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Impact of aging on pulmonary responses to acute ozone exposure in mice: role of TNFR1

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Abstract

Context—Chamber studies in adult humans indicate reduced responses to acute ozone with increasing age. Age-related changes in TNFα have been observed. TNFα induced inflammation is predominantly mediated through TNFR1.

Objective—To examine the impact of aging on inflammatory responses to acute ozone exposure in mice and determine the role of TNFR1 in age-related differences.

Materials and methods—Wildtype and TNFR1 deficient ($TNFR1^{-/-}$) mice aged 7 or 39 weeks were exposed to ozone (2 ppm for 3 h). Four hours after exposure, bronchoalveolar lavage (BAL) was performed and BAL cells, cytokines, chemokines, and protein were examined.

Results—Ozone-induced increases in BAL neutrophils and in neutrophil chemotactic factors were lower in 39- versus 7-week-old wildtype, but not ($TNFR1^{-/-}$) mice. There was no effect of TNFR1 genotype in 7-week-old mice, but in 39-week-old mice, BAL neutrophils and BAL concentrations of MCP-1, KC, MIP-2, IL-6 and IP-10 were significantly greater following ozone exposure in $TNFR1^{-/-}$ versus wildtype mice. BAL concentrations of the soluble form of the TNFR1 receptor (sTNFR1) were substantially increased in 39-week-old versus 7-week-old mice, regardless of exposure.

Discussion and conclusion—The data suggest that increased levels of sTNFR1 in the lungs of the 39-week-old mice may neutralize TNFα and protect these older mice against ozone-induced inflammation.

Keywords

Bronchoalveolar lavage; neutrophil; sTNFR1; TNFα

Introduction

Ozone (O_3) is a common air pollutant that causes significant morbidity and mortality. O_3 exposure causes cough, dyspnea, reductions in lung function, increased susceptibility to lung infections, hospitalizations, increased asthma attacks (Hazucha et al., 1996; Alexis et al., 2000; Fauroux et al., 2000; Tolbert et al., 2000; Gent et al., 2003; Triche et al., 2006; Chiu et al., 2009), and even excess deaths (Bell et al., 2005; Levy et al., 2005).

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Declaration of interest
The authors have no other declarations of interest to report.

O_3 exposure causes lung epithelial injury leading to inflammation. This inflammatory response includes the generation of numerous cytokines and chemokines, resulting in an influx of neutrophils (Hazucha et al., 1996; Johnston et al., 1999; Alexis et al., 2000; Johnston et al., 2000a; Shore et al., 2000; Shore et al., 2003; Johnston et al., 2006; Lu et al., 2006). Exposure chamber studies in human subjects indicate a reduction in the response to acute O_3 with increasing age in adult human subjects (McDonnell et al., 1993; Hazucha et al., 2003), but the mechanistic basis for this reduction has not been established. Both ourselves (Shore et al., 2002) and others (Vancza et al., 2009) have reported reductions in the inflammatory response to O_3 in young adult versus neonatal mice, but there have been no comparisons of pulmonary responsiveness to O_3 in mice older than 15 weeks. To determine whether we could model adult human age-related declines in the response to O_3 in mice, we examined the inflammatory response to acute O_3 exposure in 7- and 39-week-old mice. Although it is difficult to exactly map ages across mice and men, the 7-week-old mice can be considered similar to late adolescent/young adult humans, while the 39-week-old mice can be considered similar to middle aged humans.

Studies in both mice and humans have demonstrated a role for TNFα in responses to O_3. For example, genetic linkage studies in mice have implicated a role for a locus containing TNFα in the neutrophil influx that occurs after exposure to O_3 (Kleeberger, 2003). In humans, decrements in pulmonary function induced by O_3 exposure are augmented in subjects homozygous for the −308G polymorphism in the promoter of the TNFα gene (Yang et al., 2005). Studies using anti-TNF antibodies or TNFα receptor deficient mice, also indicate that TNFα is required for O_3-induced airway hyperresponsiveness and inflammation, but not lung injury in mice, although the requirement for TNFα appears to depend on the strength and duration of the O_3 exposure (Cho et al., 2001; Shore et al., 2001; Cho et al., 2007; Matsubara et al., 2009). The role of TNFα in the induction of cytokines and chemokines that occurs after acute O_3 has not been established, although TNFα does appear to be required for induction of IL-6 after prolonged exposure to O_3 (Cho et al., 2007).

TNFα can bind to either of two membrane bound receptors, TNFR1 and TNFR2. Most studies indicate that inflammation induced by TNFα is largely mediated through TNFR1 activation (Vandenabeele et al., 1995; Peschon et al., 1998; MacEwan, 2002; Naude et al., 2011), although TNFR2 mediated inflammation has been reported (Lucas et al., 1997; Akassoglou et al., 2003; Ramesh et al., 2003; Vielhauer et al., 2005). Soluble forms of these receptors (sTNFR1 and sTNFR2) created by cleavage from cell membranes are also present in blood and other body fluids, and have been considered by some to act as endogenous inhibitors of TNFα (Ulich et al., 1993; Hale et al., 1995; Yagi et al., 2010). Age-related elevations in serum TNFα, and in serum sTNFR1 and sTNFR2 have been reported (Dobbs et al., 1999; Brüünsgaard et al., 2003; Scalzo et al., 2009; de Gonzalo-Calvo et al., 2010), but it is unclear what the relative impact of these changes is, and whether they are relevant to the lungs. The impact of age-related changes in TNFα signaling on pulmonary responses to O_3 is essentially unknown.

To determine whether differences in TNFα signaling contributed to age-related differences in the inflammatory response to O_3, studies were performed both in wildtype (WT) mice and in mice genetically deficient in TNFR1. We chose to focus on TNFR1 because our interest was in the inflammatory response to O_3, and because most studies indicate that TNFα-induced inflammation is mediated through TNFR1 (Vandenabeele et al., 1995; Peschon et al., 1998; MacEwan, 2002; Naude et al., 2011).

In mice, increased pulmonary oxidative stress occurs during aging with an onset consistent with the older (39-week-old) mice in this study (Calvi et al., 2011). Moreover, the anti-oxidants metallothionein (MT) and heme oxygenase-1 (HO-1) are strongly induced by acute

O_3 exposure (Johnston et al., 2000b; Williams et al., 2007; Vasu et al., 2010), and have been shown to play important roles in attenuating subsequent inflammatory responses to O_3 (Hisada et al., 2000; Inoue et al., 2008). Because induction of MT-1, MT-2, and HO-1 by other stimuli has been shown to require TNFα (Oguro et al., 2002; Chiu et al., 2003; Quintana et al., 2007), we also measured the mRNA expression of MT-1, MT-2, and HO-1 to determine 1) whether age-related differences in inflammation were due to greater expression of these anti-oxidants; and 2) whether the induction of these enzymes by O_3 was TNF dependent.

