

ehp

**ENVIRONMENTAL
HEALTH
PERSPECTIVES**

ehponline.org

**Augmented Pulmonary Responses to Acute
Ozone Exposure in Obese Mice:
Roles of TNFR2 and IL13**

**Alison Suzanne Williams, Joel Andrew Mathews,
David Itiro Kasahara, Lucas Chen, Allison Patricia Wurmbrand,
Huiqing Si, and Stephanie Ann Shore**

<http://dx.doi.org/10.1289/ehp.1205880>

Online 22 February 2013



NIEHS

**National Institute of
Environmental Health Sciences**

**National Institutes of Health
U.S. Department of Health and Human Services**

Augmented Pulmonary Responses to Acute Ozone Exposure in Obese Mice: Roles of TNFR2 and IL-13

Alison Suzanne Williams, Joel Andrew Mathews, David Itiro Kasahara, Lucas Chen, Allison Patricia Wurmbrand, Huiqing Si, and Stephanie Ann Shore

Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts, USA

Corresponding author:

Stephanie Shore, Ph.D.

Harvard School of Public Health

665 Huntington Ave.

Boston, MA 02115.

Tel: 617-432-0199.

Fax: 617-432-3468.

Email: sshore@hsph.harvard.edu

Running title: TNFR2, IL-13, ozone, and obesity

Keywords: airway responsiveness; bronchoalveolar lavage; IL-5; inflammation; MIP-3 α

Acknowledgements: This work was supported by the U.S. National Institute of Health grants ES-013307, HL-084044, and ES-000002.

Financial interests: None of the authors has any actual or potential competing financial interests.

Abbreviations:

A: height of the pressure volume loops

AHR: airway hyperresponsiveness

BAL: bronchoalveolar lavage

Cpe: carboxypeptidase E

ELISA: enzyme linked immunosorbent assay

G: coefficient of lung tissue damping

G-CSF: granulocyte colony stimulating factor

H: coefficient of lung tissue elastance

IL: interleukin

LIX: LPS-induced CXC chemokine

MCP-1: monocyte chemotactic protein 1

MIP-3a: macrophage inflammatory protein 3 alpha

CCR6: C-C chemokine receptor type 6

Rn: Newtonian resistance

O₃: ozone

PMA: phorbol myristate acetate

PV: pressure volume

TLC: total lung capacity

TNF: tumor necrosis factor

WT: wildtype

ABSTRACT

Background: Acute ozone exposure results in greater inflammation and airway hyperresponsiveness (AHR) in obese versus lean mice.

Objectives: The purpose of this study was to examine the hypothesis that these augmented responses to ozone are the result of greater signaling through TNFR2 and/or IL-13.

Methods: We exposed lean wildtype (WT) and TNFR2 deficient (TNFR2^{-/-}) mice, and obese *Cpe^{fat}* and TNFR2 deficient *Cpe^{fat}* mice (*Cpe^{fat}/TNFR2^{-/-}*) to ozone (2 ppm for 3 h) either with or without treatment with anti-IL-13 or left them unexposed.

Results: Ozone-induced increases in baseline pulmonary mechanics, airway responsiveness, and cellular inflammation were greater in *Cpe^{fat}* than WT mice. In lean mice, TNFR2 deficiency ablated ozone-induced AHR, without affecting pulmonary inflammation, whereas in obese mice, TNFR2 deficiency augmented ozone-induced AHR but reduced inflammatory cell recruitment. Ozone increased pulmonary expression of IL-13 in *Cpe^{fat}* but not WT mice. Flow cytometry analysis of lung cells indicated greater IL-13 expressing CD4⁺ cells in *Cpe^{fat}* versus WT mice after ozone. In *Cpe^{fat}* mice, anti-IL-13 attenuated ozone-induced increases in pulmonary mechanics and inflammatory cell recruitment, but did not affect AHR. These effects of anti-IL-13 were not observed in *Cpe^{fat}/TNFR2^{-/-}* mice. There was no effect of anti-IL-13 in WT mice.

Conclusions: Our results indicate that pulmonary responses to ozone are not just greater, but qualitatively different in obese versus lean mice. In particular, in obese mice, ozone induces IL-13 and IL-13 synergizes with TNF via TNFR2 to exacerbate ozone-induced changes in pulmonary mechanics and inflammatory cell recruitment but not AHR in obese mice.

INTRODUCTION

Ozone (O₃), an air pollutant, causes respiratory symptoms and reductions in lung function (Alexis et al. 2000). Ozone is also a trigger for asthma: asthma-related emergency room visits increase on days of high ambient ozone (Fauroux et al. 2000; Gent et al. 2003). O₃ activates the innate immune system causing pulmonary infiltration with neutrophils and airway hyperresponsiveness (AHR) a characteristic feature of asthma (Garantziotis et al. 2009).

Two thirds of the US population is obese or overweight (National Center for Health Statistics, 2012) and obesity is a risk factor for asthma (Shore and Johnston 2006). Nevertheless, our understanding of how obesity impacts pulmonary responses to O₃ is still rudimentary. O₃-induced decrements in lung function are greater in obese and overweight than lean human subjects (Alexeeff et al. 2007; Bennett et al. 2007). Obese mice also exhibit greater pulmonary inflammation and greater AHR than lean mice after acute O₃ exposure (Johnston et al. 2006; Shore et al. 2003). The mechanistic basis for the effect of obesity on responses to O₃ is unknown.

TNF α is induced in the lung following O₃ exposure and has been implicated in responses to acute O₃ exposure (Cho et al. 2001; Matsubara et al. 2009; Shore et al. 2001; Yang et al. 2005). In obesity, serum TNF α increases (Katsuki et al. 1998; Williams et al. 2012). TNF α promoter polymorphisms that augment TNF α expression are associated with increased obesity-related risk of asthma, especially nonatopic asthma (Castro-Giner et al. 2009), suggesting that TNF α may also be relevant for obesity-related asthma. The role of TNF α in the augmented responses to acute O₃ observed in obese mice has not been established.

TNF α binds to either of two receptors, TNFR1 and TNFR2, which differ in their ability to induce inflammation and apoptosis and their affinity for cleaved versus membrane associated TNF α (Naude et al. 2011). In lean mice, O₃-induced AHR requires TNFR2 (Shore et al. 2001), though TNFR1 may also play a role (Cho et al. 2001). TNFR2 is also required for the *innate* AHR that is observed in obese mice (Williams et al. 2012), but the role of TNFR2 in the augmented responses to O₃ observed in obese mice is not established.

The first purpose of this study was to examine the hypothesis that TNFR2 is required for the augmented response to acute O₃ exposure associated with obesity. To examine this hypothesis, we bred *Cpe^{fat}* mice that are also genetically deficient in the TNFR2 receptor (*Cpe^{fat}/TNFR2^{-/-}* mice). *Cpe^{fat}* mice lack carboxypeptidase E (Cpe), an enzyme involved in appetite regulation and energy expenditure (Leibel et al. 1997). Lack of Cpe leads to obesity (Johnston et al. 2006; Johnston et al. 2010). We assessed airway responsiveness and pulmonary injury and inflammation in *Cpe^{fat}/TNFR2^{-/-}* mice along with WT, *Cpe^{fat}*, and TNFR2^{-/-} mice.

IL-13 also plays an important role in AHR. In mice, exogenous administration of IL-13 to the lungs results in AHR, and IL-13 blocking reagents inhibit allergen-induced AHR (Grunig et al. 1998; Wills-Karp et al. 1998). IL-13 may also play a role in responses to acute O₃ exposure: in lean Balb/c mice, IL-13 deficiency reduces O₃-induced AHR and inflammation (Pichavant et al. 2008; Williams et al. 2008), but the role of IL-13 in responses to O₃ in obese mice is not established. Consequently, to examine the hypothesis that IL-13 contributes to obesity-related differences in the response to O₃, we measured IL-13 expression and examined the effects of anti-IL-13 antibodies in obese and lean mice. Since TNF and IL-13 can synergize to promote the

expression of chemokines that may contribute to the effects of O₃ (Reibman et al. 2003), we examined the effect of anti-IL-13 in both TNFR2 sufficient and deficient mice.

METHODS

Animals: This study was approved by the Harvard Medical Area Standing Committee on Animals. Animals were treated humanely and with regard for alleviation of suffering. We bred *Cpe^{fat}/TNFR2^{-/-}*, *TNFR2^{-/-}*, *Cpe^{fat}*, and WT mice as previously described (Williams et al. 2012). Female mice were on a C57BL/6 background, were fed standard mouse chow diets, and were 10-12 weeks old.

