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Pulmonary Inflammation Induced by Subacute Ozone Is Augmented in Adiponectin-Deficient Mice: Role of IL-17A

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Pulmonary responses to ozone, a common air pollutant, are augmented in obese individuals. Adiponectin, an adipose-derived hormone that declines in obesity, has regulatory effects on the immune system. To determine the role of adiponectin in the pulmonary inflammation induced by extended (48–72 h) low-dose (0.3 parts per million) exposure to ozone, adiponectin-deficient (Adipo−/−) and wild-type mice were exposed to ozone or to room air. In wild-type mice, ozone exposure increased total bronchoalveolar lavage (BAL) neutrophils. Ozone-induced lung inflammation, including increases in BAL neutrophils, protein (an index of lung injury), IL-6, keratinocyte-derived chemokine, LPS-induced CXC chemokine, and G-CSF were augmented in Adipo−/− versus wild-type mice. Ozone also increased IL-17A mRNA expression to a greater extent in Adipo−/− versus wild-type mice. Moreover, compared with control Ab, anti–IL-17A Ab attenuated ozone-induced increases in BAL neutrophils and G-CSF in Adipo−/− but not in wild-type mice, suggesting that IL-17A, by promoting G-CSF release, contributed to augmented neutrophilia in Adipo−/− mice. Flow cytometric analysis of lung cells revealed that the number of CD45+/F4/80+/IL-17A+ macrophages and γ6 T cells expressing IL-17A increased after ozone exposure in wild-type mice and further increased in Adipo−/− mice. The IL-17+ macrophages were CD11c− (interstitial macrophages), whereas CD11c+ macrophages (alveolar macrophages) did not express IL-17A. Taken together, the data are consistent with the hypothesis that adiponectin protects against neutrophil recruitment induced by extended low-dose ozone exposure by inhibiting the induction and/or recruitment of IL-17A in interstitial macrophages and/or γ6 T cells. The Journal of Immunology, 2012, 188: 000–000.

A utomobile exhaust is a source of many toxic gases and particles, including ozone. Inhalation of ozone has a significant impact on human health and contributes to increased cardiovascular and respiratory mortality (1, 2). In the lung, ozone induces epithelial injury and an inflammatory response that includes a neutrophilic influx and induction of acute-phase cytokines including IL-1, IL-6, TNF-α, as well as the neutrophil chemotactic factors keratinocyte-derived chemokine (KC), MIP-2, and LPS-induced CXC chemokine (LIX) (3–12). Ozone is a trigger for asthma attacks and significantly decreases pulmonary function in asthmatic subjects (13–15). Importantly, responses to ozone are augmented in obese and overweight individuals (16, 17).

Circulating levels of adiponectin, an adipose-derived, energy regulating hormone with anti-inflammatory effects, are reduced in obese (18–20). Such declines in adiponectin contribute to many obesity-related conditions, including insulin resistance and hypertension (21, 22). Similarly, loss of the anti-inflammatory effects of adiponectin may contribute to obesity-related increases in responses to ozone. For example, macrophages are an important target cell for ozone (4, 23), and TNF-α has been shown to be required for the pulmonary neutrophilia caused by ozone (24). Adiponectin decreases LPS-induced TNF-α expression in macrophages (25, 26) while augmenting expression of anti-inflammatory molecules such as IL-10 and IL-1Ra (27) and skew macrophages from an M1 to an M2 phenotype (28). Consistent with these observations, adiponectin receptors are expressed on most circulating monocytes (29). The ozone-induced influx of neutrophils into the lungs also requires adhesion of neutrophils to endothelial cells (30), and adiponectin has been shown to enhance TNF-α-induced expression of VCAM-1, E-selectin, and ICAM-1 on endothelial cells (31). Anti-inflammatory effects of adiponectin have also been demonstrated in the lung in vivo: exogenous administration of adiponectin attenuates allergic inflammation in mice (32), whereas adiponectin deficiency augments it (33). Nevertheless, proinflammatory effects of adiponectin have also been reported (34–36). Adiponectin circulates in multimeric forms: a trimer, a hexamer composed of two trimers linked by cysteine bonds in the tails, and a high-m.w. (HMW) form composed of 12–18 monomers. These isoforms differ in their bioactivity and degradation (19, 37–39). The pro- or anti-inflammatory effects of adiponectin may depend on which adiponectin isoform is present.