We also examined a possible role of amphiregulin (Areg) in age- and TNFR1-related differences in the inflammatory response to O_3. Areg is one of a family of growth factors that act via the epidermal growth factor receptor (EGFR). Importantly, Areg is induced by O_3 exposure (Vasu et al., 2010) and others have reported roles for Areg both in promoting epithelial proliferation (Lee et al., 2011) and in limiting the pulmonary inflammation induced in a bleomycin model of pulmonary fibrosis (Fukumoto et al., 2010). Areg can also be induced by TNFα (Sisto et al., 2010).

Methods

Animals

Breeding pairs of TNFR1 deficient ($TNFR1^{-/-}$) mice were purchased from The Jackson Labs (Bar Harbor, ME). The mice have been backcrossed through at least 6 generations to C57BL/6 mice, which were therefore used as WT controls. Mice were bred in the animal facilities of the Harvard School of Public Health and were studied at either 7 or 39 weeks of age. Mice of both genders were used. It is noteworthy that alveolar development is complete by about 5 weeks of age in the mouse, although lung growth continues with somatic growth thereafter (Mund et al., 2008). The procedures were approved by the Harvard Medical Area animal use committee. Mice were on a 12 h light/dark cycle (6 AM on/6 PM off) and were exposed mid morning.

Ozone exposure

Mice were exposed to O_3 (2 ppm for 3 h) and studied 4 h after the cessation of exposure. The 4-h time point was chosen because our primary interest was in the acute inflammatory response induced by O_3: many of the cytokines and chemokines induced by O_3 peak at approximately this time point and then decline to near air exposed values by 24 h (Johnston et al., 2007). During exposure, mice were placed in individual wire mesh cages within a stainless steel and plexiglass exposure chamber. Both $TNFR1^{-/-}$ and age-matched WT mice were exposed at the same time within the same chamber. Control mice were exposed in an identical but distinct chamber to room air. Details of the O_3 exposure and monitoring system have been previously described (Johnston et al., 2005b).

Bronchoalveolar lavage

Four hours after O_3 exposure, mice were euthanized with an overdose of sodium pentobarbital. A canula was inserted into the trachea, and the lungs were lavaged with 1 ml of PBS containing 0.6 mM EDTA. The lavage was repeated, and the two samples were pooled and placed on ice until centrifuged at 2000 rpm at 4°C for 10 min. The pellet was resuspended in 1 ml of Hank's Balanced Salt solution (Sigma-Aldrich, St. Louis, MO) and total BAL cells counted using a hemacytometer. Aliquots of cells were centrifuged onto glass slides, air-dried, and stained with with Hema 3® (Biochemical Sciences, Inc., Swedesboro, NJ). Cell differentials were determined by counting 200–300 cells under 400× magnification. The BAL supernatant was frozen at −80°C until analyzed. BAL IL-6, KC, MIP-2, IP-10, MCP-1, and sTNFR1 were measured by Quantikine ELISAs or DuoSet®

ELISA development systems (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. BAL protein was measured using the Bradford protein assay procedure (Bio-Rad; Hercules, CA).

We instilled the same amount of fluid (1 ml × 2) for the BAL procedure in the 7- and 39-week-old mice. However, the 39-week-old mice were substantially larger, and likely had larger lungs and an increased amount of lung lining fluid. Therefore, there would have been greater dilution of lung lining fluid by the BAL fluid in the younger mice, leading to underestimation, relative to the 39-week-old mice, of actual BAL concentrations of cytokines and chemokines. Indeed, even in the air exposed mice, BAL concentrations of all cytokines and chemokines measured were greater in the 39-week-old than in the 7-weekold mice. Consequently, in order to compare the effect of O_3 on BAL cytokines and chemokines across age groups, we used the process described by Vancza et al. (2009) and expressed the values in O_3-exposed mice as a ratio of the air exposed values in mice of the same age and genotype. For BAL cells, we could not do this because BAL neutrophils were virtually absent in air exposed mice. Consequently, we expressed BAL cell types as total cell numbers.

RNA extraction and real time PCR

The lungs were excised and the left lung was immersed in RNAlater (Qiagen, Valencia, CA). Lung tissue was homogenized with lysis buffer containing 1% 2-mercaptoethanol using TissueLyser LT (Qiagen) at 50 Hz for 5 min. The resulting lysate was cleared by centrifugation, and total RNA precipitated with an equal volume of 70% ethanol. Total RNA was then purified using RNeasy columns (Qiagen) with a DNase II digestion step to remove genomic DNA. RNA concentration and purity was determined using a small volume spectrophotometer (Nanodrop, Thermo Scientific, Waltham, MA). 1 μg of total RNA was converted into cDNA using a commercial kit containing a combination of oligo-dt and random hexamer primers with a further cDNA sample treatment with RNase (SuperScript III for qRT-PCR, Invitrogen, Carlsbad, CA). MT-1, MT-2, HO-1, Areg, and claudin-4 (Cldn-4) mRNA were quantified using real time PCR (7300 Real-Time PCR Systems, Applied Biosystems, Carlsbad, CA) with primers described in Table 1 and SYBR-green detection. The delta Ct (ΔCt) was obtained by subtracting Ct values for 18S ribosomal RNA from the gene of interest. Changes in mRNA were expressed relative to values from the 7-week-old WT O_3 group, to obtain ΔΔCt values. Expression was calculated by $RQ = 2^{-\Delta\Delta Ct}$ (Livak et al., 2001).

Statistics

The significance of exposure, age, and genotype related changes in BAL cells, cytokines, chemokines, and in gene expression was assessed by factorial ANOVA using STATISTICA software (StatSoft®; Tulsa, OK). Fisher's least significant difference (LSD) test was used as a follow-up to determine the significance of differences between individual groups. The results are expressed as mean and SE. A p value less than 0.05 was considered significant.

Results

Body weight

The 39-week-old mice weighed significantly more than the 7-week-old mice ($p < 0.001$). However, there was no significant effect of genotype on body weight. The 7- and 39-week-old WT mice weighed 19.2 ± 0.8 and 42.0 ± 2.1 g, respectively, and the 7- and 39-week-old $TNFR1^{-/-}$ mice weighed 22.5 ± 1.1 and 45.2 ± 2.6 g, respectively.

Bronchoalveolar lavage cells

Compared to air, O_3 exposure caused a significant increase in the numbers of neutrophils ($p < 0.001$) and epithelial cells ($p < 0.001$) in the BAL and a reduction in the numbers of macrophages ($p < 0.001$) (Figure 1). No effect of O_3 on BAL lymphocytes was observed in any experimental group (data not shown). For BAL neutrophils, there were significant effects of age on the response to O_3 that varied by genotype. The number of BAL neutrophils was significantly lower in 39-week-old versus 7-week-old WT mice exposed to O_3, whereas this age-related effect was abolished in O_3-exposed TNFR1$^{-/-}$ mice (Figure 1). Similar results were obtained when the neutrophils were expressed as a percentage of total cells (data not shown).

