

# Type I Interleukin-1 Receptor Is Required for Pulmonary Responses to Subacute Ozone Exposure in Mice

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Interleukin (IL)-1, a proinflammatory cytokine, is expressed in the lung after ozone (O<sub>3</sub>) exposure. IL-1 mediates its effects through the type I IL-1 receptor (IL-1RI), the only signaling receptor for both IL-1 $\alpha$  and IL-1 $\beta$ . The purpose of this study was to determine the role of IL-1RI in pulmonary responses to O<sub>3</sub>. To that end, wild-type, C57BL/6 (IL-1RI<sup>+/+</sup>) mice and IL-1RI-deficient (IL-1RI<sup>-/-</sup>) mice were exposed to O<sub>3</sub> either subacutely (0.3 ppm for 72 h) or acutely (2 ppm for 3 h). Subacute O<sub>3</sub> exposure increased bronchoalveolar lavage fluid (BALF) protein, interferon- $\gamma$ -inducible protein (IP)-10, soluble tumor necrosis factor receptor 1 (sTNFR1), and neutrophils in IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice. With the exception of IP-10, all outcome indicators were reduced in IL-1RI<sup>-/-</sup> mice. Furthermore, subacute O<sub>3</sub> exposure increased IL-6 mRNA expression in IL-1RI<sup>+/+</sup>, but not IL-1RI<sup>-/-</sup> mice. Acute (2 ppm) O<sub>3</sub> exposure increased BALF protein, IL-6, eotaxin, KC, macrophage inflammatory protein (MIP)-2, IP-10, monocyte chemoattractant protein-1, sTNFR1, neutrophils, and epithelial cells in IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice. For IL-6, eotaxin, MIP-2, and sTNFR1, there were small but significant reductions of these outcome indicators in IL-1RI<sup>-/-</sup> versus IL-1RI<sup>+/+</sup> mice at 6 hours after exposure, but not at other time points, whereas other outcome indicators were unaffected by IL-1RI deficiency. These results suggest that IL-1RI is required for O<sub>3</sub>-induced pulmonary inflammation during subacute O<sub>3</sub> exposure, but plays a more minor role during acute O<sub>3</sub> exposure. In addition, these results suggest that the induction of IL-6 via IL-1RI may be important in mediating the effects of O<sub>3</sub> during subacute exposure.

**Keywords:** bronchoalveolar lavage; interleukin-6; macrophage; neutrophil; soluble tumor necrosis factor receptor

Exposure to ozone (O<sub>3</sub>), a principal component of photochemical smog and a powerful oxidant, exacerbates symptoms of respiratory diseases, including asthma and cystic fibrosis (1, 2). In addition, in both developed and developing countries, elevations in ambient O<sub>3</sub> concentrations are associated with increased mortality (3–5). Consequently, it is important to understand the mechanistic basis by which the respiratory system responds to O<sub>3</sub>.

In humans, exposure to O<sub>3</sub> causes substernal irritation, cough, and decrements in pulmonary function (6, 7). In many species studied, O<sub>3</sub> causes airway hyperresponsiveness (AHR) to non-specific bronchoconstrictors, such as methacholine (MCh) (6, 8–

## CLINICAL RELEVANCE

IL-1RI, the only signaling receptor for IL-1 $\alpha$  and IL-1 $\beta$ , is important for pulmonary inflammatory responses to subacute O<sub>3</sub> exposure, likely through induction of IL-6. However, IL-1RI is less important for lung inflammatory responses to acute O<sub>3</sub> exposure.

10). Inhalation of O<sub>3</sub> also leads to pulmonary injury and inflammation, including epithelial cell sloughing, lung hyperpermeability, and neutrophil emigration into the airspaces (7, 8, 11–19). This inflammatory response is characterized by increased pulmonary expression of cytokines, including IL-6, as well as chemokines, including macrophage inflammatory protein (MIP)-2, KC, interferon- $\gamma$ -inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, and eotaxin. Indeed, in rodents, many of the pulmonary manifestations of O<sub>3</sub> exposure have been attributed to the effects of these cytokines and chemokines. For example, interruption of IL-6, IP-10, KC, and MIP-2 signaling significantly diminishes O<sub>3</sub>-induced neutrophil emigration to the airspaces and/or epithelial cell injury in mice (15, 18–20). Genetic deficiency in CXCR2, the receptor for KC and MIP-2, also reduces O<sub>3</sub>-induced increases in respiratory system responsiveness to MCh (19).

Interleukin-1 (IL-1 $\alpha$  and IL-1 $\beta$ ) is also increased in lung tissue and alveolar macrophages after O<sub>3</sub> exposure (16, 21–23), and exogenous administration of IL-1 has been shown to reproduce many of the characteristic responses to O<sub>3</sub>, including AHR, neutrophil emigration into the airspaces, and lung hyperpermeability (24, 25). Moreover, IL-1 has the capacity to induce the expression of many of the cytokines and chemokines (eotaxin, IL-6, IP-10, KC, MCP-1, and MIP-2) that are generated after O<sub>3</sub> exposure in the lung (26–31). Hence, it is possible that induction of IL-1 by O<sub>3</sub> leads to generation of these other cytokines and chemokines and to the ensuing pathology.