Protocol: We exposed mice to 2 ppm O₃ for 3 hours. 24 hours after exposure, we measured pulmonary mechanics and airway responsiveness, performed bronchoalveolar lavage (BAL), harvested blood by cardiac puncture, and collected lungs for preparation of RNA. Controls for these mice were not exposed to O₃, but were otherwise treated identically and studied simultaneously. Pulmonary mechanics and airway responsiveness for these *unexposed* mice were previously reported (Williams et al. 2012). We treated other mice with anti-IL-13 antibody (2 µg/g body weight i.p.) 24 h prior to O₃ exposure. In a final cohort of O₃ exposed mice, we harvested lungs at 24 h, digested, and lung cells isolated for flow cytometry to quantitate IL-13 expressing CD4⁺ cells.

Measurement of pulmonary mechanics and airway responsiveness: We generated quasi static pressure volume (PV) curves, assessed baseline pulmonary mechanics using the forced oscillation technique, and measured airway responsiveness to aerosolized methacholine as previously described (Williams et al. 2012). We assessed Newtonian resistance (Rn), which

largely reflects the conducting airways, and the coefficients of lung tissue damping (G) and lung tissue elastance (H), which reflect changes in the small airways and pulmonary parenchyma.

Bronchoalveolar lavage: We lavaged lungs and counted BAL cells as previously described (Williams et al. 2012). We measured BAL cytokines, chemokines and hyaluronan by ELISA (R&D Systems, ebioscience, Echelon Biosciences). We measured BAL protein by Bradford assay (BioRad, Hercules, CA).

RNA extraction and Real Time PCR: We used real time PCR to quantitate IL-13 and IL-17A mRNA expression as described (Shore et al. 2011). We subtracted Ct values for a housekeeping gene, 36B4 (rplp0), a ribosomal protein, from Ct values for IL-13 or IL-17A to obtain Δ Ct values. We expressed changes in mRNA relative to values from the WT unexposed mice, using the $\Delta\Delta$ Ct method.

Flow cytometry: We flushed lungs to remove blood cells, and then excised, minced, and digested as previously described (Kasahara et al. 2012). We cultured lung cells either with or without PMA and ionomycin, in the presence of Golgi Stop (BD Bioscience), for 5 hours prior to staining for flow cytometry. We then fixed cells with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with anti-Fc γ blocking mAb (clone 93, Biolegend) and washed. We stained cells with Alexa Fluor 488-conjugated CD4 mAb (clone GK1.5, Biolegend) and Alexa Fluor 647-conjugated anti-mouse IL-13 (clone eBio13A; eBioscience). We passed cells through a BD Canto flow cytometry (BD Bioscience) and analyzed data with FlowJo software (Tree Star, Inc.).

Statistics: We used factorial ANOVA to assess the significance of differences in outcome indicators, as previously described (Williams et al. 2012). We performed analyses using Statistica software (SAS Institute, Cary, NC).

RESULTS

Body mass: There was a significant effect of Cpe ($p < 0.001$) but not TNFR2 genotype on body mass. Whether or not they were deficient in TNFR2, Cpe^{fat} mice weighed about twice as much as lean controls, consistent with previous observations (Williams et al. 2012).

Pulmonary mechanics and airway responsiveness: In WT mice, no significant differences in the PV curve of the lungs were observed in O_3 exposed versus unexposed mice (Figure 1A). However, in Cpe^{fat} mice, there was a rightward shift and widening of the PV loop indicative of increased hysteresis in O_3 exposed versus unexposed mice (Figure 1B). To quantitate these changes, we measured the area of the PV loop, and normalized it by A, the difference in volume between TLC and end expiratory lung volume (height of the PV loops in Figure 1). Area/A is a measure of the thickness of the PV loop. A was lower in obese versus lean mice, as previously reported (Williams et al. 2012), but there was no difference in A in O_3 exposed versus unexposed mice (data not shown). Consistent with the increased hysteresis induced by O_3 in Cpe^{fat} but not WT mice (Figure 1A,B), Area/A was greater in O_3 exposed versus unexposed Cpe^{fat} mice whereas O_3 had no effect on Area/A in WT mice (Figure 1C). Area/A was also greater in O_3 exposed versus unexposed $Cpe^{fat}/TNFR2^{-/-}$ mice (Figure 1C).

Even in unexposed mice, baseline Rn, G, and H were elevated in lungs of obese mice (Figure 1D,E,F). O_3 exposure had no effect on Rn in mice of any genotype, but increased baseline G and

H in Cpe^{fat} mice (Figure 1D,E,F). In contrast, no significant changes in baseline G and H were observed in O_3 -exposed versus unexposed $Cpe^{fat}/TNFR2^{-/-}$ mice.

Airway responsiveness was greater in *unexposed* Cpe^{fat} versus WT mice and this difference was abolished by TNFR2 deficiency (see Williams et al. 2012). In lean WT mice, O_3 exposure caused AHR (Figure 2A). This effect of O_3 was observed when G, a measure of the lung tissue response, was used as the outcome indicator (Figure 2A). A similar trend was observed for H, but did not reach statistical significance (see Supplemental Material, Figure S1). There was no effect of O_3 on methacholine-induced changes in Rn (Supplemental Material, Figure S1A). O_3 -induced AHR was absent in $TNFR2^{-/-}$ mice (Figure 2B). O_3 did cause AHR in Cpe^{fat} mice (Figure 2C), and the magnitude of the O_3 -induced AHR was significantly greater than in WT mice. O_3 also induced AHR in $Cpe^{fat}/TNFR2^{-/-}$ mice (Figure 2D). Indeed, O_3 -induced AHR was actually greater in $Cpe^{fat}/TNFR2^{-/-}$ than in Cpe^{fat} mice, and O_3 -induced AHR was observed even when changes in Rn were used as the outcome indicator, whereas this was not the case in mice of any other genotype (see Supplemental Material, Figure S1D). O_3 -induced AHR was also observed in Cpe^{fat} and $Cpe^{fat}/TNFR2^{-/-}$ mice when H was used as the index of response and AHR based on changes in H was also greater in $Cpe^{fat}/TNFR2^{-/-}$ than in Cpe^{fat} mice (Supplemental Material, Figure S1G,H).

Because IL-13 has been shown to contribute to O_3 -induced AHR in lean Balb/c mice (Pichavant et al. 2008; Williams et al. 2008), we determined whether there were obesity and/or TNFR2 dependent differences in IL-13 expression. O_3 caused an increase in IL-13 mRNA in obese but not lean mice (Figure 3A). There was a non-significant trend towards increased IL-13 mRNA expression in $Cpe^{fat}/TNFR2^{-/-}$ versus Cpe^{fat} mice. O_3 exposure also increased BAL IL-13 in Cpe^{fat} but not WT mice (Figure 3B). Furthermore, both the % and the total number of IL-13

expressing CD4⁺ cells were higher in *Cpe^{fat}* than WT mice exposed to O₃ (Figure 3D,E). Consequently, we also measured BAL concentrations of another Th2 cytokine, IL-5. O₃-induced increases in IL-5 were significantly greater in obese versus lean mice, and TNFR2 deficiency significantly increased BAL IL-5 in obese but not lean mice (Figure 3F) consistent with the trend observed for IL-13 (Figure 3B). IL-17A has also been linked to AHR (Kudo et al. 2012). O₃ exposure caused a significant increase in IL-17A mRNA in obese but not lean mice (Figure 3G). There was a non significant trend towards greater IL-17A in *Cpe^{fat}/TNFR2^{-/-}* than *Cpe^{fat}* mice.

To examine the functional impact of this IL-13 expression, we treated mice with antibodies to IL-13. In WT mice, there was no effect of anti-IL-13 treatment, likely because pulmonary IL-13 was negligible in these mice (Figure 3A,B). In *Cpe^{fat}* mice, anti-IL-13 reversed the effects of O₃ exposure on the PV curve (Figure 1B,C), and prevented the O₃-induced increase in baseline G and H (Figure 1 E,F), but did not affect O₃-induced AHR (Figure 2C). In *Cpe^{fat}/TNFR2^{-/-}* mice, anti-IL-13 also prevented O₃-induced changes in the PV curve (Figure 1C) but had no effect on baseline pulmonary mechanics or O₃-induced AHR (Figure 2D).