BAL cytokines and chemokines

In order to examine the mechanistic basis for the reduced O_3-induced recruitment of neutrophils that occurred in the 39-week-old wildtype mice, we measured BAL concentrations of a number of cytokines and chemokines that have been implicated in neutrophil recruitment following acute O_3 exposure (Figure 2). As described above, BAL cytokines and chemokines from O_3-exposed mice were normalized to their respective age- and genotype-matched air exposed controls (represented by the horizontal lines in Figure 2 at a “fold versus air” of 1). Factorial ANOVA indicated that compared to air, O_3 caused an increase in BAL IL-6, MCP-1, IP-10, KC, and MIP-2 ($p < 0.001$ in each case; Figure 2). In wildtype mice, a significant effect of age on the response to O_3 was observed for MCP-1, KC, and MIP-2, which were all lower in 39 versus 7-week-old mice, whereas for IL-6 and IP-10, no age-related difference was observed for O_3-exposed WT mice. However, in TNFR1 deficient mice, only BAL KC and MIP-2 were significantly lower in O_3-exposed 39 versus 7-week-old mice (Figure 2). In 7-week-old mice O_3-exposed mice, TNFR1 deficiency had no effect on BAL cytokines and chemokines except for BAL KC, which was higher in the TNFR1$^{-/-}$ than the WT mice. In contrast, in the 39-week-old O_3-exposed mice, all cytokines and chemokines were higher in the TNFR1$^{-/-}$ versus WT mice.

We also measured age-related changes in BAL concentrations of the soluble form of the TNFR1 receptor (sTNFR1) (Figure 3). Compared to air, O_3 exposure did not cause any significant change in BAL sTNFR1. However, there was a significant effect of age ($p < 0.01$). BAL sTNFR1 was substantially increased in 39-week-old versus 7-week-old mice, regardless of exposure.

Lung injury

O_3 exposure is known to cause injury to lung epithelial cells leading to increased lung permeability and an increase in BAL protein (Bhalla, 1999). As described above, BAL protein from O_3-exposed mice was normalized to their respective age- and genotype-matched air exposed controls (represented by the horizontal lines in Figure 4 at a “fold versus air” of 1). O_3 exposure caused a significant increase in BAL protein in TNFR1$^{-/-}$ but not WT mice, but the effect only reached statistical significance in the 39-week-old TNFR1$^{-/-}$ mice (Figure 4). Consistent with lack of effect of O_3 in the wildtype mice, we have previously reported that O_3 induced increases in BAL protein are minimal 4 h post-exposure (the time point used in this study), but increase substantially by 24 h after exposure (Lang et al., 2008).

Because of the impact of TNFR1 deficiency on lung epithelial barrier integrity (Figure 4), and because the tight junction protein claudin-4 has been shown to play an important role in alveolar epithelial barrier function (Wray et al., 2009; Mitchell et al., 2011) and to be impacted by TNF (Mazzon et al., 2007; Wang et al., 2009), we also measured claudin-4 mRNA expression in lung tissue from these mice (Figure 5A). Compared to air, O_3 exposure

resulted in a marked increase in claudin-4 gene expression in mice of both ages and both genotypes (Figure 5A) However, there was no significant impact of either age or genotype on claudin-4 expression.

Anti-oxidant gene expression

The mRNA expression of HO-1, MT-1, and MT-2 was markedly increased by O_3 exposure in mice of both genotypes and both ages (Figure 5B, C, D). MT-1 expression was significantly reduced in O_3-exposed 39-week-old versus 7-week-old WT mice, but there was no effect of TNFR1 deficiency on MT-1 expression in O_3-exposed mice. A similar trend was observed for MT-2, but did not reach statistical significance ($p < 0.085$). There was no significant effect of either age or TNFR1 genotype on O_3-induced HO-1 expression.

Areg expression

We also examined a possible role of Areg in age- and TNFR1-related differences in the inflammatory response to O_3. Compared to air, O_3 exposure caused a marked increase in Areg expression in mice of both ages and both genotypes (Figure 5E). Areg was induced to a greater extent in 39-week-old than in 7-week-old O_3-exposed wildtype mice, but there was no effect of TNFR1 genotype.

Discussion

Our data indicate that in mice, age-related differences in the inflammatory response to acute O_3 exposure vary with TNFR1 expression. In WT mice, the influx of BAL neutrophils was lower (Figure 1B), and BAL concentrations of MCP-1, KC, and MIP-2 were also reduced in the 39-weekold versus 7-week-old mice (Figure 2). In contrast, the decreased inflammatory responses in the older mice reverted to robust responses in TNFR1$^{-/-}$ mice (Figures 1B and 2). MT and HO-1 expression did not appear to account for the age- or TNFR1-related differences in the inflammatory response to O_3 (Figure 5). However, we did observe that BAL sTNFR1 was significantly increased in the 39 week versus 7-week-old mice (Figure 3). Taken together, the data suggest that this increase in sTNFR1 may neutralize TNF in the lung and protect the 39-weekold mice against O_3-induced inflammation.

One important technical issue requires consideration. As discussed in the Methods section, in order to correct for age-related differences in dilution of lung lining fluid by a fixed amount of instilled BAL fluid, we expressed the BAL concentrations of IL-6, MCP-1, IP-10, KC, MIP-2, and protein in the O_3-exposed mice as a ratio of the respective age- and genotype-matched air values (Figures 2 and 4), a procedure previously employed by ourselves and others in correcting for age-related differences in BAL moieties (Shore et al., 2002; Vancza et al., 2009). Hence, in Figures 2 and 4, the air exposed values have all been set to a value of “1”, represented by the horizontal lines. However, in the case of sTNFR1, we were also interested in how age might affect even the resting (air exposed) values, since sTNFR1 in the lung lining fluid may be acting to neutralize TNFα. The magnitude of the age-related difference in BAL sTNFR1 was quite marked—almost 4-fold greater in the 39 versus 7-week-old air exposed mice (Figure 3). However, an increase was expected on the basis of issues of dilution alone: in the young mice, with smaller lungs, there would have been greater dilution of lung lining fluid by the BAL fluid than in the old mice in which we instilled the same volume of fluid. To estimate how much of the apparent increase in BAL sTNFR1 in the 39- versus 7-week-old mice was simply the result of differences in dilution and how much was real, we computed the ratio of the air exposed values of the 39- versus the 7-week-old mice for all the other moieties measured in BAL fluid. The average of these ratio’s was 1.5-fold. Thus, the concentration of sTNFR1 in BAL fluid was elevated in the 39-week-old mice substantially more (4-fold) than would be expected on the basis of issues

of dilution alone (1.5-fold). A similar age-related increase in sTNFR1 has been reported in human serum (Scalzo et al., 2009).