IL-1 $\alpha$  and IL-1 $\beta$  have two cell surface receptors to which they can bind, the type I and II IL-1 receptor (IL-1RI and IL-1RII). Intracellular signaling cascades resulting in NF- $\kappa$ B activation and gene transcription occur only after IL-1RI activation, whereas IL-1 binding to IL-1RII results in no signal generation (32–34). The role of IL-1RI in the inflammatory response appears to be dependent upon the specific stimulus used. For example, IL-1RI deficiency alone ameliorates tissue injury and/or inflammation induced during glomerulonephritis, hepatic ischemia and reperfusion, or the administration of turpentine (35–38). However, pulmonary inflammation induced by stimuli including *Escherichia coli*, *Streptococcus pneumoniae*, and lipopolysaccharide is not diminished by IL-1RI deficiency (39–41).

Based on these observations, the purpose of this study was to examine the hypothesis that IL-1RI contributes to O<sub>3</sub>-induced pulmonary pathophysiology and cytokine and chemokine expression after O<sub>3</sub> exposure. Park and colleagues recently reported that exogenous administration of an IL-1 receptor antagonist

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(IL-1Ra) attenuates O<sub>3</sub>-induced AHR, cytokine/chemokine expression, neutrophil emigration into the airspaces, and epithelial cell sloughing in mice (16). The authors studied responses to acute, high-dose O<sub>3</sub> exposure (2 ppm for 3 h), but did not examine the role of IL-1 in responses to lower concentrations of O<sub>3</sub> administered over a longer period of time (subacute exposure), an exposure protocol that more closely mimics typical environmental exposures. Kleeberger and colleagues have reported that murine responses to subacute and acute O<sub>3</sub> exposure are determined by separate genetic loci (42). Thus, there is reason to believe that pulmonary responses to subacute and acute O<sub>3</sub> exposure are differentially regulated. We sought to determine if IL-1RI contributed to either or both of these responses. Accordingly, we examined O<sub>3</sub>-induced pulmonary injury and inflammation after subacute O<sub>3</sub> exposure (0.3 ppm for 72 h) in wild-type, C57BL/6 (IL-1RI<sup>+/+</sup>) mice and in mice genetically deficient in IL-1RI (IL-1RI<sup>-/-</sup>). To confirm the results of Park and co-workers (16), we also examined the effect of acute O<sub>3</sub> exposure (2 ppm for 3 h) in IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice.

## MATERIALS AND METHODS

### Animals

Male and female mice genetically deficient in IL-1RI (IL-1RI<sup>-/-</sup>) (43) were maintained and bred in a rodent barrier facility at the Harvard School of Public Health (Boston, MA). Because these animals were backcrossed onto a C57BL/6 background for at least five generations, age- and sex-matched C57BL/6J (IL-1RI<sup>+/+</sup>) mice, purchased from The Jackson Laboratory (Bar Harbor, ME) at 7 weeks of age, were used as controls. All mice were housed in micro-isolator cages within the facility, where they were given food and water *ad libitum* and exposed to a 12-hour light-dark cycle. The Harvard Medical Area Standing Committee on Animals approved all of the experimental procedures used in this study.

### Protocol

To assess pulmonary injury and inflammation, separate cohorts of mice were subjected to either a subacute (0.3 ppm for 72 h) or acute (2 ppm for 3 h) O<sub>3</sub> exposure. For subacute exposures, mice were killed, bronchoalveolar lavage was performed, and the lungs were harvested within 30 minutes after cessation of the exposure. For acute exposures, the animals were studied 3, 6, or 24 hours after removal from the exposure chamber. Genotype-matched, air-exposed controls were subjected to the same experimental protocol as their O<sub>3</sub>-exposed counterparts. In some mice, to determine the effect of IL-1RI deficiency on O<sub>3</sub>-induced changes in the pattern of breathing, we measured breathing frequency and end-expiratory pause (EEP) before and after the cessation of either subacute or acute O<sub>3</sub> exposure by placing the animals in a whole-body plethysmograph (Buxco Electronics, Inc., Wilmington, NC), as described previously (13, 14).

### O<sub>3</sub> Exposure

IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice received either a subacute (72 h) or an acute (3 h) O<sub>3</sub> exposure. For subacute (72 h) exposure, the entire micro-isolator cage, with the exception of the micro-isolator top, containing conscious IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice was placed inside a 145-L stainless steel and Plexiglas chamber, where the animals were exposed to 0.3 ppm O<sub>3</sub>. During subacute exposure, the animals had continuous access to food and water. For acute (3 h) O<sub>3</sub> exposure, conscious mice were placed in individual wire mesh cages inside the exposure chamber and exposed to 2 ppm O<sub>3</sub>. The O<sub>3</sub> doses and exposure times were chosen to allow comparison with other studies in mice (16, 17, 44–49). Furthermore, the nomenclature used to describe these O<sub>3</sub> exposure regimens, acute and subacute, has been extensively used by others (16, 17, 44–49). For room air exposures, the experimental protocol was identical to the corresponding O<sub>3</sub> exposure, except that a separate and identical exposure chamber dedicated to room air exposure was used. Exposure chamber conditions and O<sub>3</sub> generation and monitoring were previously described (19).