Pulmonary inflammation and injury: O₃ significantly increased BAL macrophages, neutrophils and protein, a marker of O₃-induced injury (Bhalla 1999) (Figure 4). Lymphocytes and eosinophils were not observed in BAL fluid of most mice regardless of exposure, genotype, or obesity status. BAL neutrophils and protein were increased in *Cpe^{fat}* versus WT mice exposed to O₃ (Figure 4). In lean mice, TNFR2 deficiency had no effect on these responses to O₃. However, in obese mice, BAL neutrophils and macrophages, but not BAL protein, were significantly reduced in TNFR2 deficient versus sufficient mice (Figure 4). The TNFR2 dependent changes in neutrophil recruitment were not the result of differences in the expression of IL-6, KC, or G-CSF (Figure 5A and Supplemental Material, Figure S2), cytokines and

chemokines reported to be important for O₃-induced neutrophil recruitment to the lungs (Johnston et al. 2005a; Johnston et al. 2005b; Kasahara et al. 2012). These chemokines were greater in obese versus lean mice exposed to O₃, but were either not affected (IL-6 and KC) or actually augmented (G-CSF) by TNFR2 deficiency in obese mice. Similarly, TNFR2 dependent changes in macrophage recruitment are not the result of changes in MCP-1, a chemokine required for O₃ induced macrophage recruitment (Zhao et al. 1998). BAL MCP-1 was greater in obese versus lean mice, but TNFR2 deficiency had no effect on BAL MCP-1 (Figure 5B). Surprisingly, O₃ induced increases in BAL TNF α was significantly reduced in obese versus lean mice (Figure 5C).

O₃ induces fragmentation of the matrix glycoprotein, hyaluronan, leading to AHR (Garantziotis et al. 2009). Such hyaluronan fragments are thought to be induced via oxidative stress caused by O₃. O₃ caused an increase in BAL hyaluronan (Figure 5D) as well as protein carbonyls (Figure 5E), a marker of oxidative stress, although protein carbonyls were only increased in obese and not lean mice. Furthermore, O₃-induced increases in BAL hyaluronan and protein carbonyls were significantly greater in obese than lean mice (Figure 5D,E), suggesting that elevations in BAL hyaluronan may contribute to greater O₃-induced AHR in obese versus lean mice. However, BAL hyaluronan does not appear to account for the greater O₃-induced AHR in *Cpe^{fat}/TNFR2^{-/-}* versus *Cpe^{fat}* because BAL hyaluronan was not different in these two strains (Figure 5D).

In *Cpe^{fat}* mice, anti-IL-13 reduced O₃-induced increases in BAL neutrophils and macrophages (Figure 4A,B), but not BAL protein (Figure 4C), whereas there was no effect of anti-IL-13 in *Cpe^{fat}/TNFR2^{-/-}* mice (Figure 4A,B). O₃-induced increases in MIP-3 α (CCL20) and LIX (CXCL5) mRNA are reduced in IL-13 deficient versus wildtype Balb/c mice, suggesting that

changes in these chemokines may contribute to effects of IL-13 deficiency on inflammatory cell recruitment (Williams et al. 2008). O₃-induced increases in BAL LIX, a neutrophil chemotactic factor, were not affected by obesity, TNFR2 deficiency, or anti-IL-13 (Figure 6A) indicating that this chemokine does not account for observed effects on BAL neutrophils (Figure 4B). However, TNFR2 deficiency did attenuate O₃-induced increases in MIP-3 α regardless of obesity status ($p < 0.001$) (Figure 6B). Anti-IL-13 also caused a significant reduction in MIP-3 α in TNFR2 sufficient ($p < 0.01$) but not deficient mice (Figure 6B), suggesting that IL-13 and TNF α synergize in the induction of MIP-3 α after O₃ exposure.

DISCUSSION

We observed greater effects of acute O₃ exposure in obese versus lean mice consistent with previous observations (Johnston et al. 2006; Shore et al. 2003). Importantly, our results also indicated obesity-related differences in the mechanisms governing the pulmonary effects of O₃ (see Table 1 for summary). In lean mice, TNFR2 deficiency reduced O₃-induced AHR but had no effect on O₃-induced inflammation, whereas in obese mice, TNFR2 deficiency enhanced O₃-induced AHR while attenuating O₃-induced inflammation (Figures 2,4). In lean mice, IL-13 was not induced by O₃, and there was no impact of anti-IL-13 on responses to O₃. However, in obese mice, O₃ did induce IL-13 expression (Figure 3). Importantly, obesity-related differences in IL-13 expression accounted for the ability of O₃ to induce changes in the PV curve of lung and to increase baseline G and H in obese *Cpe^{fat}* but not lean WT mice (Figure 1). IL-13 also contributed to greater recruitment of inflammatory cells to the lungs of *Cpe^{fat}* versus WT mice (Figure 4). Moreover, in obese mice, IL-13 and TNFR2 appeared to synergize to exacerbate O₃-induced inflammation and changes in pulmonary mechanics, since anti-IL-13 reduced

inflammatory cell recruitment and G and H in TNFR2 sufficient but not TNFR2 deficient *Cpe^{fat}* mice (Figure 4). MIP-3 α expression may have contributed to the TNFR2/IL-13 synergy that promoted inflammatory cell recruitment, since TNFR2 deficiency attenuated O₃-induced increases in BAL MIP3 α in untreated mice but not in mice treated with anti-IL-13 (Figure 6). Thus obesity-related differences in the induction of IL-13 after O₃ exposure not only confers unique and/or augmented responses to O₃ on the obese mice, but also appears to account for some of the obesity-related differences in the impact of TNFR2 deficiency in obese mice, since TNFR2 can synergize with IL-13 in obese but not lean mice.

Our results demonstrating O₃-induced AHR in WT but not TNFR2^{-/-} mice (Figure 2) confirm previous reports indicating that TNFR2 is required for O₃-induced AHR in lean mice (Cho et al. 2001; Shore et al. 2001). Circulating TNF α is increased in obese versus lean mice and the *innate* AHR characteristic of obese mice is reduced when these mice are TNFR2 deficient (Williams et al. 2012) (the unexposed mice in Figure 2C,D). Hence, we expected that TNFR2 deficiency might also attenuate O₃-induced AHR in obese mice and might even ablate obesity-related differences in the impact of O₃ on AHR. We did observe a reduction in O₃-induced changes in baseline pulmonary mechanics in *Cpe^{fat}/TNFR2^{-/-}* versus *Cpe^{fat}* mice (Figure 1E,F). However, O₃-induced AHR was actually greater in *Cpe^{fat}/TNFR2^{-/-}* versus *Cpe^{fat}* mice (Figure 2). This greater O₃-induced AHR was not the result of greater obesity in the *Cpe^{fat}/TNFR2^{-/-}* mice: body mass was the same in the two groups. O₃-induced increases in BAL TNF α were lower in *Cpe^{fat}* versus WT mice (Figure 5C). While this reduction might explain a reduced impact of TNFR2 deficiency in obese mice, it cannot explain the observed reversal in the direction of the impact of TNFR2 deficiency on O₃-induced AHR.

Because others have reported reduced O₃-induced AHR in lean IL-13^{-/-} versus WT Balb/c mice and greater O₃-induced AHR in IL-13 transgenic mice (Pichavant et al. 2008; Williams et al. 2008), we examined the role of IL-13 in O₃-induced AHR in obese mice. O₃ caused a significant increase in pulmonary IL-13 mRNA expression and BAL IL-13 in *Cpe^{fat}* but not WT mice (Figure 3A,B). CD4⁺ T cells appeared to be the source of this IL-13 (Figure 3C-E), and another CD4⁺ T cell derived cytokine, IL-5, was also induced by O₃ exposure in obese mice (Figure 3F).

To determine whether IL-13 contributed to obesity- and/or TNFR2-dependent changes in the response to O₃, we treated mice with anti-IL-13 prior to O₃ exposure. Anti-IL-13 attenuated the O₃-induced increase in the hysteresis of the PV curve that was induced by O₃ exposure in obese mice (Figure 1B,C). Lung surfactant is important in limiting lung hysteresis and IL-13 reduces the pulmonary expression of surfactant protein C (Ito and Mason 2010), which is important for the surface activity of surfactant. Coupled with the observations that O₃-induced changes in lung hysteresis were not observed in WT mice (Figure 1A,C) which lacked IL-13 (Figure 3), the results suggest that O₃ caused changes in pulmonary surfactant activity in *Cpe^{fat}* mice and that these changes were mediated by IL-13. Changes in lung hysteresis in obese mice may be aggravated by the ability of O₃ exposure to cause oxidation of phospholipids important for surfactant activity (Pulfer and Murphy 2004). O₃-induced changes in baseline G and H, measures of the lung tissue, were also ablated by anti-IL-13 treatment (Figure 1). Thus obesity-related differences in the ability of O₃ to induce IL-13 expression (Figure 3) appear to account for the ability of O₃ to induce changes in baseline pulmonary mechanics in *Cpe^{fat}* but not WT mice. TNFR2 deficiency also reduced O₃-induced changes in pulmonary mechanics in *Cpe^{fat}* mice and anti-IL-13 had no effect on mechanics in *Cpe^{fat}/TNFR2^{-/-}* mice (Figure 1) suggesting that synergy between IL-13 and TNFR2 contributes to these changes.

