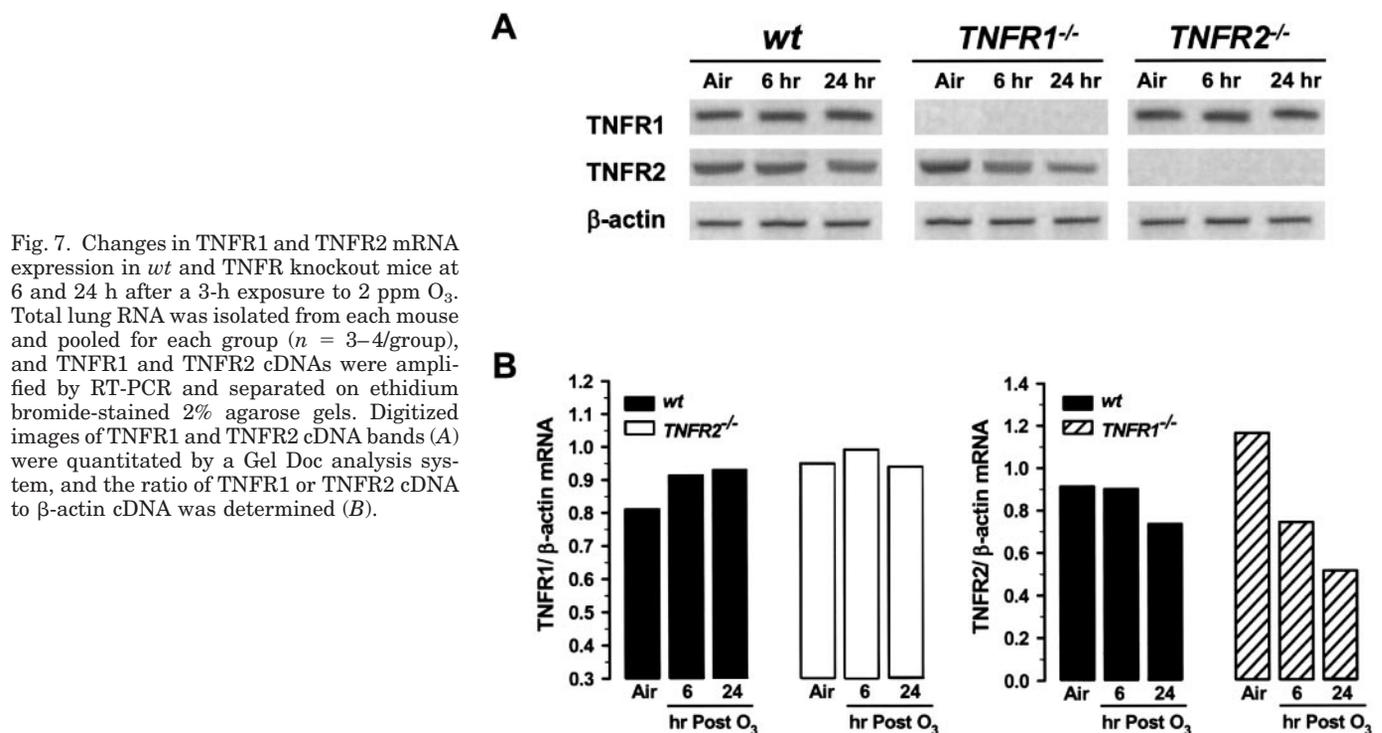
Fig. 7. Changes in TNFR1 and TNFR2 mRNA expression in *wt* and TNFR knockout mice at 6 and 24 h after a 3-h exposure to 2 ppm O_3. Total lung RNA was isolated from each mouse and pooled for each group ($n = 3$–4/group), and TNFR1 and TNFR2 cDNAs were amplified by RT-PCR and separated on ethidium bromide-stained 2% agarose gels. Digitized images of TNFR1 and TNFR2 cDNA bands (*A*) were quantitated by a Gel Doc analysis system, and the ratio of TNFR1 or TNFR2 cDNA to β-actin cDNA was determined (*B*).

sels, perivascular regions, and interstitium (Fig. 5D). The epithelial hyperplasia in alveoli and terminal bronchioles evident in O₃-exposed *wt* mice was less noticeable in *TNFR2*(−/−) mice after O₃ exposure.