### Bronchoalveolar Lavage

The animals were prepared for bronchoalveolar lavage (BAL) as previously described (19). The lungs were lavaged twice with 1 ml of ice-cold lavage buffer, PBS containing 0.6 mM EDTA. During each lavage, the lavage buffer was instilled and retrieved twice, and both lavagates subsequently pooled and stored on ice. The lavagate was spun, the supernatant collected and stored at –80°C, and the total BAL fluid (BALF) cells and differentials were determined as previously described (19). The total BALF protein concentration was determined spectrophotometrically according to the Bradford protein assay procedure (Bio-Rad Laboratories, Hercules, CA). The concentrations of BALF eotaxin, IP-10, IL-6, KC, MCP-1, MIP-2, and soluble tumor necrosis factor receptor 1 (sTNFR1) were determined with either enzyme-linked immunosorbent assay (ELISA) kits or DuoSet ELISA development systems (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. These outcome indicators were measured because their levels increase in BALF after O<sub>3</sub> exposure and/or their expression has been reported to be regulated by IL-1 (18, 26–31). Consequently, it is plausible that their expression is altered by IL-1RI deficiency after O<sub>3</sub> exposure. Finally, because there were no significant differences in any of the BALF outcome indicators examined in mice exposed to room air for 3 versus 72 hours, the data for all air-exposed mice were pooled.

### RNA Extraction and Real-Time Reverse-Transcription PCR

For measurement of IL-1β and IL-1α mRNA expression, RNA was extracted from frozen lung tissue and purified, and reverse transcription (RT) performed as previously described (18, 50, 51). RT reactions were diluted with 30 μl of water and stored at –80°C. Quantitative real-time RT-PCR was performed using an iCycler iQ real-time PCR detection system and iQ SYBR Green supermix in accordance with the manufacturer's instructions (Bio-Rad Laboratories). Primer sets and product sizes for murine IL-1β and β-actin were previously described (18, 50). Primer sets and the product size for IL-1α were as follows: IL-1α (forward, 5'-CAA ACT GAT GAA GCT CGT CA-3' and reverse, 5'-TCT CCT TGA GCG CTC ACG AA-3' [225 bp]) (52). Generation of positive controls for construction of standard curves was performed as previously described (50). For each gene, analyzed, melting curve analysis yielded a single peak consistent with one PCR product. For IL-1β and IL-1α, changes in mRNA expression in response to O<sub>3</sub> exposure were assessed relative to changes in β-actin mRNA transcript copy number. There was no difference in β-actin levels between room air- and O<sub>3</sub>-exposed mice.

For measurement of IL-6 and ICAM-1 mRNA, total lung RNA was extracted and purified using the RNeasy miniprotocol (Qiagen, Valencia, CA) and RNase-free DNase set (Qiagen). Real-time RT-PCR was performed using the iScript one-step RT-PCR kit for probes (Bio-Rad Laboratories) and the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories). Primers and TaqMan probes were designed using the Beacon Designer software (PREMIER Biosoft International; Palo Alto, CA). The primers used were previously described (41). Fold-induction values (versus IL-1RI<sup>+/+</sup>, room-air exposed mice) were normalized to the content of 18S rRNA (53).

### Statistical Analysis

The effect of IL-1RI deficiency and O<sub>3</sub> on mRNA levels and BALF parameters, were assessed by factorial ANOVA. For the analyses on BALF cells, values were logarithmically transformed to conform to a normal distribution. The effect of O<sub>3</sub> and IL-1RI deficiency on the pattern of breathing was assessed by repeated measures ANOVA. STATISTICA software (StatSoft, Tulsa, OK) was used to perform all statistical analyses. The results are expressed as the arithmetic mean ± SEM, except for BALF cells, which are expressed as the geometric mean ± SEM. *n* is the number of mice per treatment group. A *P* value less than 0.05 was considered significant.

## RESULTS

### Effect of Subacute and Acute O<sub>3</sub> Exposure on IL-1α and IL-1β mRNA Expression

Real-time RT-PCR was used to determine IL-1α and IL-1β mRNA expression in the lungs of wild-type (IL-1RI<sup>+/+</sup>) mice

after room air or O<sub>3</sub> exposures (Figure 1). Transcripts for both IL-1 $\alpha$  and IL-1 $\beta$  were expressed in the lungs of air-exposed, wild-type (IL-1RI<sup>+/+</sup>) mice. Subacute O<sub>3</sub> exposure (0.3 ppm for 72 h) significantly increased the mRNA levels of both IL-1 $\alpha$  and IL-1 $\beta$  when compared with their respective, air-exposed controls. After acute exposure to 2 ppm O<sub>3</sub> for 3 hours, IL-1 $\alpha$  mRNA expression was significantly increased only 24 hours after the cessation of O<sub>3</sub> exposure, while IL-1 $\beta$  mRNA expression was unchanged at any time point.

#### Effect of IL-1RI Deficiency on Pulmonary Injury and Inflammation after Subacute O<sub>3</sub> Exposure

To assess the impact of IL-1RI deficiency on pulmonary injury and inflammation after subacute O<sub>3</sub> exposure (0.3 ppm for 72 h), we examined BALF outcome indicators from IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice. After room air exposure, the levels of BALF sTNFR1 as well as the number of BALF macrophages were significantly reduced in IL-1RI<sup>-/-</sup> versus IL-1RI<sup>+/+</sup> mice (Figure 2). Subacute O<sub>3</sub> exposure (0.3 ppm for 72 h) caused a significant increase in the concentrations of BALF protein, IP-10, sTNFR1, and the number of BALF neutrophils in both genotypes (Figure 2). However, the levels of BALF protein and sTNFR1 as well as the number of BALF neutrophils were significantly reduced in IL-1RI<sup>-/-</sup> compared with IL-1RI<sup>+/+</sup> mice. Subacute O<sub>3</sub> exposure also caused an increase in BALF macrophages in IL-1RI<sup>+/+</sup>, whereas no change was observed in IL-1RI<sup>-/-</sup> mice. There was no effect of this O<sub>3</sub> exposure regimen or of genotype on the number of BALF epithelial cells. As we have previously reported that this O<sub>3</sub> exposure regimen does not produce detectable levels of BALF IL-6 or KC (18), their levels were not measured.