In contrast to the effects on baseline pulmonary mechanics (Figure 1), we observed no effect of anti-IL-13 treatment on O₃-induced AHR in *Cpe^{fat}* mice (Figure 2C), nor was there any effect on AHR in *Cpe^{fat}/TNFR2^{-/-}* mice (Figure 2D), indicating that the enhanced AHR in *Cpe^{fat}/TNFR2^{-/-}* mice was not the result of increased IL-13 signaling. Thus, other factors must account for obesity-related increases in O₃-induced AHR and for the augmented AHR observed in *Cpe^{fat}/TNFR2^{-/-}* mice. IL-5 can induce AHR even in the absence of eosinophils (Borchers et al. 2001) (eosinophils were not observed in these animals). IL-5 was augmented in ozone exposed *Cpe^{fat}* versus WT mice and further augmented in *Cpe^{fat}/TNFR2^{-/-}* mice (Figure 3F), consistent with the changes in AHR (Figure 2). IL-17A can also induce AHR (Kudo et al. 2012). IL-17A was expressed after O₃ exposure (Figure 3G), especially in the obese mice, and could also contribute to the augmented O₃-induced AHR observed in these mice (Figure 2).

In lean mice, TNFR2 deficiency had no effect on O₃-induced inflammatory cell recruitment (Figure 4), consistent with previous observations (Cho et al. 2001; Shore et al. 2001). In contrast, in obese mice, TNFR2 deficiency reduced O₃-induced neutrophil and macrophage recruitment and ablated obesity-related differences in these outcomes (Figure 4). Anti-IL-13 treatment also had no effect on O₃-induced inflammation in lean WT mice, which lacked IL-13 (Figure 3A,B), but significantly reduced O₃-induced inflammatory cell recruitment in obese *Cpe^{fat}* mice (Figure 4A,B). A similar reduction in O₃-induced inflammation occurs in lean IL-13 deficient Balb/c mice (Williams et al. 2008), which are more Th2 prone than the C57BL/6 mice used in the present study. Importantly, in obese mice, TNFR2 and IL-13 appeared to interact to promote inflammatory cell recruitment, since anti-IL-13 treatment had no effect on BAL neutrophils or macrophages in *Cpe^{fat}/TNFR2^{-/-}* mice even though these mice had at least as much IL-13 expression as *Cpe^{fat}* mice (Figure 4A,B). This interaction may occur at the level of MIP-3 α

expression. In obese *Cpe^{fat}* mice, both TNFR2 deficiency and anti-IL-13 treatment resulted in a significant decrease in BAL MIP-3 α (Figure 6B), consistent with observations of others that TNF α and IL-13 can both induce the expression of MIP-3 α in airway epithelial cells (Reibman et al. 2003). Importantly, anti-IL-13 inhibited MIP-3 α expression in TNFR2 sufficient but not deficient *Cpe^{fat}* mice (Figure 6B). Dendritic cells and T cells typically express CCR6, the receptor for MIP-3 α , but neutrophils can be induced to express CCR6 in the presence of TNF α (Yamashiro et al. 2000). In addition, MIP-3 α can induce migration of IL-17A expressing cells that may contribute to neutrophil recruitment (Li et al. 2011). IL-17A was induced by O₃ (Figure 3G).

O₃-induced changes in lung function (i.e. G and H) and cellular inflammation occurred in concert (Table 1). O₃-induced increases in BAL neutrophils and in both G and H were greater in *Cpe^{fat}* versus WT mice. Furthermore, both TNFR2 deficiency and anti-IL-13 reduced O₃-induced changes in G and H as well as BAL neutrophils in obese mice (Table 1). The results suggest that effects of O₃ on lung function and inflammation may be mechanistically related. In contrast, O₃-induced AHR and inflammation were dissociated. For example, TNFR2 deficiency and anti-IL-13 either augmented or had no effect on O₃-induced but attenuated O₃-induced inflammation in obese mice.

Two technical issues that require consideration. First, obese mice have a slightly higher minute ventilation (Shore and Johnston 2006) and consequently a slightly higher inhaled dose of O₃. Their lungs are also smaller (Williams et al. 2012), so that the dose per gram of lung tissue may be higher. We do not think this issue contributed substantially to the outcome, since neither TNFR2 deficiency nor anti-IL-13 had any effect on lung volume (data not shown), but both

treatments reduced augmented effects of O₃ on BAL cells and pulmonary mechanics in obese mice. Second, we used the same volume of fluid for lavage in obese and lean mice. Because the lungs of the obese mice were smaller and presumably had correspondingly less lung lining fluid, there should have been greater dilution of substances in that lining fluid and thus lower concentrations of BAL moieties in the obese than the lean mice. In fact, the opposite was true for most BAL cytokines/chemokines examined (Figures 3,5,6, and Supplemental Material, Figure S2). Thus issues related to normalization of the BAL procedure are unlikely to explain the observed results.

In summary, our results indicated differences in the mechanisms regulating pulmonary responses to O₃ in lean and obese mice. In particular, TNFR2 deficiency had opposing effects on O₃-induced AHR in lean and obese mice. In addition, O₃ increased the pulmonary expression of IL-13 in obese but not lean mice, and this IL-13 appeared to account for the augmented ability of O₃ to induce changes in pulmonary mechanics and inflammation in obese mice via synergistic effects with TNFR2. The majority of the population of the United States is either obese or overweight. Our results emphasize the need for improved understanding of the effects of O₃ in this population.

References

- Alexeeff SE, Litonjua AA, Suh H, Sparrow D, Vokonas PS, Schwartz J. 2007. Ozone Exposure and Lung Function: Effect Modified by Obesity and Airways Hyperresponsiveness in the VA Normative Aging Study. *Chest* 132(6):1890-1897.
- Alexis N, Urch B, Tarlo S, Corey P, Pengelly D, O'Byrne P, et al. 2000. Cyclooxygenase metabolites play a different role in ozone-induced pulmonary function decline in asthmatics compared to normals. *Inhal Toxicol* 12(12):1205-1224.
- Bennett WD, Hazucha MJ, Folinsbee LJ, Bromberg PA, Kissling GE, London SJ. 2007. Acute pulmonary function response to ozone in young adults as a function of body mass index. *Inhal Toxicol* 19(14):1147-1154.
- Bhalla DK. 1999. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *J Toxicol Environ Health B Crit Rev* 2(1):31-86.
- Borchers MT, Crosby J, Justice P, Farmer S, Hines E, Lee JJ, et al. 2001. Intrinsic AHR in IL-5 transgenic mice is dependent on CD4+ cells and CD49d-mediated signaling. *American Journal of Physiology - Lung Cellular and Molecular Physiology* 281(3):L653-L659.
- Castro-Giner F, Kogevinas M, Imboden M, de Cid R, Jarvis D, Machler M, et al. 2009. Joint effect of obesity and TNFA variability on asthma: two international cohort studies. *Eur Respir J* 33(5):1003-1009.
- Cho HY, Zhang LY, Kleeberger SR. 2001. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-alpha receptors. *Am J Physiol Lung Cell Mol Physiol* 280(3):L537-L546.
- Fauroux B, Sampil M, Quenel P, Lemoullec Y. 2000. Ozone: a trigger for hospital pediatric asthma emergency room visits. *Pediatr Pulmonol* 30(1):41-46.
- Garantziotis S, Li Z, Potts EN, Kimata K, Zhuo L, Morgan DL, et al. 2009. Hyaluronan mediates ozone-induced airway hyperresponsiveness in mice. *J Biol Chem* 284(17):11309-11317.
- Gent JF, Triche EW, Holford TR, Belanger K, Bracken MB, Beckett WS, et al. 2003. Association of low-level ozone and fine particles with respiratory symptoms in children with asthma. *Jama* 290(14):1859-1867.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282(5397):2261-2263.

- Ito Y, Mason RJ. 2010. The effect of interleukin-13 (IL-13) and interferon-gamma (IFN-gamma) on expression of surfactant proteins in adult human alveolar type II cells in vitro. *Respir Res* 11:157.
- Johnston RA, Mizgerd JP, Shore SA. 2005a. CXCR2 is essential for maximal neutrophil recruitment and methacholine responsiveness after ozone exposure. *Am J Physiol Lung Cell Mol Physiol* 288(1):L61-67.
- Johnston RA, Schwartzman IN, Flynt L, Shore SA. 2005b. Role of interleukin-6 in murine airway responses to ozone. *Am J Physiol Lung Cell Mol Physiol* 288(2):L390-397.
- Johnston RA, Theman TA, Shore SA. 2006. Augmented Responses to Ozone in Obese Carboxypeptidase E-Deficient Mice. *Am J Physiol Regul Integr Comp Physiol* 290:R126-133.
- Johnston RA, Zhu M, Hernandez CB, Williams ES, Shore SA. 2010. Onset of obesity in carboxypeptidase E-deficient mice and effect on airway responsiveness and pulmonary responses to ozone. *J Appl Physiol* 108(6):1812-1819.
- Kasahara DI, Kim HY, Williams AS, Verboon NG, Tran J, Si H, et al. 2012. Pulmonary inflammation induced by subacute ozone is augmented in adiponectin-deficient mice: role of IL-17A. *J Immunol* 188(9):4558-4567.
- Katsuki A, Sumida Y, Murashima S, Murata K, Takarada Y, Ito K, et al. 1998. Serum levels of tumor necrosis factor-alpha are increased in obese patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* 83(3):859-862.
- Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, et al. 2012. IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat Med* 18(4):547-554.
- Leibel RL, Chung WK, Chua SC, Jr. 1997. The molecular genetics of rodent single gene obesities. *J Biol Chem* 272(51):31937-31940.
- Li Z, Burns AR, Miller SB, Smith CW. 2011. CCL20, gammadelta T cells, and IL-22 in corneal epithelial healing. *Faseb J* 25(8):2659-2668.
- Matsubara S, Takeda K, Jin N, Okamoto M, Matsuda H, Shiraishi Y, et al. 2009. Vgamma1+ T cells and tumor necrosis factor-alpha in ozone-induced airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 40(4):454-463.