TNFR mRNA Expression

Subacute O₃ exposure. To ensure the absence of TNFR1 (p55) and TNFR2 (p75) mRNA expression in *TNFR1*(−/−) and *TNFR2*(−/−) mice, respectively, and to determine whether transcriptional activation of the *TNFR* gene is associated with activation of TNF-α signaling in O₃-sensitive mice, TNFR1 and TNFR2 mRNA expression was determined by RT-PCR in murine lung tissues after 3, 24, and 48 h of 0.3 ppm O₃ exposure. The normal mouse lung constitutively expressed both TNFR1 and TNFR2 mRNA (Fig. 6A). As expected, no TNFR1 mRNA was detected in the *TNFR1*(−/−) mice exposed to either air or O₃. Similarly, TNFR2 mRNA was not detected in *TNFR2*(−/−) mice exposed to either air or O₃. The basal expression of TNFR1 mRNA in *TNFR2*(−/−) mice (25%) and TNFR2 mRNA in *TNFR1*(−/−) mice (fourfold) was higher than that in *wt* mice (Fig. 6B). However, O₃ enhanced the steady-state expression of TNFR1 and TNFR2 mRNAs only in *wt* mice as early as 3 h of exposure (56% and threefold, respectively), and mRNA levels remained elevated at 48 h of exposure in these mice. After a 24-h O₃ exposure, the expression of TNFR1 mRNA in *TNFR2*(−/−) mice and TNFR2 mRNA in *TNFR1*(−/−) mice was decreased to levels lower than those in *wt* mice. At 48 h of exposure, the level of TNFR1 mRNA in *TNFR2*(−/−) mice was slightly elevated but did not exceed that of *wt* mice.

Acute O₃ exposure. Lung TNFR mRNA expression levels were determined by RT-PCR 6 and 24 h after a 3-h exposure to 2 ppm O₃. As determined in the subacute O₃ exposure experiment, the basal expression of TNFR1 mRNA in *TNFR2*(−/−) mice and TNFR2 mRNA in *TNFR1*(−/−) mice was higher than those in *wt* mice (Fig. 7). The differences in TNFR2 expression between *wt* and *TNFR1*(−/−) mice after acute and subacute air exposure may be attributed to exposure (chamber) effects or, more likely, may simply reflect between-experiment variation in gene expression and quantitation. Within-experiment RT-PCR results (Figs. 6 and 7) were highly reproducible. In *wt* mice, O₃ caused a slight elevation (~14%) in TNFR1 mRNA expression 6 and 24 h postexposure, although the increased TNFR1 mRNA level did not exceed the basal expression level of TNFR1 mRNA in *TNFR2*(−/−) mice (Fig. 7B). O₃ did not enhance TNFR2 mRNA in *wt* mice. There was no marked change in TNFR1 mRNA in *TNFR2*(−/−) mice after O₃ exposure. However, the basal level of TNFR2 mRNA in *TNFR1*(−/−) mice was decreased by 24 h postexposure to a level lower than that in *wt* mice. TNFR1 mRNA was not detected in *TNFR1*(−/−) mice, and TNFR2 mRNA was not detected in *TNFR2*(−/−) mice (Fig. 7A).

DISCUSSION

Results of this study indicated that TNFR-mediated responses play a key role in the O₃-induced injury and hyperreactivity in pulmonary airways of the mouse. Mice lacking TNFR1, TNFR2, or both were markedly less sensitive than *wt* mice to lung inflammation and epithelial damage by subacute O₃ exposure (0.3 ppm for 48 h). On acute exposure to 2 ppm O₃ (3 h), airway hyperreactivity to ACh in *wt* mice was diminished in mice lacking either TNFR1 or TNFR2, independent of pulmonary inflammation and epithelial injury. These observations strongly suggested that molecular and cellular mechanisms triggered by TNFR are critical in the development of pulmonary O₃ toxicity in mice and supported TNF-α as a subacute O₃ susceptibility gene in murine lungs. The current findings also implied that independent mechanisms control 1) lung inflammatory and permeability changes in response to subacute O₃ and 2) pulmonary inflammation induced by two levels of O₃ (i.e., subacute and acute). In addition, O₃-induced lung hyperpermeability may not be dependent on TNF-α signaling regardless of O₃ levels.