Acute O_3 exposure caused an increase in neutrophils and epithelial cells and a reduction in macrophages in BAL fluid. The increase in neutrophils is the classic manifestation of cellular inflammation with O_3 exposure, while the increase in epithelial cells likely reflects epithelial injury. The reduction in macrophages is thought to represent cell activation leading to increased adherence to the pulmonary epithelium, making them more difficult to remove with lavage. Importantly, we observed a reduction in neutrophil recruitment following O_3 exposure in 39 versus 7-week-old wildtype mice after O_3 exposure (Figure 1B). To address the mechanistic basis for this reduction in BAL neutrophils, we measured BAL concentrations of IL-6, KC, MIP-2, IP-10, and MCP-1 (Figure 2). Each of these cytokines and chemokines has been implicated in the influx of neutrophils that occurs following acute O_3 exposure in mice (Zhao et al., 1998; Michalec et al., 2002; Johnston et al., 2005a; Johnston et al., 2005b; Lang et al., 2008). BAL KC, MIP-2, and MCP-1 were each reduced in 39 versus 7-week-old wildtype mice exposed to O_3 (Figure 2), suggesting that reductions in these chemokines may be contributing to the reduction in neutrophil recruitment that occurred in the 39-week-old mice. Elder et al. (Elder et al., 2000) also reported a reduction in O_3-induced neutrophil recruitment to the lung in aged (22-month-old) versus young (10-week-old) rats, and Stiles et al. (1988) reported fewer centriacinar lesions in the lungs of O_3-exposed aged (15-month-old) versus young adult (8-week-old) rats. Hamade et al. (Hamade et al., 2010) reported that the bradycardia induced by O_3 exposure was reduced in older (1 year) versus younger (5 months) mice. However, to our knowledge, this is the first report of the impact of increasing age on O_3-induced pulmonary inflammation in adult mice.

Reduced pulmonary responses to O_3 have also been observed in older human subjects. In a study utilizing 240 human nonsmokers aged 18–60 years, Hazucha et al. observed that the decline in FEV_1 that was induced by acute O_3 exposure in a chamber study was smaller in older (>35 years) than in younger (≤ 35 years) subjects (Hazucha et al., 2003). In another chamber study, McDonnell et al. (1993) also observed smaller O_3-induced reductions in FEV_1 in older adult human subjects, but the oldest individuals in their study were only 32 years of age. In contrast, Korrick et al. (1998) observed no age-related differences in decline in FEV_1 in a study of over 500 individuals exposed to ambient O_3 while hiking Mount Washington. While such observations appear to be at variance with the *greater* susceptibility of older individuals to O_3 induced respiratory related emergency room visits and hospital admissions (Delfino et al., 1997; Moolgavkar et al., 1997; Yang et al., 2003), it is important to recognize that these studies (Delfino et al., 1997; Moolgavkar et al., 1997; Yang et al., 2003), included not just individuals healthy enough to participate in chamber studies or extended hikes, but also individuals who may have had underlying respiratory complications.

We used a concentration of 2 ppm O_3 in this study. This concentration is higher than the ambient O_3 concentrations that would be observed even in the most polluted cities in the world. However, in mice, such concentrations are routinely used by many investigators in studying the acute effects of O_3 exposure on respiratory outcomes (Slade et al., 1997; Johnston et al., 2000b; Cho et al., 2001; Kierstein et al., 2008; Matsubara et al., 2009; Williams et al., 2009), because rodents are much less susceptible to O_3 than humans (Hatch et al., 1994; Slade et al., 1997). Hatch et al. (1994) estimated, based on the concentration of ozone reaction product accumulated in BAL cells and fluids from stable isotopically labeled O_3, that rodents require much higher concentrations of O_3 to achieve the same O_3 dose as humans. Indeed, because of such species differences, Slade et al. (1997) have estimated that the O_3 exposure we used here is roughly equivalent in dose to that employed by Hazucha et

al. (2003) and McDonnell et al. (1993) in their examination of age-related differences in the acute response to O_3 in humans, even though the concentration differed by about 5-fold.

We cannot rule out the possibility that the reduced inflammation observed in the 39-week-old mice (Figure 1B and 2) is the result of a lower inhaled dose of O_3. The inhaled dose is the product of exposure time, exposure concentration, and minute ventilation during the exposure (Weister, 1987). Dauger et al. reported no differences in minute ventilation normalized for body weight between room air exposed 30-, 100-, and 210-day-old mice (Dauger et al., 2003). However, in rodents, minute ventilation declines during O_3 exposure, which may account for at least part of their reduced susceptibility to O_3 described above. Arito et al. (1997) measured O_3-induced reductions in ventilation in 4–6 versus 20–22-month-old rats, and found that the magnitude of O_3-induced reductions in ventilation was greater in the younger animals. Since reducing ventilation also reduces the inhaled dose of O_3, the inhaled dose of the older rats would have been higher than that of the younger rats, which would be predicted to result in greater inflammation. We have also reported age-related differences in this effect of O_3 in mice, although the age span we studied (2–12 weeks of age) was quite different from that used by Arito et al. (Shore et al., 2000). In contrast to their results, we reported that very young (2-week-old) mice have little reduction (hence a higher net dose of O_3) compared to 12-week-old mice, in which minute ventilation declined by more than 60% during a 3 h 2 ppm exposure such as employed here (Shore et al., 2000). The age-related effect appeared to plateau between 4 and 8 weeks of age, but we did not measure effects of O_3 on ventilation in mice older than 12 weeks.

Others have reported that laboratory rodents housed as these mice were, with *ad libitum* feeding and lack of exercise, typically become overweight as they age (Martin et al., 2010). We considered the possibility that age-related increases in adiposity might be contributing to the age-dependent responses to O_3 that we observed (Figures 1 and 2). However, we have previously reported increased responses to acute O_3 exposure in obese mice (Shore et al., 2003; Johnston et al., 2006; Lu et al., 2006), whereas reduced responses to O_3 were observed in the older mice in this study (Figures 1 and 2). Moreover, although no direct markers of adiposity were assessed, body weight was not different in the wildtype and TNFR1 deficient 39-week-old mice, even though their responses to O_3 were substantially different (Figures 1 and 2). Thus, the data suggest that age, rather than adiposity, accounts for the reduced responses to O_3 observed in 39- versus 7-week-old mice.