- National Center for Health Statistics. 2012. Healthy People 2010 Final Review. Available: http://www.cdc.gov/nchs/data/hpdata2010/hp2010_final_review.pdf [accessed Feb 13, 2013].
- Naude PJ, den Boer JA, Luiten PG, Eisel UL. 2011. Tumor necrosis factor receptor cross-talk. *FEBS J*.
- Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, et al. 2008. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* 205(2):385-393.
- Pulfer MK, Murphy RC. 2004. Formation of biologically active oxysterols during ozonolysis of cholesterol present in lung surfactant. *J Biol Chem* 279(25):26331-26338.
- Reibman J, Hsu Y, Chen LC, Bleck B, Gordon T. 2003. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *American journal of respiratory cell and molecular biology* 28(6):648-654.
- Shore SA, Johnston RA. 2006. Obesity and asthma. *Pharmacol Ther* 110(1): 83-102.
- Shore SA, Rivera-Sanchez YM, Schwartzman IN, Johnston RA. 2003. Responses to ozone are increased in obese mice. *J Appl Physiol* 95(3):938-945.
- Shore SA, Schwartzman IN, Le Blanc B, Murthy GG, Doerschuk CM. 2001. Tumor necrosis factor receptor 2 contributes to ozone-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 164(4):602-607.
- Shore SA, Williams ES, Chen L, Benedito LA, Kasahara DI, Zhu M. 2011. Impact of aging on pulmonary responses to acute ozone exposure in mice: role of TNFR1. *Inhalation toxicology* 23(14):878-888.
- Williams AS, Chen L, Kasahara DI, Si H, Wurmbrand AP, Shore SA. 2012. Obesity and airway responsiveness: Role of TNFR2. *Pulmonary pharmacology & therapeutics*.
- Williams AS, Nath P, Leung SY, Khorasani N, McKenzie AN, Adcock IM, et al. 2008. Modulation of ozone-induced airway hyperresponsiveness and inflammation by interleukin-13. *Eur Respir J* 32(3):571-578.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. 1998. Interleukin-13: central mediator of allergic asthma [see comments]. *Science* 282(5397):2258-2261.
- Yamashiro S, Wang JM, Yang D, Gong WH, Kamohara H, Yoshimura T. 2000. Expression of CCR6 and CD83 by cytokine-activated human neutrophils. *Blood* 96(12):3958-3963.

Yang IA, Holz O, Jorres RA, Magnussen H, Barton SJ, Rodriguez S, et al. 2005. Association of tumor necrosis factor-alpha polymorphisms and ozone-induced change in lung function. *Am J Respir Crit Care Med* 171(2):171-176.

Zhao Q, Simpson LG, Driscoll KE, Leikauf GD. 1998. Chemokine regulation of ozone-induced neutrophil and monocyte inflammation. *Am J Physiol* 274(1 Pt 1):L39-46.

Table 1. Effect of IL-13 blockade or TNFR2-deficiency on ozone-induced increases in airway responsiveness, BAL neutrophils, and pulmonary mechanics in lean and obese mice

Intervention	Lean mice			Obese mice		
	Airway responsiveness	BAL neutrophils	Pulmonary mechanics	Airway responsiveness	BAL neutrophils	Pulmonary mechanics
IL-13 blockade	no change	no change	no change	no change	decrease	decrease
TNFR2 deficiency	decrease	no change	no change	increase	decrease	decrease

Outcomes (no change, increase, decrease) indicate the qualitative change in the response to ozone relative to the absence of IL-13 blockade or TNFR2 deficiency). BAL: bronchoalveolar lavage

FIGURE LEGENDS

Figure 1. O₃-induced changes in pulmonary mechanics. Mice were studied 24 h after O₃ exposure. Pressure volume (PV) curves of WT (A) and *Cpe^{fat}* (B) mice that were unexposed, exposed to ozone, or treated with anti-IL-13 24 h prior to ozone exposure. Shown are the area of the PV curve normalized for volume A, the difference between total lung capacity and end expiratory volume (i.e. the height of the PV curves) (C), and baseline values for airway resistance (Rn) (panel D), the coefficient of lung tissue damping, G (panel E), and the coefficient of lung tissue elastance, H (panel F). Results are mean \pm SE of data from 6-9 mice in each group. * p<0.05 versus unexposed genotype-matched lean mice. #p<0.05 versus TNFR2 genotype matched lean mice with the same exposure; & p<0.05 versus ozone exposed genotype matched mice not treated with anti-IL-13.

Figure 2. Airway responsiveness in mice that were unexposed, exposed to ozone, or treated with anti-IL-13 24 h prior to ozone exposure. Methacholine induced changes in G, a measure of the lung tissue response, are shown. Panels A, B, C, and D show results for WT, TNFR2^{-/-}, *Cpe^{fat}*, and *Cpe^{fat}/TNFR2^{-/-}* mice respectively. Results are mean \pm SE of data from 6-9 mice per group. * p<0.05 for ozone exposed versus unexposed genotype-matched lean mice. #p<0.05 versus TNFR2 genotype matched lean mice with the same exposure; † p<0.05 versus obesity-matched TNFR2 sufficient mice with the same ozone exposure; & p<0.05 versus ozone exposed genotype matched mice not treated with anti-IL-13.

Figure 3. (A) Pulmonary mRNA expression of IL-13 and (B) BAL IL-13 concentration in mice that were either unexposed or exposed to ozone. (C) Example of flow cytometry data from one

Cpe^{fat} mouse showing histograms of CD4⁺ lung cells stained with isotype control antibody or stained with anti-IL-13 and either unstimulated or stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 5 hours to induce cytokine expression. Shown also are the % (D) and total number (E) of PMA and ionomycin stimulated CD4⁺ cells isolated from lungs of ozone exposed mice that expressed IL-13. (F) BAL IL-5 concentrations and (G) IL-17A mRNA expression in unexposed and ozone exposed mice. Results are mean \pm SE of data from 4-8 mice per group. Results for IL-13 and IL-17A mRNA are normalized to 36B4 expression. * $p < 0.05$ versus unexposed genotype-matched lean mice. # $p < 0.05$ versus TNFR2 genotype matched lean mice with the same exposure; † $p < 0.05$ versus obesity-matched TNFR2 sufficient mice with the same ozone exposure

Figure 4. Bronchoalveolar lavage (BAL) macrophages (A), neutrophils (B), and protein (C) in mice that were unexposed, exposed to ozone, or treated with anti-IL-13 24 hours prior to ozone exposure. Results are mean \pm SE of data from 4-9 mice per group. * $p < 0.05$ versus unexposed genotype-matched lean mice. # $p < 0.05$ versus TNFR2 genotype matched lean mice with the same exposure; † $p < 0.05$ versus obesity-matched TNFR2 sufficient mice with the same ozone exposure; & $p < 0.05$ versus ozone exposed genotype matched mice not treated with anti-IL-13.

Figure 5. BAL G-CSF (A), MCP-1 (B), TNF α (C) hyaluronan (D), protein carbonyls (E), in mice that were either unexposed or exposed to ozone. * $p < 0.05$ versus unexposed genotype-matched lean mice. # $p < 0.05$ versus TNFR2 genotype matched lean mice with the same exposure; † $p < 0.05$ versus obesity-matched TNFR2 sufficient mice with the same ozone exposure

Figure 6. BAL LIX (A) and MIP-3 α (B) in mice that were unexposed, exposed to ozone, or treated with anti-IL-13 24 h prior to ozone exposure. Results are mean \pm SE of data from 5-8 mice per group. * $p < 0.05$ versus unexposed genotype-matched lean mice; † $p < 0.05$ versus obesity-matched TNFR2 sufficient mice with the same ozone exposure; & $p < 0.05$ versus ozone exposed genotype matched mice not treated with anti-IL-13.

