Modulation of ozone-induced airway hyperresponsiveness and inflammation by interleukin-13

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ABSTRACT: The present study aimed to determine whether the T-helper cell type 2-derived cytokines, interleukin (IL)-4 and -13, can modulate the lung response to ozone exposure.

IL-13^{-/-}, IL-4/13^{-/-} and IL-13-overexpressing transgenic (Tg) mice were exposed to ozone (3 ppm; 3 h) or air. Wild-type (Wt) Balb/c mice and transgenic-negative littermates (IL-13Wt) were used as controls for gene-deficient and IL-13Tg mice, respectively.

IL-4/13^{-/-} and IL-13^{-/-} mice developed a lesser degree of ozone-induced airway hyperresponsiveness (AHR) while IL-13Tg mice developed a greater degree of AHR compared with ozone-exposed wild-type or IL-13Wt mice, respectively. Ozone caused a time-dependent increase of bronchoalveolar lavage (BAL) neutrophils and macrophages in wild-type mice, maximal at 20–24 h, which was attenuated in the IL-13^{-/-} and IL-4/13^{-/-} mice. In IL-13Tg mice, there was a greater increase in BAL neutrophils after ozone exposure compared with IL-13Wt mice. Using quantitative real-time PCR, ozone-induced mRNA expression for IL-6 and keratinocyte chemokine was further enhanced in IL-13^{-/-} and IL-4/13^{-/-} mice, and was inhibited in IL-13Tg mice. Macrophage inflammatory protein (MIP)-3 α /CCL20 expression was enhanced after ozone exposure in wild-type mice, inhibited in IL-13^{-/-} and IL-4/13^{-/-} mice, while in IL-13Tg mice it was enhanced. A similar pattern of expression was observed with lipopolysaccharide-induced cytokine (LIX/CXCL5/ENA-78) expression.

In conclusion, interleukin-13 augments ozone-induced airway hyperresponsiveness and neutrophilic inflammation, possibly through modulation of certain cytokines induced by ozone exposure.

KEYWORDS: Airways hyperresponsiveness, interleukin-4, interleukin-13, neutrophils, ozone

High levels of ambient ozone, a powerful oxidant pollutant gas, pose a significant threat to respiratory health and are linked to the worsening of symptoms and increased hospitalisations of patients with obstructive lung disease, such as asthma and chronic obstructive pulmonary disease [1–7]. Ozone exposure induces airway hyperresponsiveness (AHR) to bronchoconstrictor agents and lung neutrophilia in many species, including humans [8–13]. The mechanisms underlying ozone-induced increase in lung resistance (RL) and inflammation are unclear. The influx of neutrophils may be due to their recruitment by pro-inflammatory cytokines and chemokines known to be upregulated by exposure to ozone in both rodents or humans, such as cytokine-induced neutrophil chemoattractant, macrophage inflammatory protein (MIP)-2 (CXCL2), tumour necrosis factor α , interleukin (IL)-1 β , IL-8 (CXCL8), keratinocyte chemokine (KC; CXCL1) and IL-6

[14–19]. In addition, there is increased expression of MIP-3 α /CCL20 and lipopolysaccharide-induced CXC chemokine (LIX; CXCL5) in lungs of mice exposed to ozone (by gene microarray analysis; data not shown).

Several studies have indicated that ozone exposure may induce or amplify the allergic response. Thus, ozone exposure of healthy or allergic asthmatic individuals can lead to an eosinophilic inflammation in the upper airways [20]. Furthermore, ozone exposure can increase the response of the lower airways of asthma patients to the airway or eosinophilic response to allergen [21, 22]. In animal models, positive interactions between ozone exposure and components of the allergic response have also been reported [23]. In the current study, the interaction of ozone with the asthmatic response was investigated by examining the role of IL-4 and -13, two structurally related cytokines involved in

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STATEMENT OF INTEREST

None declared.