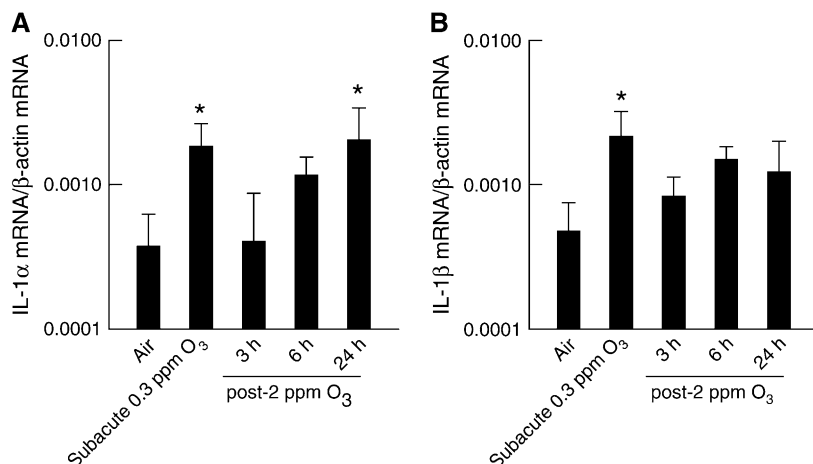
After subacute O<sub>3</sub> exposure, BALF protein, sTNFR1, and neutrophils were reduced in IL-1RI<sup>-/-</sup> versus IL-1RI<sup>+/+</sup> mice. Qualitatively similar results were observed in IL-6<sup>-/-</sup> versus IL-6<sup>+/+</sup> mice after exposure to the same O<sub>3</sub> exposure regimen (0.3 ppm O<sub>3</sub> for 72 h) (18). Because IL-1 has the capacity to stimulate IL-6 expression (54), it is conceivable that the results observed in IL-1RI<sup>-/-</sup> mice were due to a reduced ability of IL-1RI<sup>-/-</sup> mice to produce IL-6. To examine this possibility, we measured IL-6 mRNA expression in IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice after room air and subacute O<sub>3</sub> exposure (Figure 3A). (Note that BALF IL-6 levels after these exposures are below the limit of detection of our assay [18].) After room air exposure, there was no difference in IL-6 mRNA expression between IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice. However, subacute O<sub>3</sub>

exposure significantly increased IL-6 mRNA expression in IL-1RI<sup>+/+</sup> but had no effect on IL-6 expression in IL-1RI<sup>-/-</sup> mice. In addition, intercellular adhesion molecule-1 (ICAM-1) expression is important in the migration of neutrophils to sites of inflammation in some settings (55). However, there was no effect of genotype or exposure on ICAM-1 mRNA expression (Figure 3B). Thus, IL-6, but not ICAM-1, was induced by subacute O<sub>3</sub> exposure, and IL-6 expression required IL-1RI.

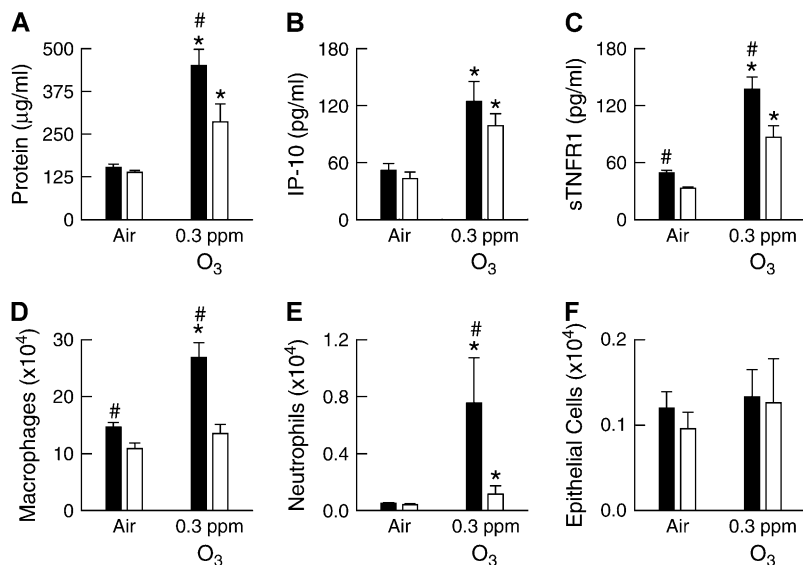
#### Effect of IL-1RI Deficiency on Pulmonary Injury and Inflammation after Acute O<sub>3</sub> Exposure

To assess the impact of IL-1RI deficiency on acute (2 ppm for 3 h) O<sub>3</sub> exposure, we measured various BALF outcome indicators in IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice 3, 6, and 24 hours after cessation of O<sub>3</sub> exposure (Figures 4 and 5). For IL-1RI<sup>+/+</sup> mice, the response to O<sub>3</sub> followed two types of time courses depending on the outcome indicator. For IL-6, eotaxin, KC, MIP-2, and IP-10, BALF levels were increased above air-exposed controls at 3 and/or 6 hours after the cessation of O<sub>3</sub> exposure and then began to resolve by 24 hours after exposure (Figure 4). For protein, MCP-1, and sTNFR1, levels after O<sub>3</sub> exposure were elevated above air-exposed controls at either 3 and/or 6 hours after exposure and continued to remain elevated or even increased further at 24 hours after exposure. Compared with IL-1RI<sup>+/+</sup> mice, IL-1RI deficiency resulted in decreased BALF sTNFR1 both in air- and O<sub>3</sub>-exposed mice. IL-1RI deficiency also resulted in a decrease in BALF IL-6, eotaxin, and MIP-2 6 hours after cessation of O<sub>3</sub> but not at other times. There was no effect of IL-1RI deficiency on O<sub>3</sub>-induced changes in protein, KC, IP-10, or MCP-1.