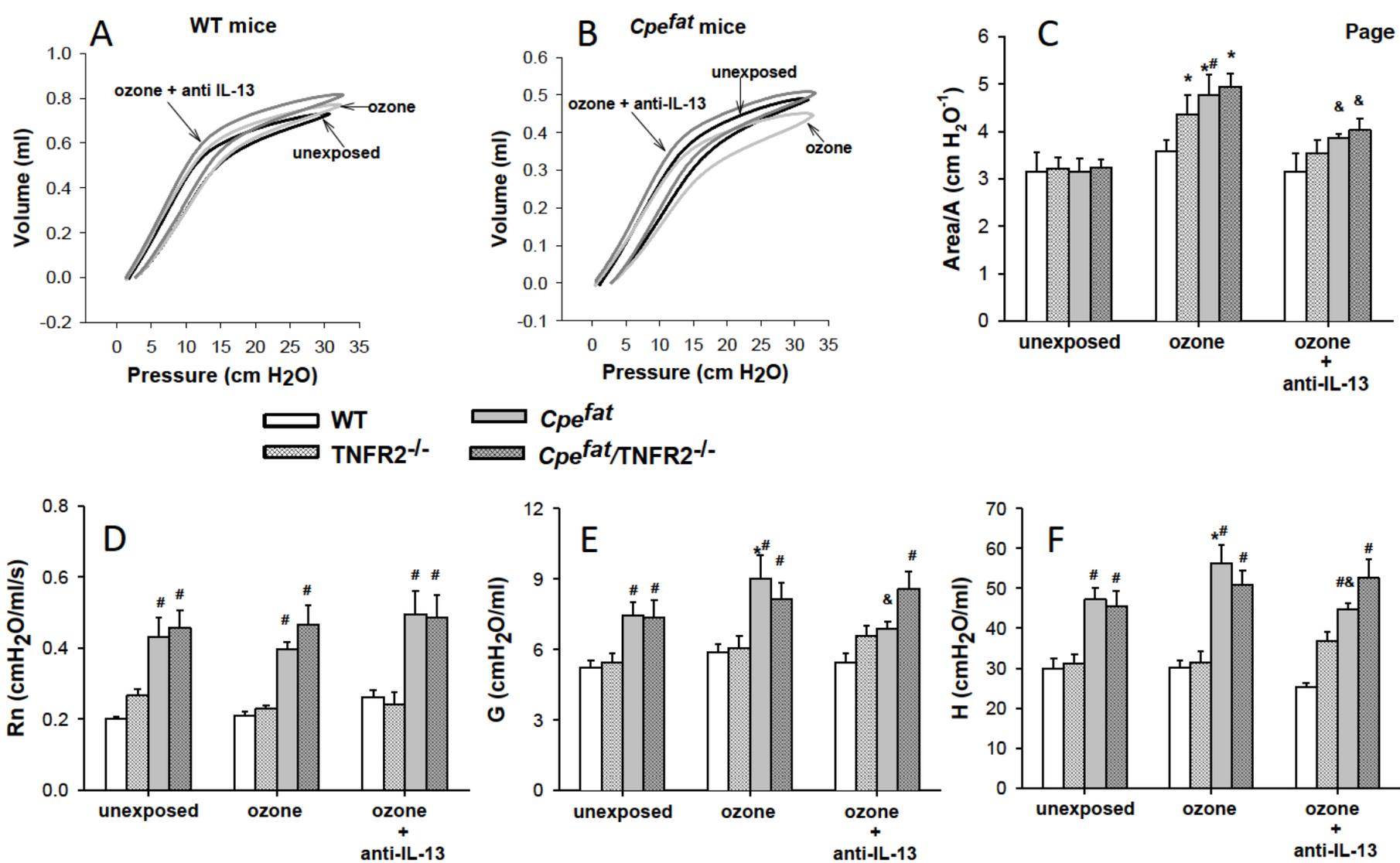


Figure 1

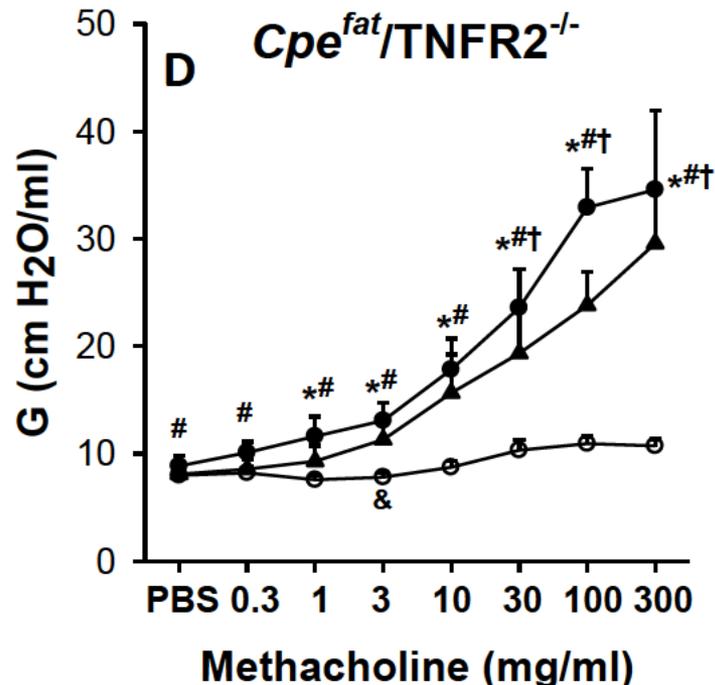
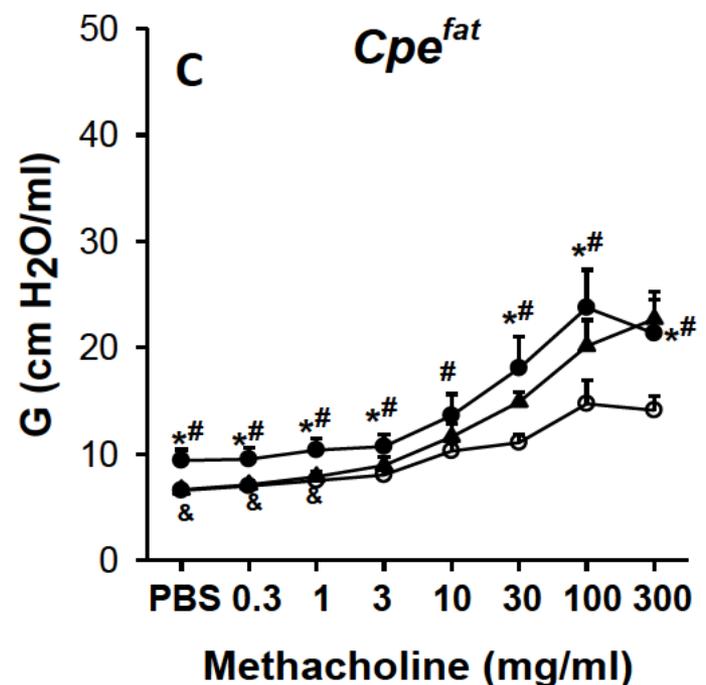
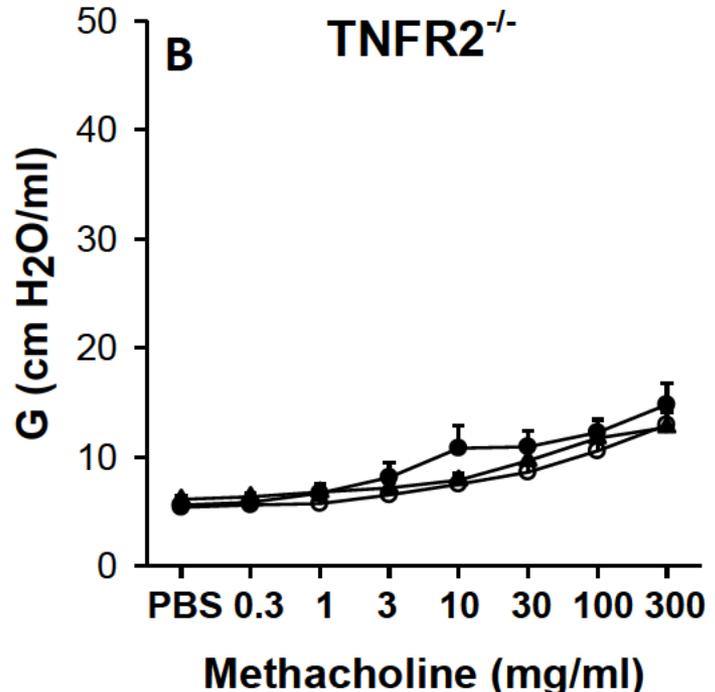
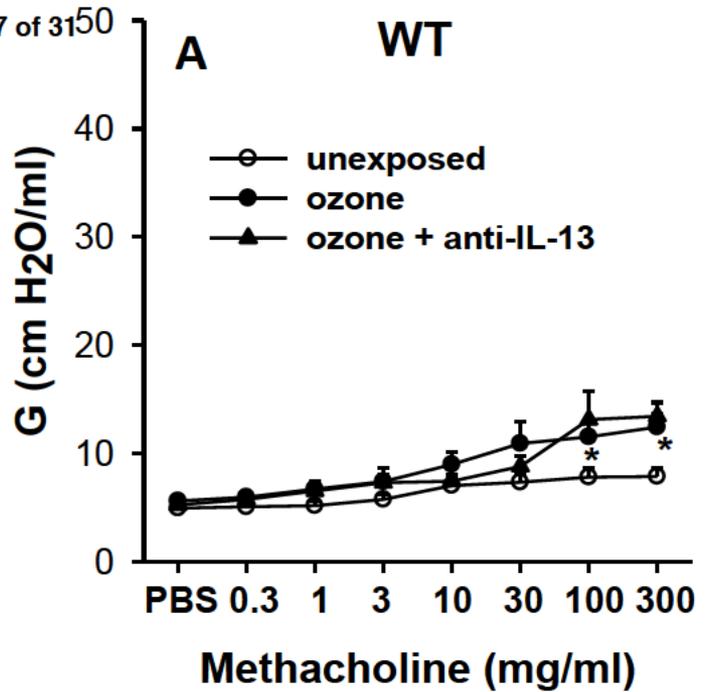