T-helper cell type 2-mediated immune responses [24], in the lung response to ozone exposure. IL-13 has been particularly implicated in causing many aspects of asthma including AHR [25], airway inflammation [26] and airway wall remodelling [27]. The present approach was to study the effect of ozone exposure in mice in which either IL-13 alone or both IL-4 and -13 have been disrupted, and in mice in which IL-13 has been overexpressed. The present data indicate that IL-13 is an important modulator of ozone-induced AHR and lung inflammation.

METHODS

Generation of IL-13^{-/-}, double IL-4/13^{-/-} and IL-13Tg mice

IL-13^{-/-} mice were created by the disruption of exon 1 in the mouse IL-13 gene [28]. IL-4/13^{-/-} mice were generated by the simultaneous disruption of the mouse IL-13 and IL-4 genes, as reported previously [24]. Knockout mice were identified using PCR analysis of genomic DNA, and wild-type (Wt) Balb/c mice were used as controls. Transgenic (Tg) mice over-expressing IL-13 (IL-13Tg) were created by cloning a 6 kb BamHI-digested DNA fragment of the complete IL-13 gene upstream of the human cluster of differentiation 2 locus control region [29]. Transgenic mice were identified using PCR analysis of genomic DNA and negative-transgenic littermates (IL-13Wt) were used as controls.

Study design and methods

Two separate studies were performed. In the first study, IL-13^{-/-} and IL-4/13^{-/-} and wild-type Balb/c mice (Harlan, Bicester, Oxon, UK) were investigated; in the second study, IL-13Tg and IL-13Wt were examined. In both studies, groups of mice were exposed to either ozone (3 ppm, 3 h) or air. Mice were exposed to ozone produced by an ozonizer (Model 500 Ozoniser; Sander, Wuppertal, Germany), mixed with air for 3 h at a concentration of 3 ppm within a sealed Perspex® container. Ozone concentration was continually monitored with an ozone probe (ATi Technologies, Oldham, UK). Bronchoalveolar lavage (BAL) was performed 3 h after exposure and lung tissues were collected for mRNA extraction for real-time PCR. Other groups of mice were studied at 20–24 h after exposure with measurement of RL and airway responsiveness to acetylcholine (ACh; Sigma, Dorset, UK), followed by BAL.

Assessment of AHR

At 20–24 h after exposure, mice were anaesthetised with an intraperitoneal injection of anaesthetic solution containing midazolam and hypnorm (0.315 mg·mL⁻¹ fentanyl citrate and

10 mg·mL⁻¹ fluanisone). Mice were tracheostomised and ventilated (250 breaths·min⁻¹ with tidal volume of 250 µL). Mice were monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS, Bordon, UK). Transpulmonary pressure was assessed using an oesophageal catheter (EMMS). Instantaneous calculation of RL was obtained. Increasing concentrations (4–256 mg·mL⁻¹) of ACh were administered with an ultrasonic nebuliser, and RL was recorded for a 5-min period following each concentration. RL after each concentration was expressed as percentage change from baseline RL measured following nebulised PBS. The provocative concentration of ACh required to increase RL by 150% from baseline (PC150) was calculated.

BAL

Briefly, either 3 or 20–24 h after exposure, following an overdose of anaesthetic, mice were lavaged with six 0.5-mL aliquots of PBS *via* the endotracheal tube, which was retrieved as the BAL fluid [30]. Total cell counts and differential cell counts from cytospin preparations stained by May-Grünwald-Giemsa stain were determined under an optical microscope (Olympus BH2; Olympus Optical Company Ltd, Tokyo, Japan). At least 400 cells were counted and identified as macrophages, eosinophils, lymphocytes or neutrophils according to standard morphology.

cDNA synthesis, reverse transcription and real-time PCR

Whole-lung tissue samples were homogenised under liquid nitrogen and RNA was extracted using RNeasy mini kit (Qiagen, Crawley, UK). RNA (5 µg per sample) was used to synthesise single-stranded complementary DNA (cDNA) using random hexamers and an avian myeloblastosis virus reverse transcriptase (Promega, Southampton, UK). The cDNA generated was used as a template in subsequent real-time PCR analyses. Transcript levels were determined by real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) using SYBR Green PCR Master Mix Reagent (Qiagen). The murine forward and reverse primers (0.5 µM) used are specified in table 1. Cycling conditions were as follows. Step 1: 15 min at 95°C; step 2: 20 s at 94°C; step 3: 20 s at 60°C; step 4: 20 s at 72°C, with step 2 to step 4 repeated for 45 cycles. The standard curves used to determine relative expression for each primer were obtained by performing real-time PCR for a diluted sample, for example, to 1:1, 1:10, 1:100 and 1:1000. Gene expression was normalised to glyceraldehyde 3-phosphate dehydrogenase.