If obesity-related declines in adiponectin contribute to the augmented effects of ozone in the obese, we would expect the anti-inflammatory effects of adiponectin to dominate in the setting of ozone exposure. Consequently, adiponectin deficiency would be expected to augment ozone-induced inflammation. In contrast, we...
have reported that adiponectin-deficient (Adipo−/−) mice exposed to acute ozone (2 ppm [parts per million] for 3 h) had decreased neutrophilic inflammation and decreased induction of cytokines and chemokines compared with wild-type mice (40). The acute ozone exposure regimen is frequently used (11, 12, 40–43), because it induces a robust response in mice, which, as with other rodents, have a reduced sensitivity to ozone compared with humans (44). However, elevated atmospheric ozone tends to persist not for hours but for several days or even weeks, the time scale of typical weather patterns (45). Kleeberger et al. (7) have developed a “subacute” model where mice are exposed to lower concentrations (0.3 ppm) for longer periods of time (48–72 h), and many investigators use this as a more realistic model of ozone exposure (3, 6, 7, 11, 46). Importantly, the factors that determine pulmonary responses to short-duration high-dose (acute) ozone exposure to ozone are not the same as those that impact responses to longer lower dose (subacute) ozone (3, 8, 24, 47). For example, TNF-α is required for the pulmonary neutrophilia induced by subacute but not acute ozone exposure in mice (3, 8, 48). Such differences suggest that the impact of adiponectin deficiency on pulmonary responses to subacute versus acute ozone exposure might be different. Therefore, we exposed wild-type and Adipo−/− mice to ozone (0.3 ppm during 24–72 h) or to air and examined pulmonary inflammation and injury. Our data derived using subacute exposure model demonstrated increased ozone-induced inflammation in Adipo−/− mice, indicating that anti-inflammatory activities of adiponectin do indeed dominate under these prolonged exposure conditions.

IL-17A is involved in neutrophil recruitment to the lung by both infectious and noninfectious agents (49–51). In particular, we have reported that IL-17A is induced in the lung by following three exposures to ozone (1 ppm for 3 h) with 2-d intervals between exposures (51). Importantly, blocking IL-17A, either by genetic deletion or via neutralizing Abs, inhibited the neutrophil influx induced by ozone in this model (51). A source of IL-17A in this situation was invariant NKT cells. To determine whether IL-17A also contributes to the augmented inflammation observed following subacute ozone in Adipo−/− mice, we treated mice with IL-17A neutralizing Abs.

**Materials and Methods**

**Animals**

Adipo−/− mice from Dr. Y. Matsuzawa (Sumitomo Hospital, Osaka, Japan) (52) were bred in-house. Gender- and age-matched C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were used as wild-type controls. All protocols were approved by the Harvard Medical Area Standing Committee on Animals. Mice were 11–13 wk old at the time of exposure.

**Ozone exposure**

Mice were housed in microisolator cages, with their HEPA filter removed, and given with water and food ad libitum. The cages were then placed in a 145-l stainless steel and Plexiglas exposure chamber. Mice were exposed for up to 72 h to 0.3 ppm ozone as described previously (6, 7). Ozone was generated from medical grade oxygen by an in-house electric ozone generator. Ozone levels were continuously monitored as described before (40). Air-exposed mice were exposed concurrently, in a separate, but identical chamber.