In IL-1RI<sup>+/+</sup> mice, BALF macrophages transiently decreased at 3 and 6 hours after O<sub>3</sub> exposure before returning to pre-exposure levels at 24 hours after exposure (Figure 5), likely reflecting increased activation of macrophages and increased adhesion, making them less easy to wash out of the lungs. In contrast, BALF neutrophils and epithelial cells increased continuously from 3 to 24 hours after O<sub>3</sub> exposure. IL-1RI deficiency resulted in a small, but significant, decrease in BALF macrophages in air-exposed mice, as noted earlier. A similar trend was observed at each time point after O<sub>3</sub> exposure, but was significant only at 3 hours after exposure. IL-1RI deficiency did not reduce O<sub>3</sub>-induced changes in BALF neutrophils or epithelial cells. In fact, at 6 hours after O<sub>3</sub> exposure, BALF neutrophils were actually increased in IL-1RI<sup>-/-</sup> versus IL-1RI<sup>+/+</sup> mice.



**Figure 1.** (A) IL-1 $\alpha$  and (B) IL-1 $\beta$  mRNA expression in lung tissue from room air- or O<sub>3</sub>-exposed wild-type (C57BL/6) mice. Lungs were harvested 30 minutes after cessation of exposure to 0.3 ppm O<sub>3</sub> for 72 hours, or 3, 6, or 24 hours after cessation of exposure to 2 ppm O<sub>3</sub> for 3 hours. Lungs from air-exposed controls were obtained at the same time. mRNA transcript copy number was normalized to  $\beta$ -actin transcript copy number.  $n = 7-13$  mice for each group. \* $P < 0.05$  compared with air-exposed controls.



**Figure 2.** The concentrations of protein, interferon- $\gamma$ -inducible protein (IP)-10, soluble tumor necrosis factor receptor 1 (sTNFR1), as well as the total number of macrophages, neutrophils, and epithelial cells in the bronchoalveolar lavage fluid (BALF) of wild-type, C57BL/6 (IL-1RI<sup>+/+</sup>; solid bars) and IL-1RI-deficient (IL-1RI<sup>-/-</sup>; open bars) mice 30 minutes after the cessation of a 72-hour exposure to either room air or 0.3 ppm ozone (O<sub>3</sub>).  $n = 8-26$  for each treatment group. \* $P < 0.05$  compared with genotype-matched, air-exposed controls. # $P < 0.05$  compared with IL-1RI<sup>-/-</sup> mice within the same exposure group.

### Effect of IL-1RI Deficiency on O<sub>3</sub>-Induced Changes in the Pattern of Breathing

We have previously reported that exposure of mice to 2 ppm O<sub>3</sub> for 3 hours results in a decrease in minute ventilation, particularly a marked reduction in breathing frequency resulting from an increased end-expiratory pause (EEP) (14). Genotype-related differences in the effect of O<sub>3</sub> on breathing pattern could affect the inhaled dose of O<sub>3</sub>, since this dose is the product of minute ventilation, O<sub>3</sub> concentration, and duration of exposure (56). Consequently, we used whole-body plethysmography to examine O<sub>3</sub>-induced changes in breathing frequency and EEP in IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice. There was no difference in breathing frequency or EEP in air-exposed IL-1RI<sup>+/+</sup> versus IL-1RI<sup>-/-</sup> mice (Table 1). Subacute O<sub>3</sub> exposure had no effect on breathing pattern in mice of either genotype (Table 1). Breathing frequency was significantly decreased, and EEP was significantly increased in the 3-hour period immediately following cessation of exposure to 2 ppm O<sub>3</sub> (Table 1), but there was no effect of genotype on either of these outcome indicators. Thus, it is unlikely that differences in the pattern of breathing account for the genotype-related differences in pulmonary injury and inflammation observed between IL-1RI<sup>-/-</sup> and IL-1RI<sup>+/+</sup> mice.

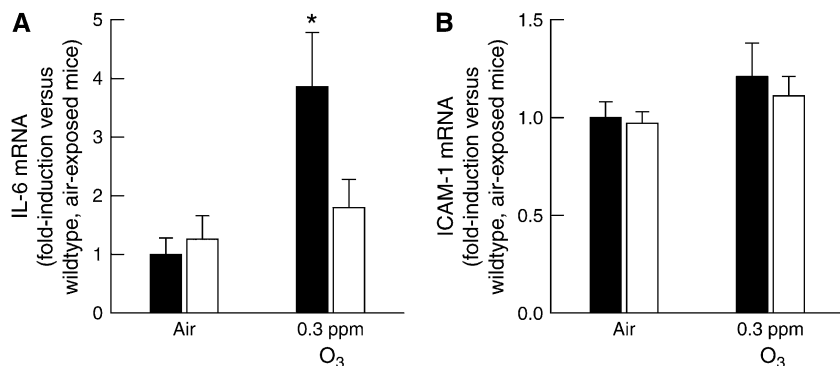
### DISCUSSION

The results of this study indicate that deficiency of IL-1RI, the only signaling receptor for both IL-1 $\alpha$  and IL-1 $\beta$ , attenuates O<sub>3</sub>-