TABLE 1 Real-time PCR primers		
Primer	Forward	Reverse
GAPDH	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
KC	CGCTGCTGCTGCTGGCCACCA	GGCTATGACTTCGGTTTGGGTGCAG
IL-6	CACAGAGGATACCACTCCCAACA	TCCACGATTCCAGAGAAACA
LIX/CXCL5	CTGTCTTGTAAGCAACCAATAGC	CAGAAGCAGCAAGCAACTACGA
MIP-3α/CCL20	CAGAAGCAGCAAGCAACTACGA	CTGTCTTGTAAGCAACCAATAGC
GAPDH: glyceraldehyde 3-phosphate dehydrogenase; KC: keratinocyte chemokine; IL: interleukin ; LIX/CXCL5: lipopolysaccharide-induced CXC chemokine; MIP-3α/CCL20: macrophage inflammatory protein 3α

FIGURE 1. Concentration–response curves in response to a, b) acetylcholine (ACh) and c, d) -log provocative concentration of ACh required to increase lung resistance (RL) by 150% from baseline (PC₁₅₀) of interleukin (IL)-13^{-/-}, IL-4/13^{-/-}, wild-type, IL-13^{+/+} and IL-13Wt mice exposed to air or to ozone. Data are presented as mean + SEM (a and b). —: mean; *: p<0.05; ***: p<0.001 compared with air-exposed mice of the same strain; #: p<0.05, ##: p<0.01 compared with ozone-exposed wild-type mice.

Measurement of BAL IL-6 and KC levels

Levels of IL-6 and KC were measured in BAL fluid using a DuoSet ELISA kit (R&D Systems, Abington, UK) according to the manufacturer's instructions.

Data analysis

Data are presented as mean ± SEM. For multiple comparisons of different groups, the Kruskal–Wallis test for ANOVA was used, followed by the Dunn's test for comparison between two individual groups or the Mann–Whitney test. A p-value <0.05 was considered significant.

RESULTS

AHR

There were no significant differences in the baseline RL values following PBS challenge in each experimental group. Following ozone exposure, there was an increase in airway responsiveness in all groups as denoted by a shift of the concentration response curve to the left (fig. 1a and b). However, IL-4/13^{-/-} and IL-13^{-/-} mice developed a lesser degree of AHR when compared with wild-type mice. In IL-13^{-/-} mice, the increase in airway responsiveness was particularly attenuated (-log PC₁₅₀ 1.083±0.1 and 1.58±0.1 after ozone in wild-type and IL-13^{-/-} mice, respectively; p<0.001; fig. 1c). In all three strains, ozone

induced a significant degree of AHR. Conversely, AHR after ozone exposure in IL-13Tg mice was significantly increased compared with ozone-exposed wild-type mice (-log PC₁₅₀ of 0.957±0.04 and 1.239±0.09 for IL-13Tg and IL-13Wt, respectively; p<0.01; fig. 1d).

Lung inflammation

Exposure to ozone caused a time-dependent increase in total cells, neutrophils and macrophages recovered in BAL fluid in wild-type mice, with a maximal effect at 20–24 h (figs 2 and 3). There was a significant time-dependent attenuation of the total numbers of BAL cells observed at 20–24 h after ozone exposure but not after 3 h in IL-13^{-/-} and IL-4/13^{-/-} mice (fig. 2). Conversely, in IL-13^{+/+} transgenic mice, the total number of BAL cells was significantly higher than that observed in IL-13Wt mice at both 3 and 20–24 h after ozone exposure (fig. 2). The numbers of neutrophils in the IL-13^{-/-} mice following ozone exposure at 20–24 h were also significantly reduced compared with wild-type mice; this was also similar in IL-4/13^{-/-} mice at 20–24 h after exposure to ozone (fig. 3b). In IL-13^{+/+} transgenic mice, the neutrophil counts were increased at 20–24 h after ozone exposure compared with IL-13Wt mice (fig. 3b). In IL-4/13^{-/-} mice, the decrease in BAL