Whereas O_3-induced neutrophil influx into the lungs was reduced in 39- versus 7-week-old wildtype mice, this was not the case in $TNFR1^{-/-}$ deficient mice (Figure 1B). Indeed, although there was little impact of TNFR1 deficiency in the 7-week-old mice, in the 39-week-old mice TNFR1 deficiency enhanced neutrophil influx (Figure 1B) and increased many of the BAL cytokines and chemokines examined. We were surprised by these observations, since in mice, both TNF inhibition and TNFR1 or TNFR2 deficiency have been reported to inhibit the neutrophilic inflammation induced by exposure to 0.3 ppm O_3 for 48–72 h in this strain of mice (Kleeberger et al., 1997; Cho et al., 2001), although the nature of that exposure was quite different from what was employed in this study. We have previously reported that neither TNFR1 nor TNFR2 deficiency affects the neutrophil influx observed 24 h after exposure to 2 ppm O_3 for 3 h (Shore et al., 2001), the same type of exposure employed here. Those data were obtained in young adult mice and hence are consistent with the lack effect of TNFR1 deficiency on neutrophil recruitment observed in the 7-week-old mice reported here. However, the augmented inflammatory response observed with TNFR1 deficiency in the 39-week-old mice was unexpected, since it was opposite in direction to what would be expected if the pro-inflammatory actions of TNFα were involved in mediating these inflammatory responses to O_3.

The anti-oxidants, MT and HO-1, are each induced by acute O_3 exposure (Johnston et al., 2000b). Moreover, induction of these enzymes by O_3 is functionally important: O_3-induced inflammation is augmented in mice genetically deficient in MT-1 (Inoue et al., 2008), or in rats in which HO-1 is chemically inhibited (Hisada et al., 2000). TNFα is capable of inducing MT-1, MT-2, and HO-1 (Kaji et al., 1993; Sato et al., 1994). Indeed, induction of MT-1 and MT-2 after traumatic brain injury is reduced in mice deficient in TNFR1 (Quintana et al., 2007). Similarly, TNFR1$^{-/-}$ deficient mice had reduced liver expression of HO-1 and associated increased injury in a model of acetaminophen induced hepatotoxicity (Chiu et al., 2003). TNF is also required for induction of HO-1 in liver following LPS (Oguro et al., 2002). Based on these observations, we reasoned that greater expression of these anti-oxidants in the 39-week-old mice might be contributing to their protection against O_3-induced inflammation (Figures 1 and 2) and that TNFR1 deficiency might reduce expression of these anti-oxidants, resulting in loss of this protection. Our data do not support this hypothesis. In O_3-exposed mice, both MT-1 and MT-2 were reduced in the 39-week-old versus 7-week-old mice while HO-1 was not impacted, and TNFR1 deficiency did not affect expression of any of these enzymes (Figure 5).

We observed greater induction of Areg mRNA in 39 versus 7-week-old wildtype mice exposed to O_3, whereas no age-related difference was observed in TNFR1$^{-/-}$ mice (Figure 5E). Areg is one of a family of ligands that act on the EGFR receptor, and Areg has been reported to increase airway epithelial proliferation (Lee et al., 2011), which could be important in protection against the epithelium damaging effects of O_3. Indeed, exogenous administration of Areg protects mice against the pulmonary inflammation induced by bleomycin exposure (Fukumoto et al., 2010). We do not know whether there were differences in Areg protein as well as mRNA expression in the 39-versus 7-week-old mice. However, it is conceivable that the augmented expression of Areg in 39-week-old mice (Figure 5E) may be contributing to the relative protection of the 39-week-old mice against O_3-induced inflammation (Figures 1 and 2), and that loss of this protective agerelated increase in Areg expression in TNFR1$^{-/-}$ mice may be contributing to their augmented responses to O_3.

We observed an age-related increase in sTNFR1 in the lung lining fluid (Figure 3). sTNFR1 is the extracellular domain of the p55 TNF receptor (TNFR1) that is released from the cell surface by proteolytic cleavage, via the enzyme TACE (ADAM17), that is also responsible for shedding of TNFα from the cell surface. We do not know the mechanistic basis for this age-related increase in sTNFR1, but it may be related to oxidative stress, since others have reported elevations in oxidative stress in lungs of mice with aging (Calvi et al., 2011), and since oxidative stress promotes activation of TACE (Brill et al., 2009). Importantly, sTNFR1 can exert anti-inflammatory actions by neutralizing TNFα (Yagi et al., 2010). Administration of sTNFR1 with TNFα blocks the neutrophil influx into the peritoneal cavity induced by i.p. injection of TNFα alone (Hale et al., 1995). Similarly, co-injection of endotoxin and sTNFR1 intratracheally attenuates the pulmonary neutrophilia induced by endotoxin alone (Ulich et al., 1993). Such effects of endotoxin are in part mediated by TNFα. Thus, the observation that BAL sTNFR1 was elevated in 39- versus 7-week-old mice (Figure 3), may explain why neutrophil influx and induction of neutrophil chemotactic factors induced by O_3 were limited in the 39-week-old mice (Figures 1B and 2). This hypothesis is supported by the observation that when TNFR1 was deleted, neutrophil influx and BAL chemokine levels were restored in the 39-week-old mice. The data are consistent with the hypothesis that deletion of TNFR1 led to increased TNFα and increased activation of the remaining TNF receptor, TNFR2. Although the TNFR1 dominates over TNFR2 for many inflammatory effects of TNFα (Vandenabeele et al., 1995; Peschon et al., 1998; MacEwan, 2002; Naude et al., 2011). TNFR2 can still activate NF-κB (MacEwan, 2002; Gupta et al., 2005), a transcription factor important for expression of many of the

chemokines induced by O_3 Indeed, TNFR2 but not TNFR1 is required for several types of inflammatory conditions. For example, TNFR2 is required for renal injury and inflammation in a mouse model of acute renal failure induced by cisplatin, whereas TNFR1 is not (Ramesh et al., 2003). TNFR2 but not TNFR1 is required for the induction of glomerular injury and inflammation in a mouse model of glomerulonephritis (Vielhauer et al., 2005). TNFα acting on TNFR2 receptors in brain microvessels is also required for upregulation of ICAM1 and leukocyte recruitment in a model of cerebral malaria (Lucas et al., 1997). Similarly, we have previously reported that TNFR2 is required for O_3-induced airway hyperresponsiveness in mice, whereas TNFR1 is not (Shore et al., 2001). Thus, it is possible that increased inflammatory responses to O_3 observed in TNFR1 deficient mice are being mediated by TNFR2.