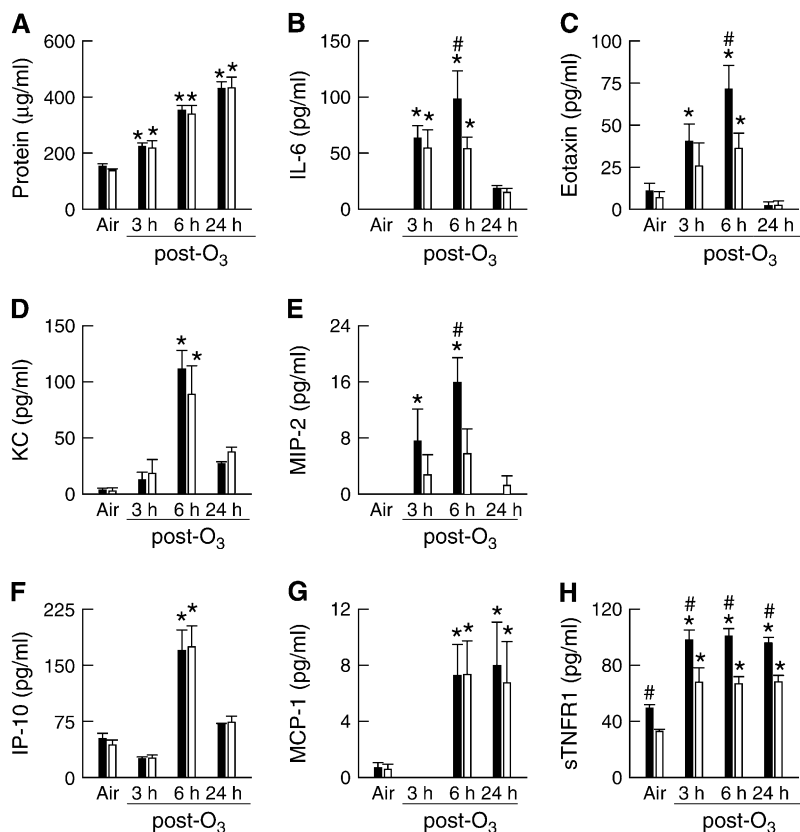
induced pulmonary injury and inflammation after subacute O<sub>3</sub> exposure (0.3 ppm for 72 h) (Figure 2), while it has minimal effects on pulmonary injury and inflammation after acute O<sub>3</sub> exposure (Figures 4 and 5). Furthermore, our data suggest that the amelioration of O<sub>3</sub>-induced pulmonary injury and inflammation in IL-1RI<sup>-/-</sup> mice may be due to reduced IL-6 expression in these animals.

Both IL-1 $\alpha$  and IL-1 $\beta$  mRNA were expressed in the lungs of wild-type (IL-1RI<sup>+/+</sup>) mice after room air exposure (Figure 1). Changes in the expression of both IL-1 $\alpha$  and IL-1 $\beta$  mRNA occurred with O<sub>3</sub> exposure, but the effects were dependent on the nature of the O<sub>3</sub> exposure regimen. For example, expression of IL-1 $\alpha$  mRNA increased after both subacute and acute O<sub>3</sub> exposure, whereas expression of IL-1 $\beta$  increased only after subacute O<sub>3</sub> exposure. Consistent with these results, we have previously reported that acute O<sub>3</sub> exposure (2 ppm for 3 h) had no effect on IL-1 $\beta$  mRNA expression measured 4 or 24 hours after the cessation of exposure (50). Macrophages are one source of IL-1 induced by O<sub>3</sub> exposure (21), but other cells, including epithelial and smooth muscle cells, have the capacity to produce IL-1 (57-61), and it is conceivable that IL-1 is released by different cell types during acute versus subacute O<sub>3</sub> exposure, accounting for the differences in IL-1 expression.

After subacute O<sub>3</sub> exposure, IL-1RI deficiency significantly attenuated the levels of BALF protein and sTNFR1 as well as the number of BALF macrophages and neutrophils (Figure 2), suggesting that lung hyperpermeability and pulmonary inflammation are dependent upon IL-1RI signaling under these



**Figure 3.** (A) IL-6 and (B) intercellular adhesion molecule (ICAM)-1 mRNA expression in lung tissue from room air- or O<sub>3</sub>-exposed, wild-type C57BL/6 (IL-1RI<sup>+/+</sup>; solid bars) and IL-1RI-deficient (IL-1RI<sup>-/-</sup>; open bars) mice. Lungs were harvested 30 minutes after cessation of exposure to 0.3 ppm O<sub>3</sub> for 72 hours. Lungs from air-exposed controls were obtained at the same time. mRNA levels for each treatment group were normalized for 18S rRNA and expressed as the fold-induction of the values determined for air-exposed, wild-type C57BL/6 (IL-1RI<sup>+/+</sup>) mice.  $n = 8-10$  mice for each group. \* $P < 0.05$  compared with air-exposed controls.