FIGURE 2. Total bronchoalveolar lavage (BAL) cells after exposure to air (□: 3 h; ■: 20–24 h) or ozone (■: 3 h; ■: 20–24 h) in wild-type (Wt), interleukin (IL)-13^{-/-}, IL-4/13^{-/-}, IL-13Wt and IL-13^{+/+} mice. Data are presented as mean + SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared with air-exposed mice of same strain at same time-point; #: $p = 0.05$ compared with ozone-exposed wild-type mice at same time-point; †: $p = 0.05$ compared with ozone-exposed wild-type mice at same time-point.

neutrophils at 20–24 h after ozone exposure was not significantly decreased compared with wild-type mice. BAL macrophage numbers were increased at 20–24 h after ozone exposure in wild-type or IL-13Wt mice; this effect was attenuated at 24 h after ozone exposure in IL-13^{-/-} and IL-4/13^{-/-} mice (fig. 3a). In IL-13^{+/+} mice, the BAL macrophage numbers were not increased compared with IL-13Wt mice (fig. 3a).

Real-time PCR

Quantitative real-time PCR was performed in lungs from IL-13^{-/-}, IL-4/13^{-/-} and in IL-13Tg mice collected at 3 h after ozone exposure. There was significantly increased mRNA expression for IL-6, KC/CXCL1, MIP-3 α /CCL20 and LIX/CXCL5/ENA-78 in ozone-exposed wild-type and IL-13Wt mice (fig. 4). In ozone-exposed IL-13^{-/-} and IL-4/13^{-/-} mice, mRNA expression for IL-6 was further increased but this was not significant (fig. 4a); the expression was nonsignificantly different in IL-13Tg mice, compared with respective ozone-exposed IL-13Wt controls (fig. 4a). A similar pattern was observed with ozone-induced expression of KC/CXCL1. KC/CXCL1 mRNA expression was significantly less in ozone-exposed IL-13Tg mice compared with IL-13Wt mice (fig. 4d); however, there was no significant increase in KC in IL-13^{-/-} and IL-4/13^{-/-} mice after ozone exposure (fig. 4c). MIP-3 α /CCL20 expression was not significantly different in IL-13^{-/-} and IL-4/13^{-/-} mice after ozone exposure compared with wild-type mice (fig. 4b) but LIX/CXCL5/ENA-78 expression in ozone-exposed knockout mice was less than in ozone-exposed wild-type mice ($p < 0.05$ in IL-4/13^{-/-} and $p = 0.057$ in IL-4^{-/-} mice; fig. 4e). Ozone-induced MIP-3 α /CCL20 and CXCL5/LIX/ENA-78 expression was nonsignificantly changed in IL-13Tg mice, compared with IL-13Wt (fig. 4b and e).

BAL ELISA for IL-6 and KC

BAL levels of IL-6 and KC increased significantly after ozone exposure at 24 h (fig. 5a and b). In both IL-13^{-/-} and IL-4/13^{-/-} mice, the levels of IL-6 and KC were increased compared with

FIGURE 3. Bronchoalveolar lavage (BAL) a) macrophage and b) neutrophil cell numbers after exposure to air (□: 3 h; ■: 20–24 h) or ozone (■: 3 h; ■: 20–24 h) in wild-type (Wt), interleukin (IL)-13^{-/-}, IL-4/13^{-/-}, IL-13Wt and IL-13^{+/+} mice. Data are presented as mean + SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared with air-exposed mice of same strain at same time-point. #: $p < 0.05$; ##: $p < 0.01$ compared with ozone-exposed wild-type mice at same time-point. †: $p = 0.05$ compared with ozone-exposed wild-type mice at same time-point.

those in wild-type mice, but the difference was only significant for IL-6 levels in IL-13^{-/-} mice (fig. 5a and b). In IL-13^{+/+} transgenic mice, the level of IL-6 was not significantly lower after ozone compared with IL-13Wt mice (fig. 5a); the level of KC was lower but this was not significant (fig. 5b).