In summary, the pulmonary inflammation induced by acute exposure to 2 ppm O_3 for 3 hours was reduced in 39- versus 7-week-old mice. The age-related reduction in BAL neutrophils, cytokines, and chemokines was largely abolished in TNFR1$^{-/-}$ mice. The data suggest that increased levels of sTNFR1 present in the lungs of the older mice may neutralize TNF and protect them against O_3-induced inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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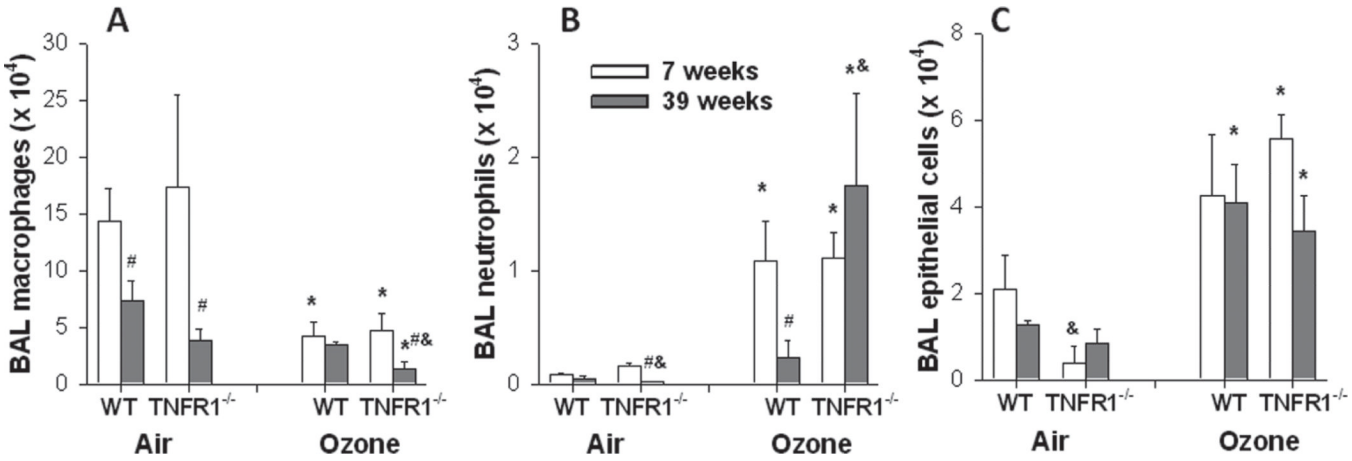

Figure 1.

Effect of age on the number of macrophages (A), neutrophils (B), and epithelial cells (C) in bronchoalveolar lavage (BAL) of WT and TNFR1$^{-/-}$ mice exposed to air or ozone (O3) (2 ppm for 3 h). Mice were studied 4 h after O3 exposure. Results are mean ± SE of data from 3 to 6 mice in each group. $^{*}p < 0.05$ versus genotype and age-matched mice exposed to air; $^{\#}p < 0.05$ versus genotype and exposure matched 7-week-old mice; $^{\&}p < 0.05$ versus exposure and age-matched WT mice.

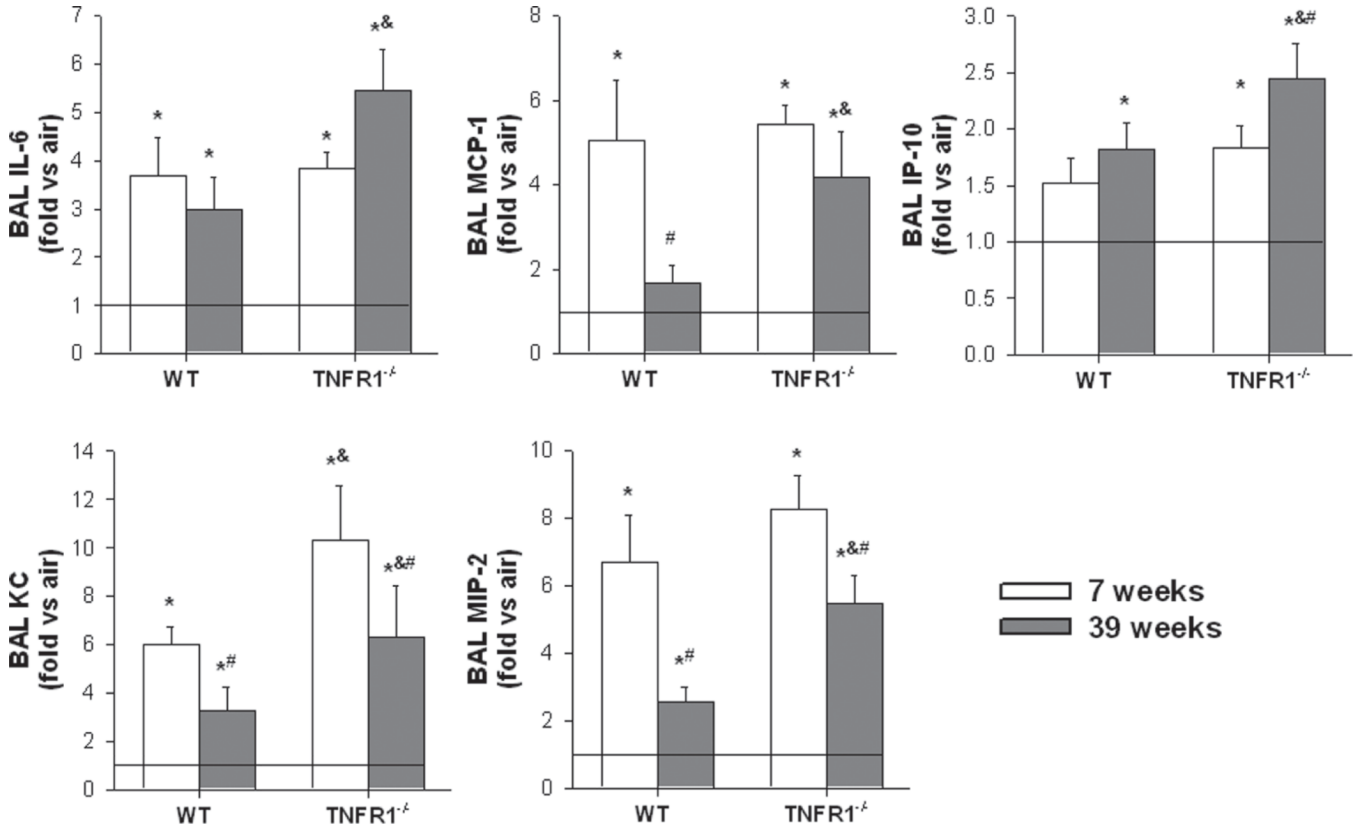

Figure 2.

Effect of age on bronchoalveolar lavage (BAL) concentrations of IL-6 (A), MCP-1 (B), IP-10 (C), KC (D), and MIP-2 (E), in WT and TNFR1$^{-/-}$ mice exposed to O3. Results are mean ± SE of data from 3 to 6 mice in each group. The data are expressed as fold change relative to genotypeand age-matched air controls, which are represented by the horizontal line. *$p < 0.05$ versus genotype and age-matched mice exposed to air; $^{\#}p < 0.05$ versus genotype and exposure matched 7-week-old mice; $^{\&}p < 0.05$ versus exposure and age-matched WT mice.