**Figure 4.** The concentrations of protein, IL-6, eotaxin, KC, macrophage inflammatory protein (MIP)-2, IP-10, MCP-1, and sTNFR1 in the BALF of wild-type, C57BL/6 (IL-1RI<sup>+/+</sup>; solid bars) and IL-1RI-deficient (IL-1RI<sup>-/-</sup>; open bars) mice 3, 6, or 24 hours after the cessation of a 3-hour exposure to either room air or 2 ppm ozone (O<sub>3</sub>). The concentrations of BALF protein, IP-10, and sTNFR1 from air-exposed mice are identical to those found in Figure 2 and are included here to demonstrate the effect of O<sub>3</sub> on our outcome indicators. *n* = 5–26 for each treatment group. \**P* < 0.05 compared with genotype-matched, air-exposed controls. #*P* < 0.05 compared with IL-1RI<sup>-/-</sup> mice within the same exposure group.

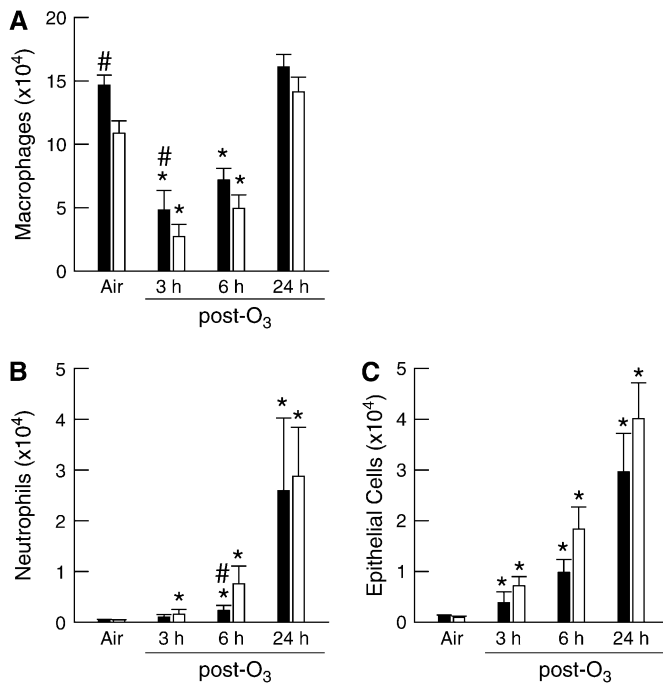
exposure conditions. We have previously reported that IL-6 deficiency, like IL-1RI deficiency, also diminishes subacute O<sub>3</sub>-induced increases in BALF protein, sTNFR1, and the number of BALF neutrophils (18). IL-1 has the capacity to stimulate IL-6 expression (54), and we have shown here that IL-1RI deficiency has the capacity to reduce the expression of IL-6 after subacute O<sub>3</sub> exposure (Figure 3). Thus, it is conceivable that in the setting of subacute O<sub>3</sub> exposure, the role of IL-1 may be to induce IL-6 expression and/or release, leading to reductions in the effects of IL-6 in IL-1RI<sup>-/-</sup> animals, rather than by IL-1 directly impacting outcome indicators such as BALF protein and neutrophils.

There were also small, but significant, decreases in the levels of BALF sTNFR1 and the number of BALF macrophages in IL-1RI<sup>-/-</sup> mice exposed to room air. These results suggest that IL-1RI influences either the expression and/or membrane cleavage of sTNFR1 in the lung in addition to the recruitment of macrophages to the airspaces under baseline conditions. However, the factor(s) mediating these effects are not known.

In contrast to subacute O<sub>3</sub> exposure, our data suggest that IL-1RI signaling has a much more limited role in the pulmonary injury and inflammation observed following acute (3 h) O<sub>3</sub> exposure. IL-6, eotaxin, and MIP-2 were significantly decreased in IL-1RI<sup>-/-</sup> mice versus IL-1RI<sup>+/+</sup> mice (Figure 4), but the effects were only observed 6 hours after exposure and not at other time points, and changes in other inflammatory outcomes, including lung hyperpermeability, neutrophil recruitment, epithelial cell sloughing, KC, MCP-1, and IP-10, occurred independently of IL-1RI signaling. From the data presented, we cannot conclude whether the reductions in IL-6, eotaxin, and MIP-2 are a direct or an indirect result of IL-1RI deficiency. Curiously, even though there was evidence for a role of IL-1RI in the effects of 2 ppm O<sub>3</sub> on IL-6, eotaxin, and MIP-2 at 6

hours, neither IL-1 $\alpha$  nor IL-1 $\beta$  mRNA expression was increased by O<sub>3</sub> exposure at this time. However, there was mRNA expression of both cytokines even in the air-exposed mice, suggesting that basal expression of IL-1 may be acting as a co-stimulus with some other factor that is induced by O<sub>3</sub>. We have also observed increased pulmonary IL-1RI mRNA expression in mice 4 hours after acute O<sub>3</sub> exposure (2 ppm for 3 h) (unpublished microarray observations from our laboratory), a finding consistent with those of Park and colleagues (16). Thus, differences in the acute inflammatory response to O<sub>3</sub> between IL-1RI<sup>+/+</sup> and IL-1RI<sup>-/-</sup> mice (Figures 4 and 5) may be the result of basal levels of IL-1 signaling through increased IL-1RI. In addition, even though changes in mRNA were not observed at 6 hours (Figure 1), IL-1 $\alpha$  and IL-1 $\beta$  protein levels could be elevated—for example, via changes in proteolytic or post-transcriptional processing and/or secretion. We did attempt to measure the levels of IL-1 $\alpha$  and IL-1 $\beta$  in BALF after O<sub>3</sub> exposure, but the levels of both cytokines were below the detection limits of our assay.