DISCUSSION

In the present study, deletion of IL-13 or of IL-4 and IL-13 in mice was shown to protect against ozone-induced AHR and inhibit neutrophil and macrophage accumulation in the BAL fluid. By contrast, IL-13 overexpression exacerbated ozone-induced AHR and lung neutrophilia. Additionally, it was shown that ozone-induced expression of IL-6, KC/CXCL1, CCL20/MIP-3 α and CXCL5/LIX were influenced by deletion of IL-13 or by overexpression of IL-13. Therefore, IL-13 can modulate the airway and inflammatory response to ozone. The contribution of IL-4 was unclear from the results observed in IL-4/13^{-/-} mice, and can only be resolved by studying IL-4^{-/-} mice.

The mechanisms of ozone-induced AHR are unknown but it is possible that ozone may directly cause an increase in airway smooth muscle (ASM) contractility to form the basis of AHR.

FIGURE 4. mRNA expression of a) interleukin (IL)-6, b) Macrophage inflammatory protein (MIP)-3α/CCL20 c,d) keratinocyte chemokine (KC), and e) lipopolysaccharide-induced CXC chemokine (CXCL5/LIX) expressed as a ratio of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), after exposure to air (□) or ozone (■) in wild-type (Wt), IL-13^{-/-}, IL-4/13^{-/-}, IL-13Wt and IL-13^{+/+} mice. Data are presented as mean + SEM. *: p<0.05; **: p<0.01 compared with air-exposed wild-type mice; #: p<0.05; †: p=0.057 compared with ozone-exposed wild-type mice.

Preliminary data from the present authors' laboratory has shown that bronchi from ozone-exposed mice show increased isometric-contraction responses to acetylcholine *ex vivo* and that this increased contractility together with the increased AHR are abolished by pre-treatment of mice to an antioxidant, N-acetylcysteine (data not shown; F.X. Blanc, Imperial College, London, UK; personal communication). Similarly, the effect of IL-13 in increasing AHR may be through a direct action on the ASM, since IL-13 has been shown to directly increase ASM contractility through the activation of IL-13 receptors identified on ASM cells [31]. IL-13 increases carbachol- and KCl-induced maximal force of murine tracheal rings [32]. IL-13 also reduces isoproterenol-induced relaxation as measured by cell stiffness in human ASM cells [31]. The potential for IL-13 to increase

bronchial responsiveness *in vivo* has also been demonstrated in mice where direct instillation of IL-13 into the lungs causes AHR [33]. In addition, in IL-13^{-/-} mice, allergen-induced AHR is inhibited [34], while in IL-13Tg mice, it is enhanced [25].

Interestingly, the present observations support the notion that IL-13 positively influenced the degree of ozone-induced lung neutrophilia. IL-13 is not a known chemoattractant for neutrophils but for eosinophils; this is an effect that may be mediated through the release of the eosinophil chemokine, eotaxin/CCL13 [35, 36]. It has been reported that patients with allergic rhinitis exposed to ozone experimentally produce an eosinophilic response [37, 38]. It is possible that the lack of an eosinophilic response in the mice is due to the fact that the

FIGURE 5. Bronchoalveolar (BAL) levels of a) interleukin (IL)-6 and b) keratinocyte chemokine (KC) after exposure to air (□) or ozone (■) in wild-type (Wt), interleukin (IL)-13^{-/-}, IL-4/13^{-/-}, IL-13Wt and IL-13^{+/+} mice. Data are presented as mean + SEM. *: $p < 0.05$; **: $p < 0.01$ compared with air-exposed wild-type mice; #: $p < 0.05$ compared with ozone-exposed wild-type mice.