Figure 2

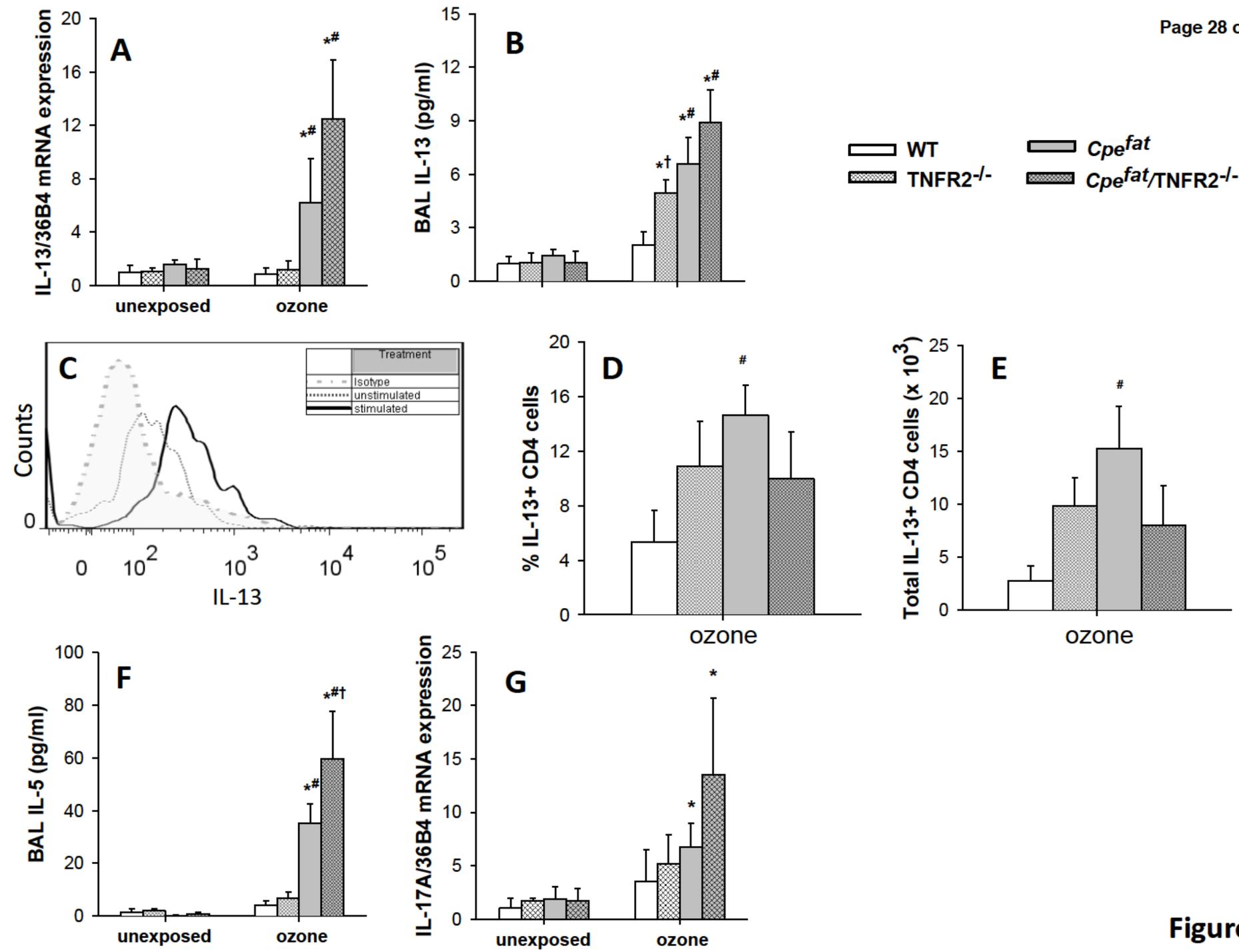


Figure 3

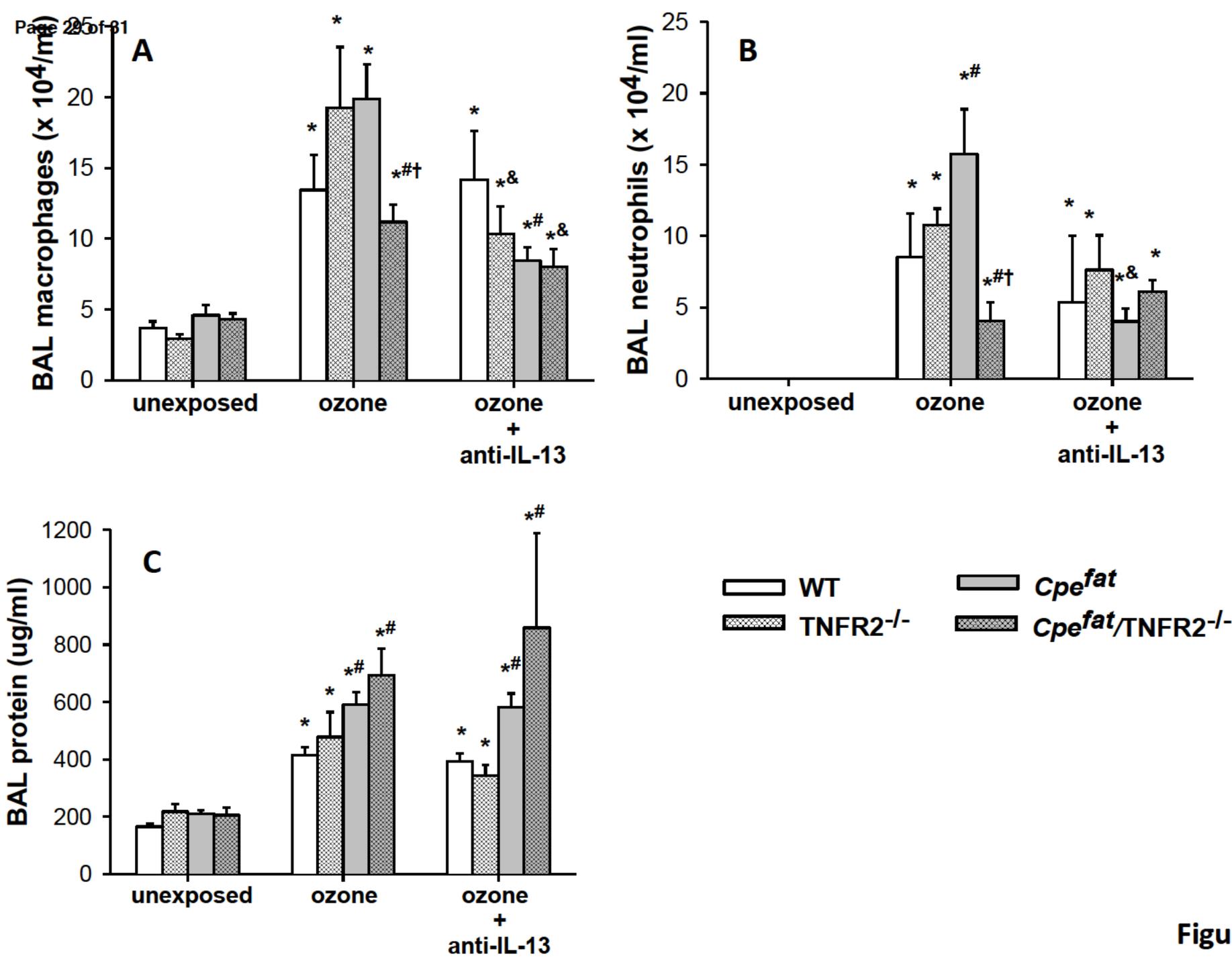


Figure 4