In contrast to the minimal role for IL-1RI in the response to acute 2 ppm O<sub>3</sub> exposure observed in this study, Park and colleagues have reported that exogenous administration of IL-1 $\alpha$  attenuates increases in lung KC, MIP-2, TNF- $\alpha$ , epithelial cell sloughing, and neutrophils induced by the same exposure protocol (16). From these data, Park and colleagues concluded that IL-1 plays a substantial role in mediating pulmonary responses to acute 2 ppm O<sub>3</sub> exposure. Park and colleagues reported increased expression of IL-1 $\beta$ , not IL-1 $\alpha$ , mRNA with this protocol (16), whereas we observed the opposite (Figure 1). We do not know what accounts for this difference, but it is possible that the results reflect differences in other exposure conditions. Notably, the study of Park and coworkers (16) was performed at a high altitude (Denver, CO). It is conceivable



**Figure 5.** The total number of macrophages, neutrophils, and epithelial cells in the BALF of wild-type, C57BL/6 (IL-1RI<sup>+/+</sup>; solid bars) and IL-1RI-deficient (IL-1RI<sup>-/-</sup>; open bars) mice 3, 6, or 24 hours after the cessation of a 3-hour exposure to either room air or 2 ppm ozone (O<sub>3</sub>). The data shown here from air-exposed mice are identical to those found in Figure 2 and are included here to demonstrate the effect of O<sub>3</sub> on our outcome indicators.  $n = 8-26$  for each treatment group. \* $P < 0.05$  compared with genotype-matched, air-exposed controls. # $P < 0.05$  compared with IL-1RI<sup>-/-</sup> mice within the same exposure group.

that hypoxia *per se* or other climatic conditions (low humidity) may have contributed to the different outcomes. Furthermore, it is possible that the functional differences observed between our study and the study of Park and colleagues are a result of differences in IL-1 expression. That is, we observed an increase in IL-1 $\alpha$  expression after acute (3 h) 2 ppm O<sub>3</sub> exposure, whereas Park and colleagues observed an increase in IL-1 $\beta$  expression (16). The IL-1-dependent functional changes observed after O<sub>3</sub> exposure in the study of Park and coworkers may only be dependent on increased IL-1 $\beta$  expression. Such changes would not be manifest in our study since we observed an increase in only IL-1 $\alpha$ , and not IL-1 $\beta$ , expression.

**TABLE 1. EFFECT OF IL-1RI DEFICIENCY ON THE PATTERN OF BREATHING FOLLOWING SUBACUTE AND ACUTE ROOM AIR OR O<sub>3</sub> EXPOSURE**

Genotype (Exposure)	Breathing Frequency (breaths/min)	End-Expiratory Pause (ms)
IL-1RI <sup>+/+</sup> (air)	345 ± 15	40.4 ± 5.2
IL-1RI <sup>-/-</sup> (air)	367 ± 13	29.4 ± 3.1
IL-1RI <sup>+/+</sup> (subacute 0.3 ppm O <sub>3</sub> )	411 ± 12	25.9 ± 1.9
IL-1RI <sup>-/-</sup> (subacute 0.3 ppm O <sub>3</sub> )	359 ± 11	32.1 ± 5.3
IL-1RI <sup>+/+</sup> (acute 2 ppm O <sub>3</sub> )	159 ± 15*	236.7 ± 35.4*
IL-1RI <sup>-/-</sup> (acute 2 ppm O <sub>3</sub> )	161 ± 16*	223.0 ± 37.2*

Results are the mean ± SEM for 7–24 mice in each group. Measurements were made between 30 and 45 minutes after the cessation of subacute room air or O<sub>3</sub> (0.3 ppm) exposure and between 0.5 and 3 hours after the cessation of acute room air or O<sub>3</sub> (2 ppm) exposure.

\*  $P < 0.05$  compared with genotype-matched, air-exposed controls.

As described above, we observed only a very limited role of IL-1RI in the pulmonary injury and inflammation observed after acute, high-dose O<sub>3</sub> exposure (Figures 4 and 5). Previous reports also rule out major contributions from IL-6, TNF- $\alpha$ , and Toll-like receptor 4 in acute inflammatory responses to 2 ppm O<sub>3</sub> (14, 17, 18, 47). Although abrogation of any one of these mediators alone has minimal or no effects on O<sub>3</sub>-induced pulmonary injury and inflammation, it is possible that they may be acting in concert to produce the inflammatory effects of O<sub>3</sub>. Supporting this postulate, whereas interrupting signaling from either IL-1 or TNF- $\alpha$  fails to compromise neutrophil recruitment elicited by *Escherichia coli*, *Streptococcus pneumoniae*, and lipopolysaccharide in the lungs, the combined interruption of both IL-1 and TNF- $\alpha$  signaling significantly decreases neutrophil recruitment elicited by any of these three stimuli (39, 62–64).

In conclusion, our data indicate that IL-1RI contributes prominently to the pulmonary inflammation observed after subacute O<sub>3</sub> exposure (0.3 ppm for 72 h), whereas its role declines during acute (2 ppm) O<sub>3</sub> exposure. Furthermore, IL-1RI induction of IL-6 may contribute to the pulmonary inflammation induced during subacute exposure.

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