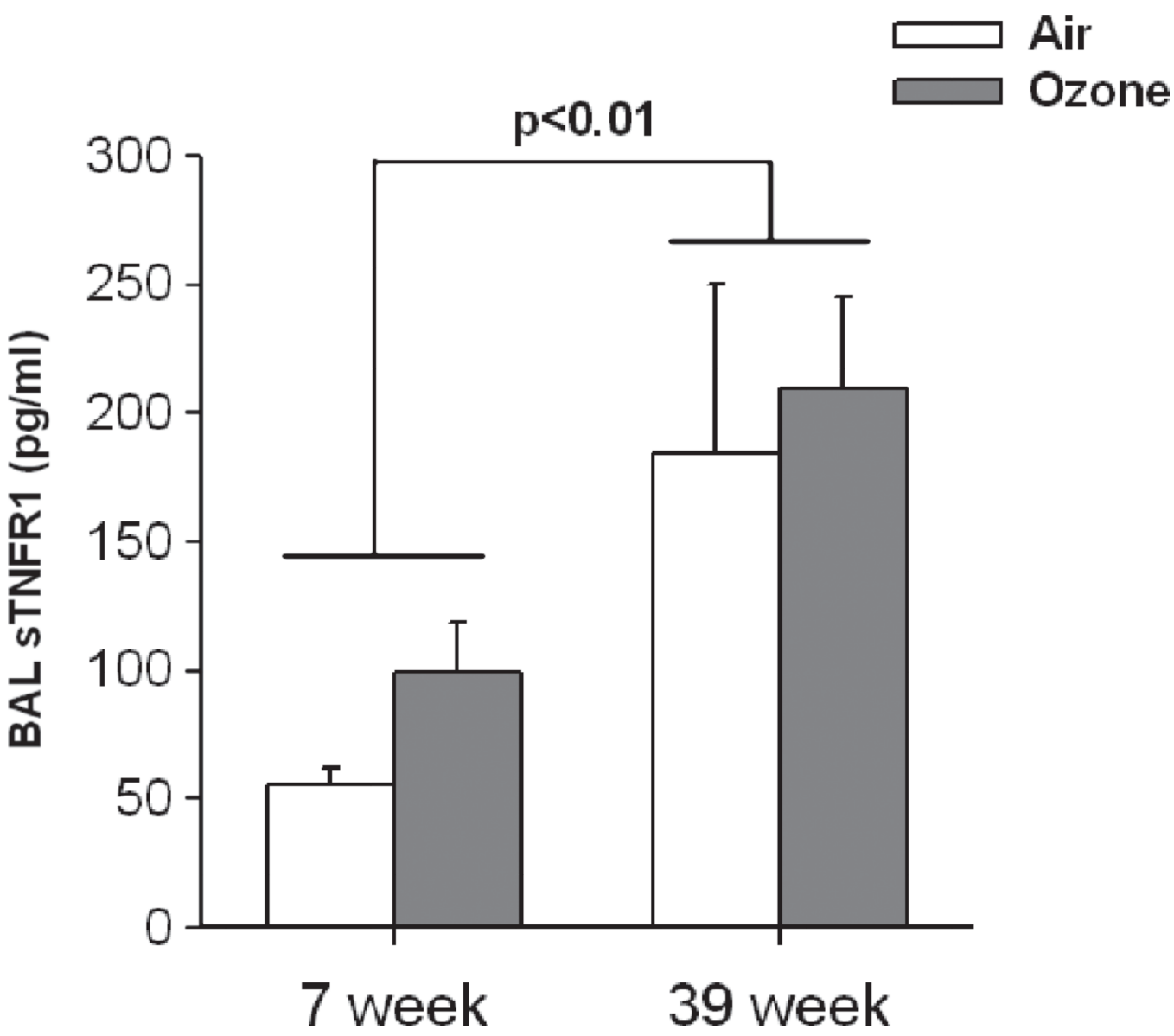

Figure 3.
Effect of age on bronchoalveolar lavage (BAL) concentrations of soluble form of TNFR1 (sTNFR1) in WT mice exposed to air or O_3. Results are mean ± SE of data from 3 to 6 mice in each group. There was a significant increase in sTNFR1 in the older mice.