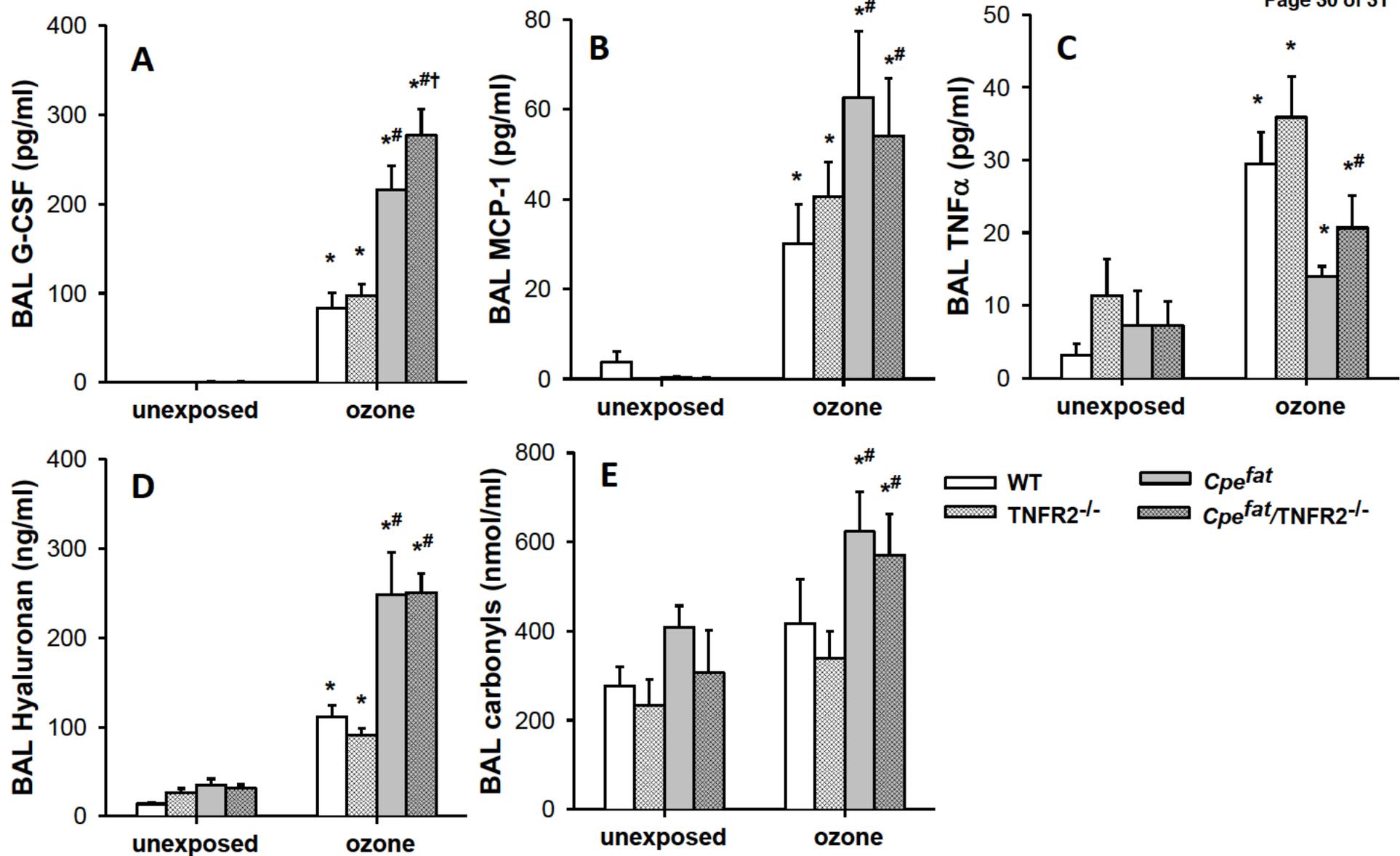


Figure 5

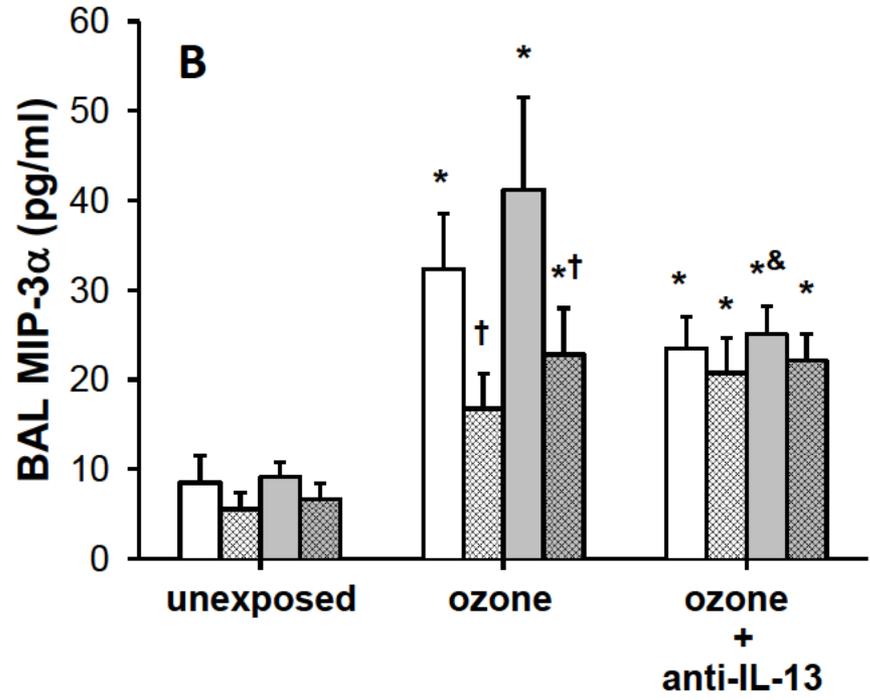
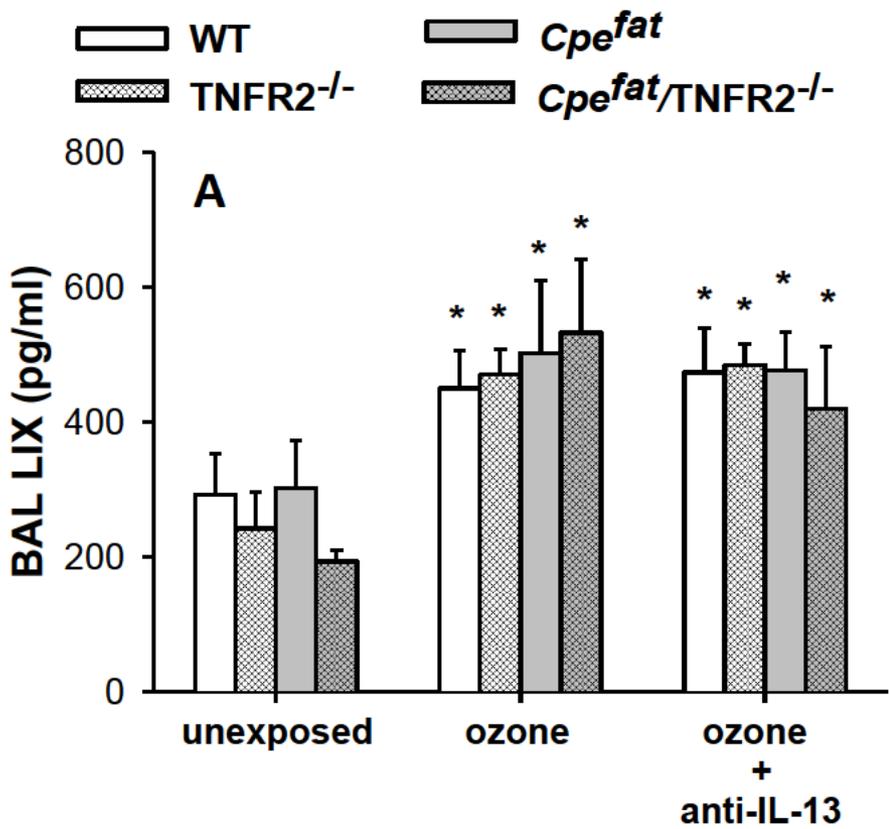


Figure 6