bone marrow is not primed to produce eosinophils, particularly in the IL-13^{-/-} mice. One mechanism for the increased neutrophilic response may relate to the potential for IL-13 to increase oxidative stress in the lungs, thus contributing to the oxidant stimulus of ozone. IL-13 and IL-4 are potent inducers of nicotinamide adenine dinucleotide phosphate oxidase enzyme, dual oxidase-1, in the airway epithelium [39]. IL-13 enhances blood monocyte superoxide production in response to phorbol myristate acetate [40]; however, another study has indicated that IL-13 inhibits protein kinase C-triggered respiratory burst in human monocytes [41]. More recently, IL-13 has been reported to increase superoxide production from human bronchial epithelial cells [42]. Therefore, the possibility that IL-13 can contribute to oxidative stress or worsen the consequences of oxidative stress deserves further study in the present model. There may also be other mechanisms of enhancement including at the level of tissue permeability or T-cell activation.

The present data regarding the measurement of cytokine expression in the lungs of ozone-exposed mice may provide a clue as to the mechanisms by which IL-13 may modulate ozone-induced AHR and inflammation. These results show a complex pattern of changes of the level of expression of these cytokines, but must be interpreted as preliminary since they

were examined at only one time-point (3 h after exposure) and there was variability in the responses that precluded significance in many of the comparisons using numbers of observations that were sufficiently powerful to answer the primary questions on AHR and inflammation. In the present study, only four of many cytokines that are known to be upregulated by ozone exposure were chosen, namely the pro-inflammatory cytokines KC and LIX/CXCL5/ENA-78, both neutrophil chemoattractants and activators, while IL-6 has pro- and anti-inflammatory activities and MIP-3 α /CCL20 is a chemoattractant for dendritic cells.

Using quantitative real-time PCR, the present authors showed that there was a nonsignificant increase in IL-6 and KC/CXCL1 expression in IL-13^{-/-} mice, while LIX/CXCL5/ENA-78 was significantly inhibited in IL-13^{-/-} and IL-4/13^{-/-} mice after ozone-exposure. IL-6 expression was decreased nonsignificantly in IL-13^{+/+} mice after ozone exposure. The modulation of IL-6 gene expression, observed in the knock-out and transgenic strains, was similar to that observed of IL-6 protein levels in BAL fluid. These results suggest that IL-13 expression may modulate ozone-induced expression of IL-6. Previous studies have shown that bradykinin-induced increase in IL-6 protein and mRNA is inhibited by IL-13 in human ASM cells and that IL-6 expression was also found to be bradykinin B₂ receptor-dependent [43]. Interestingly, IL-6 has been shown to play a pro-inflammatory role in ozone-induced inflammation [44].

In conclusion, the present authors demonstrate an important role for interleukin-13 in ozone-induced airway hyperresponsiveness and inflammation. Inhibition of interleukin-13 may, therefore, prevent the inflammation and airway hyperresponsiveness in nonallergic airway diseases where oxidative stress is a key component, such as in asthma and chronic obstructive pulmonary disease.

REFERENCES

- Balmes JR, Aris RM, Chen LL, *et al.* Effects of ozone on normal and potentially sensitive human subjects. Part I: Airway inflammation and responsiveness to ozone in normal and asthmatic subjects. *Res Rep Health Eff Inst* 1997; 78: 1–37.
- Vagaggini B, Carnevali S, Macchioni P, *et al.* Airway inflammatory response to ozone in subjects with different asthma severity. *Eur Respir J* 1999; 13: 274–280.
- Stenfors N, Pourazar J, Blomberg A, *et al.* Effect of ozone on bronchial mucosal inflammation in asthmatic and healthy subjects. *Respir Med* 2002; 96: 352–358.
- Galán I, Tobías A, Banegas JR, Aránguez E. Short-term effects of air pollution on daily asthma emergency room admissions. *Eur Respir J* 2003; 22: 802–808.
- Eiswerth ME, Douglass Shaw W, Yen ST. Impacts of ozone on the activities of asthmatics: revisiting the data. *J Environ Manage* 2005; 77: 56–63.
- Peel JL, Tolbert PE, Klein M, *et al.* Ambient air pollution and respiratory emergency department visits. *Epidemiology* 2005; 16: 164–174.
- Medina-Ramón M, Zanobetti A, Schwartz J. The effect of ozone and PM₁₀ on hospital admissions for pneumonia and chronic obstructive pulmonary disease: a national multicity study. *Am J Epidemiol* 2006; 163: 579–588.