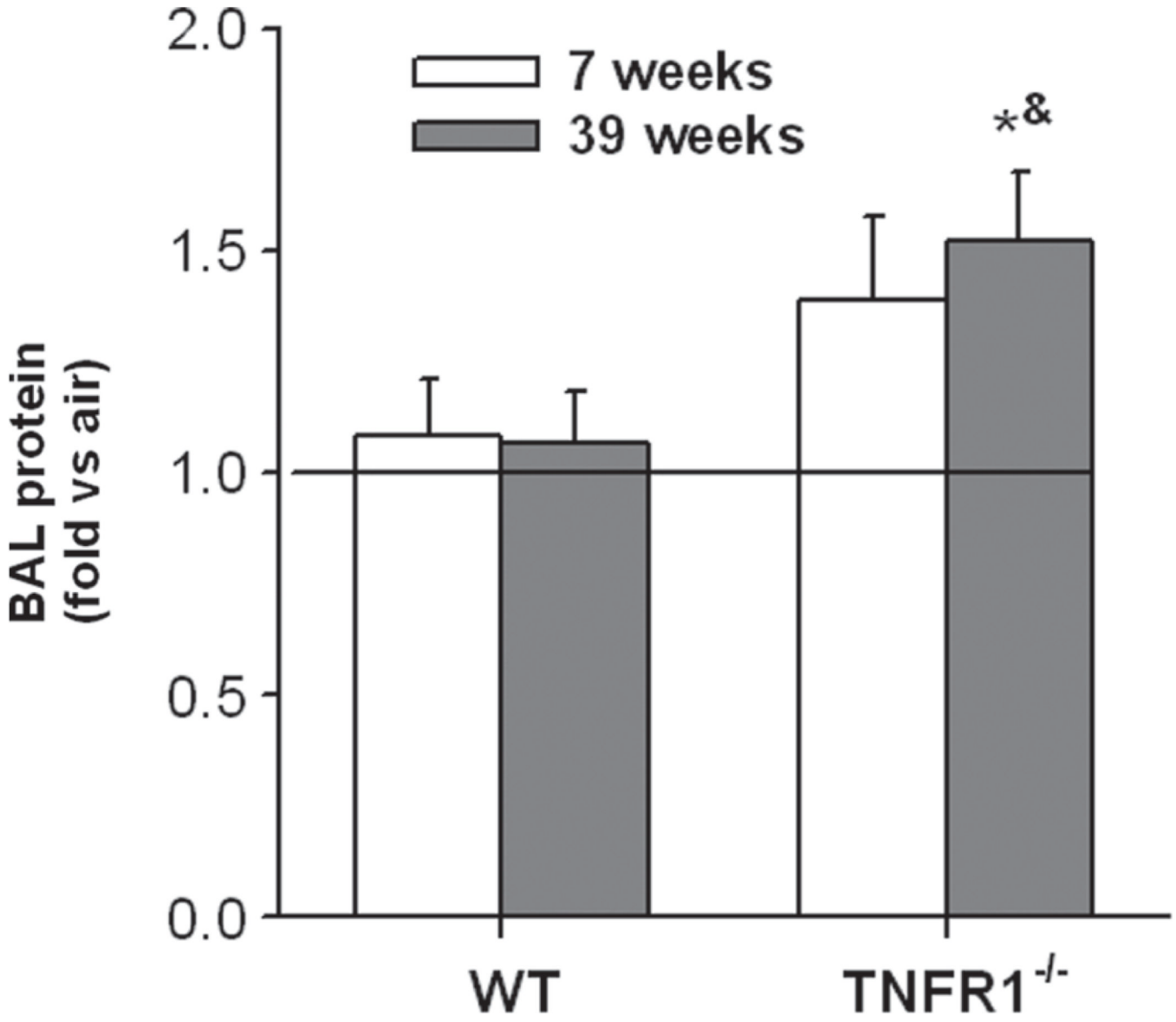

Figure 4.

Effect of age on bronchoalveolar lavage protein concentration in WT and TNFR1$^{-/-}$ mice exposed to O3. Results are mean ± SE of data from 3 to 6 mice in each group, and are expressed as fold change relative to the genotype- and age-matched air controls which are represented by the horizontal line. *$p < 0.05$ versus genotype and age-matched mice exposed to air; $^{\&}p < 0.05$ versus exposure and age-matched WT mice.

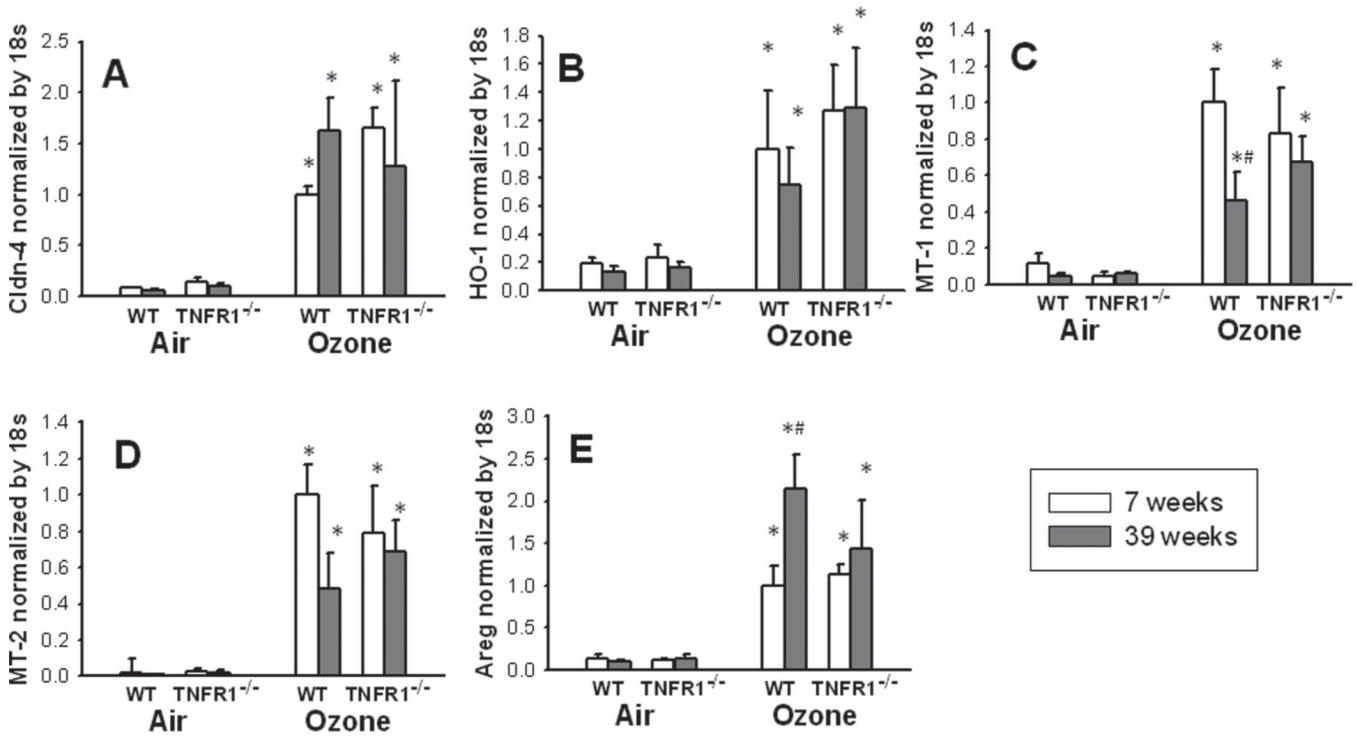

Figure 5.

Effect of age and TNFR1 deficiency on pulmonary mRNA expression of claudin-4 (Cldn-4) (A), heme oxygenase-1 (HO-1) (B), metallothionein-1 (MT-1) (C), metallothionein-2 (MT-2) (D), and amphiregulin (Areg) (E). Results are mean ± SE of data from 3 to 6 mice in each group. Gene expression was normalized for 18S and expressed relative to the 7-week-old wildtype (WT) O3 exposed group. $^{*}p < 0.05$ versus genotype and age-matched mice exposed to air; $^{\#}p < 0.05$ versus 7-week-old genotype and exposure matched mice.

Table 1

Primers used for PCR.

Gene	Primers
MT-1	Forward: CACGACTTCAACGTCCTGAG
	Reverse: TGCACTTGCAGTTCTTGCAG
MT-2	Forward: CGATGGATCCTGCTCCTG
	Reverse: ACTTGTCGGAAGCCTCTTTG
HO-1	Forward: AAGCCGAGAATGCTGAGTTC
	Reverse: TCCAGGGCCGTGTAGATATG
Claudin-4	Forward: AACATCGTCACGGCACAGAC
	Reverse: CGAGCATCGAGTCGTACATC
Amphiregulin	Forward: TTCATGGCGAATGCAGATAC
	Reverse: TGTCATCCTCGCTGTGAGTC
18S ribosomal RNA	Forward: GTAACCCGTTGAACCCCATT
	Reverse: CCATCCAATCGGTAGTAGCG