TNF-α is recognized as a key mediator in the pathogenesis of various inflammatory disorders. Numerous studies (27, 29, 30) have demonstrated that TNF-α plays a pivotal role in acute lung injury and inflammation induced by environmental toxicants such as bacterial endotoxin and silica. However, only a few studies (e.g., Refs. 19, 31) have examined the deleterious effects of TNF-α on airway responses to O₃. The specific roles of the two TNFRs in TNF-α-mediated biological activities triggered by inflammatory stimuli such as lipopolysaccharide have been investigated using receptor-specific antibodies or ligands and receptor knockout mice. TNFR1 is known as the primary signaling receptor through which the majority of inflammatory and cytotoxic responses attributed to TNF-α occur (3). In contrast, TNF-α-mediated T cell apoptosis and proliferation or skin necrosis are directed through TNFR2 (38). Additionally, TNFR2 has been postulated to function as a TNF-α antagonist by neutralizing TNF-α and as a TNF-α agonist by recruiting and delivering TNF-α to facilitate the interaction between TNF-α and TNFR1 at the cell surface (ligand passing) (32, 39). In vivo studies with silica exposure (29, 30) demonstrated the importance of TNFR signaling in lung inflammation, injury, and fibrosis. However, little is known about the behavior of TNFR1 and TNFR2 and TNF-α signaling events in the pathogenesis of airway O₃ toxicity. In the present study, a similar magnitude of attenuation in inflammation and epithelial injury responses to subacute O₃ exposure was observed in the absence of either TNFR1 or TNFR2. The same trend was detected in the airway hyperreactivity response to acute O₃ exposure. The present study also revealed that the suppressive effects on pulmonary injury and inflammation in the double-receptor knockout mice [*TNFR1/TNFR2*(−/−)] exposed to subacute O₃ were not greater than those in the single-receptor knockout mice.

Our experiments were not designed to understand the detailed mechanisms of the two receptors mediating pulmonary O_3 toxicity. However, results suggested that both TNFR1 and TNFR2 can independently modulate specific pulmonary responses to O_3 (i.e., epithelial injury, inflammation, and hyperreactivity) in mice. Although the lack of additive suppression in double-receptor knockout mice seems not to support the ligand-passing concept in which TNFR1-mediated responses are enhanced by TNFR2, we cannot rule out a possible overlap between signaling pathways under two receptors in the development of O_3-induced pulmonary injury. Further studies will be necessary to elucidate the role of intracellular signaling events triggered by two receptors in the pathogenesis of O_3-induced airway injury and hyperreactivity.

The pattern of TNFR gene expression in the lungs of *wt* (upregulation) and receptor knockout (downregulation) mice by subacute O_3 exposure was correlated with the pulmonary injury responses in both genotypes of mice. The relationship between TNFR gene expression and pulmonary injury has been reported in a recent study (30) in which the inducible expression of TNFR2 mRNA was believed to contribute to the silica- and bleomycin-induced lung fibrosis. Results of the present study suggested that upregulation of the steady-state levels of TNFR mRNA precedes the TNFR-dependent pulmonary injury responses. There was an elevation in basal TNFR1 mRNA expression in mice lacking TNFR2, and the converse was also observed. It appears that the two receptors in mouse lungs act in a compensatory manner in the absence of the other. To our knowledge, this is the first report that supports this concept, and the mechanisms through which compensation occurs have not been characterized. It is also unclear why O_3 downregulated TNFR1 or TNFR2 mRNA expression in the lungs of single-knockout mice. It is possible that the absence of signaling mediated by one TNFR may lead to dysregulation of the other receptor gene during lung injury processes. The gene encoding TNFR2 is known to be more readily inducible than the gene encoding TNFR1 (5), and we observed greater inducible levels of TNFR2 mRNA than of TNFR1 mRNA in *wt* mice exposed to subacute O_3.

The present study also demonstrated that O_3-induced permeability changes in murine lungs are not attributed to TNFR-mediated responses. The apparent dissociation between inflammatory and hyperpermeability responses to O_3 has been suggested by a number of previous studies. For example, Reinhart et al. (35) induced neutrophilic inflammation in rats (with 1% rabbit serum) before acute exposure to 0.8 ppm O_3 and found that enhanced neutrophilic infiltration did not augment the pulmonary hyperpermeability with inhaled O_3. In another study, mice were treated with cyclophosphamide, colchicine, or indomethacin to inhibit or deplete neutrophils before acute exposure to 2 ppm O_3, and all treatments had no effect on O_3-induced lung hyperpermeability (17). Although the mechanisms of O_3-induced changes in lung permeability are not understood, two separate studies (14, 26) with