- 8 Fabbri LM, Aizawa H, Alpert SE, *et al.* Airway hyperresponsiveness and changes in cell counts in bronchoalveolar lavage after ozone exposure in dogs. *Am Rev Respir Dis* 1984; 129: 288–291.
- 9 Koto H, Salmon M, Haddad el-B, Huang TJ, Zagorski J, Chung KF. Role of cytokine-induced neutrophil chemoattractant (CINC) in ozone-induced airway inflammation and hyperresponsiveness. *Am J Respir Crit Care Med* 1997; 156: 234–239.
- 10 Zhang LY, Levitt RC, Kleeberger SR. Differential susceptibility to ozone-induced airways hyperreactivity in inbred strains of mice. *Exp Lung Res* 1995; 21: 503–518.
- 11 Cho HY, Zhang LY, Kleeberger SR. Ozone-induced lung inflammation and hyperreactivity are mediated *via* tumor necrosis factor- α receptors. *Am J Physiol Lung Cell Mol Physiol* 2001; 280: L537–L546.
- 12 Moore PE, Church TL, Chism DD, Panettieri RA Jr, Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L847–L853.
- 13 Seltzer J, Bigby BG, Stulbarg M, *et al.* O₃-induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J Appl Physiol* 1986; 60: 1321–1326.
- 14 Inoue H, Aiziwa H, Nakano H, *et al.* Nitric oxide synthase inhibitors attenuate ozone-induced airway inflammation in guinea pigs. Possible role of interleukin-8. *Am J Respir Crit Care Med* 2000; 161: 249–256.
- 15 Haddad el-B, Salmon M, Koto H, Barnes PJ, Adcock I, Chung KF. Ozone induction of cytokine-induced neutrophil chemoattractant (CINC) and nuclear factor- κ b in rat lung: inhibition by corticosteroids. *FEBS Lett* 1996; 379: 265–268.
- 16 Bhalla DK, Reinhart PG, Bai C, Gupta SK. Amelioration of ozone-induced lung injury by anti-tumor necrosis factor- α . *Toxicol Sci* 2002; 69: 400–408.
- 17 Bhalla DK, Gupta SK. Lung injury, inflammation, and inflammatory stimuli in rats exposed to ozone. *J Toxicol Environ Health A* 2000; 59: 211–228.
- 18 Johnston CJ, Stripp BR, Reynolds SD, Avissar NE, Reed CK, Finkelstein JN. Inflammatory and antioxidant gene expression in C57BL/6J mice after lethal and sublethal ozone exposures. *Exp Lung Res* 1999; 25: 81–97.
- 19 Pendino KJ, Shuler RL, Laskin JD, Laskin DL. Enhanced production of interleukin-1, tumor necrosis factor- α , and fibronectin by rat lung phagocytes following inhalation of a pulmonary irritant. *Am J Respir Cell Mol Biol* 1994; 11: 279–286.
- 20 Peden DB, Setzer RW Jr, Devlin RB. Ozone exposure has both a priming effect on allergen-induced responses and an intrinsic inflammatory action in the nasal airways of perennially allergic asthmatics. *Am J Respir Crit Care Med* 1995; 151: 1336–1345.
- 21 Kehrl HR, Peden DB, Ball B, Folinsbee LJ, Horstman D. Increased specific airway reactivity of persons with mild allergic asthma after 7.6 hours of exposure to 0.16 ppm ozone. *J Allergy Clin Immunol* 1999; 104: 1198–1204.
- 22 Vagaggini B, Taccola M, Cianchetti S, *et al.* Ozone exposure increases eosinophilic airway response induced by previous allergen challenge. *Am J Respir Crit Care Med* 2002; 166: 1073–1077.
- 23 Wagner JG, Hotchkiss JA, Harkema JR. Enhancement of nasal inflammatory and epithelial responses after ozone and allergen coexposure in Brown Norway rats. *Toxicol Sci* 2002; 67: 284–294.
- 24 McKenzie GJ, Fallon PG, Emson CL, Grecis RK, McKenzie AN. Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *J Exp Med* 1999; 189: 1565–1572.
- 25 Kuperman DA, Huang X, Koth LL, *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002; 8: 885–889.
- 26 Pope SM, Brandt EB, Mishra A, *et al.* IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism. *J Allergy Clin Immunol* 2001; 108: 594–601.
- 27 Zhu Z, Homer RJ, Wang Z, *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999; 103: 779–788.
- 28 McKenzie GJ, Emson CL, Bell SE, *et al.* Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 1998; 9: 423–432.
- 29 Emson CL, Bell SE, Jones A, Wisden W, McKenzie AN. Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. *J Exp Med* 1998; 188: 399–404.
- 30 Nath P, Eynott P, Leung SY, Adcock IM, Bennett BL, Chung KF. Potential role of c-Jun NH₂-terminal kinase in allergic airway inflammation and remodelling: effects of SP600125. *Eur J Pharmacol* 2005; 506: 273–283.
- 31 Laporte JC, Moore PE, Baraldo S, *et al.* Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am J Respir Crit Care Med* 2001; 164: 141–148.
- 32 Tliba O, Deshpande D, Chen H, *et al.* IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. *Br J Pharmacol* 2003; 140: 1159–1162.
- 33 Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004; 202: 175–190.
- 34 Nath P, Leung SY, Williams AS, *et al.* Complete inhibition of allergic airway inflammation and remodelling in quadruple IL-4/5/9/13^{-/-} mice. *Clin Exp Allergy* 2007; 37: 1427–1435.
- 35 Abonyo BO, Alexander MS, Heiman AS. Autoregulation of CCL26 synthesis and secretion in A549 cells: a possible mechanism by which alveolar epithelial cells modulate airway inflammation. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L478–L488.
- 36 Yamamoto S, Kobayashi I, Tsuji K, *et al.* Upregulation of interleukin-4 receptor by interferon- γ : enhanced interleukin-4-induced eotaxin-3 production in airway epithelium. *Am J Respir Cell Mol Biol* 2004; 31: 456–462.
- 37 Holz O, Mücke M, Paasch K, *et al.* Repeated ozone exposures enhance bronchial allergen responses in subjects with rhinitis or asthma. *Clin Exp Allergy* 2002; 32: 681–689.
- 38 Peden DB, Setzer RW Jr, Devlin RB. Ozone exposure has both a priming effect on allergen-induced responses and

- an intrinsic inflammatory action in the nasal airways of perennially allergic asthmatics. *Am J Respir Crit Care Med* 1995; 151: 1336–1345.
- 39** Harper RW, Xu C, Eiserich JP, *et al.* Differential regulation of dual NADPH oxidases/oxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett* 2005; 579: 4911–4917.
- 40** Katsifa H, Tsaparidou S, Diza E, Gil-Lamaignere C, Walsh TJ, Roilides E. Effects of interleukin-13 on antifungal activity of human monocytes against *Candida albicans*. *FEMS Immunol Med Microbiol* 2001; 31: 211–217.
- 41** Sozzani P, Cambon C, Vita N, *et al.* Interleukin-13 inhibits protein kinase c-triggered respiratory burst in human monocytes. Role of calcium and cyclic AMP. *J Biol Chem* 1995; 270: 5084–5088.
- 42** Suresh V, George SC. IL-13 induces formation of reactive oxygen species in lung epithelial cells. *Am J Respir Crit Care Med* 2007; 175: A175.
- 43** Huang CD, Tliba O, Panettieri RA Jr, Amrani Y. Bradykinin induces interleukin-6 production in human airway smooth muscle cells: modulation by Th2 cytokines and dexamethasone. *Am J Respir Cell Mol Biol* 2003; 28: 330–338.
- 44** Johnston RA, Schwartzman IN, Flynt L, Shore SA. Role of interleukin-6 in murine airway responses to ozone. *Am J Physiol Lung Cell Mol Physiol* 2005; 288: L390–L397.