rodents pretreated by a platelet-activating factor (PAF) receptor antibody indicated that the PAF receptor mediates pulmonary permeability and inflammation induced by acute O_3. More recently, genetic linkage analysis of the lung hyperpermeability response to subacute O_3 exposure identified a significant QTL on chromosome 4 (20). A candidate susceptibility gene, Toll-like receptor-4 (*Tlr4*), was identified within the QTL and tested. In that study, an association of differential O_3 sensitivity in resistant (C3) and susceptible (C3H/HeOuJ) mice with a polymorphism in the coding region of *Tlr4* was demonstrated. Furthermore, an opposite pattern of *Tlr4* mRNA expression was shown in C3H/HeOuJ (upregulation) and C3 (downregulation) mice after subacute O_3 exposure, consistent with the hypothesis that decreased expression of *Tlr4* conferred resistance to O_3-induced hyperpermeability. Further studies of *Tlr4* signal pathways may provide a better understanding of the molecular mechanisms of O_3-induced pulmonary hyperpermeability.

Using simple breeding experiments and genetic cosegregation analyses, Kleeberger et al. (18) demonstrated that separate genetic mechanisms control inflammatory responses induced in mice by acute and subacute O_3 exposures. That study implied that mechanisms that confer differential susceptibility to acute exposure are not necessarily predictive of susceptibility to subacute exposure. Results of the present study suggest that TNFR signaling mechanisms are fundamental to the pathogenesis of inflammation and epithelial injury induced by subacute but not by acute O_3 exposures and thus support the previous observations. This dissociation of mechanisms underlying injury responses by different O_3 exposures is perhaps not surprising because the magnitude and the peak time of the inflammatory response vary with O_3 concentration and exposure duration (6). The lack of TNFR gene induction in *wt* mice exposed to 2 ppm O_3 (as opposed to subacute exposure results) also suggests that mechanisms other than TNF-α signaling are elicited directly by O_3 and/or by subsequent transient neutrophilic inflammation and lung injury. Studies with PAF receptor antagonists (14, 26) and mast cell-deficient mice (25) suggested that mast cells contributed to a significant part of the lung inflammation and epithelial injury induced by 2 ppm O_3, perhaps by releasing soluble mediators including PAF. However, further studies are necessary to understand the precise molecular and cellular mechanisms of lung responses to acute O_3 exposure.

O_3-induced airway hyperreactivity has been demonstrated in a number of species (4), but the mechanisms are not completely understood. It has been demonstrated that parasympathetic nerves mediate O_3-induced hyperreactivity to histamine, ACh, and methacholine in guinea pigs and dogs (see Ref. 45). Furthermore, it has been hypothesized that dysfunction of inhibitory M_2 muscarinic receptors is critical to the development of O_3-induced hyperreactivity in the guinea pig (45). Depletion of polymorphonuclear neutrophils abolished the O_3 effect on reactivity and sug-

gested that inflammatory cell infiltration was a necessary component to hyperreactivity (10). In the mouse, however, polymorphonuclear neutrophil depletion did not affect O$_3$-induced hyperreactivity to ACh (46). The differences between studies may be related to species specificity of M$_2$ receptor involvement in the development of hyperreactivity. In the present study, mice lacking TNFR signaling did not develop airway hyperreactivity due to acute O$_3$ and suggested that TNF-α has an important role in the hyperreactivity induced by this exposure. The potential role of TNF-α in airway responsiveness has been previously demonstrated. Exogenous recombinant human TNF-α induced methacholine hyperresponsiveness and inflammation in human subjects (40), and anti-TNF-α antibody significantly inhibited lipopolysaccharide-induced airway hyperresponsiveness to ACh in rats (15). It has been postulated that the effect of TNF-α may be mediated through inducible nitric oxide synthase, which, in turn, regulates mediators such as interleukin-8 (or the mouse homolog macrophage inflammatory protein-2) (13), but further studies are necessary to clarify these mechanisms.

In summary, we have demonstrated that TNFR-mediated responses play a critical role in pulmonary inflammation, epithelial injury, and airway hyperreactivity induced by O$_3$ in mice. The results strongly support *Tnf* as a pulmonary susceptibility gene in inflammatory and epithelial responses induced by subacute O$_3$ exposure in mice. The inflammatory mechanisms stimulated by acute O$_3$ exposure differed from those with subacute O$_3$ exposure, and TNF-α signaling did not contribute to the acute inflammation in the mouse lungs. Although the pulmonary inflammation and epithelial injury induced by subacute O$_3$ exposure are largely but not completely mediated through TNF-α signaling, we cannot exclude the role of other candidate genes in the lung injury and inflammatory responses. Further studies are needed to clarify the TNFR-mediated signal transduction mechanisms and downstream effector genes through which TNF-α exerts lung injury and inflammation in response to subacute O$_3$ exposure.

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