**Protocols**

In the first cohort of experiments, Adipo−/− and wild-type mice were exposed to air or 0.3 ppm ozone for up to 72 h (subacute ozone). At the end of exposure, mice were euthanized with an overdose of pentobarbital. The chest cavity was opened, venous blood was drawn by a cardiac puncture of the right ventricle, and serum was prepared. The trachea was then cannulated, and bronchoalveolar lavage (BAL) was performed using two instillations of 1 ml PBS (pH 7.4). The left lung lobe was harvested and immersed in RNA later solution (Qiagen, Valencia, CA) for 24 h and then transferred to a −20˚C freezer until isolation of total RNA was performed. In the second set of experiments, Adipo−/− and wild-type mice were treated with an i.p. injection of 100 μg in 100 μl of either anti–IL-17 neutralizing Ab (Rat IgG2A, clone 50104, MAB421; R&D Systems, Minneapolis, MN) or isotype control Ab (clone 54447, MAB006; R&D Systems) resuspended in sterile saline. After 30 min, mice were exposed to 0.3 ppm ozone for 48 h, and then blood, BAL fluid, and lungs were harvested as described above. In the third series of experiments, Adipo−/− and wild-type mice were exposed to ozone or air for 72 h. The animals were then euthanized, the lungs were harvested, and the cells were isolated for flow cytometry to identify cellular sources of IL-17A.

**Bronchoalveolar lavage**

BAL fluid was spun at 1500 rpm at 4˚C. The cell pellet was resuspended with 1 ml PBS, and total cells were counted using a hemocytometer. Cytospin preparations were obtained, stained with H&E, and used to obtain differential cell counts on at least 300 cells. BAL fluid was aliquoted and stored at −80˚C until assayed for total protein (a measure of ozone-induced lung epithelial injury), adiponectin, and inflammatory cytokines and chemokines.

**ELISA**

Total adiponectin, MIP-2, KC, IL-1 receptor antagonist (IL-1Ra), IL-6, TNF-α, G-CSF, and LIX in the BAL fluid or serum was measured by commercially available ELISA kits (R&D Systems and BD Biosciences, San Jose, CA). BAL protein was measured by Bradford assay (Bio-Rad, Hercules, CA).

**Western blots**

BAL and serum adiponectin isoforms were resolved by a nonreducing Western blot method as described previously (40). Briefly, 30 μl BAL fluid in SDS sample buffer was separated on 1.5 mm 4–12% PAGE in MES buffer (Invitrogen, Carlsbad, CA), transferred to a nitrocellulose membrane, and probed with anti-adiponectin Ab. Adiponectin isoforms were identified via their position relative to m.w. standards.

**Real-time PCR**

The left lung was homogenized and total RNA purified using a RNeasy Column kit (Qiagen) and treated with DNase II to remove genomic DNA. RNA amount and purity was determined at 260 and 280 nm by using a small volume spectrophotometer (Nanodrop; Thermo Scientific, Waltham, MA). A total of 1 μg RNA was converted into cDNA using Super Script III First-strand amplification kit for qRT-PCR (Invitrogen). IL-17A, IL-10, and Inflammatory IL-17A neutralizing Abs. cells were identified via their position relative to m.w. standards.

**Flow cytometry**

After exposing mice to 0.3 ppm ozone or air as described above, BAL was performed, and the lungs were flushed by instilling 10 ml ice-cold PBS into the right ventricle after sectioning the wall of the left ventricle. The lungs were then harvested and processed with PBS containing 2% FCS. Lung tissue was minced and incubated with collagenase type 4 (0.6 mg/ml; Worthington Biochemical) and GolgiStop (BD Biosciences) in RPMI 1640 medium at 37˚C. Harvested cells were counted and preincubated with anti-Fcγ blocking mAb (2.4G2) and washed. Cells were then stained with anti-mouse PE–Texas Red-conjugated CD45 (clone 50104, MAB421; Invitrogen) to identify hematopoetic cells. To examine lymphocytes, cells were stained with PerCP/Cy5.5-conjugated CD3 (clone 145-2C11; BD Biosciences), CD4 T cells were identified with Alexa Fluor 750-conjugated CD4 mAb (clone RM4-5; eBioscience), and γδ T cells were identified with anti-mouse PE-conjugated TCRγδ mAb (clone GL3; eBioscience). To examine macrophages, cells were stained with FITC-conjugated F4/80 mAb (clone BM8; BioLegend), Alexa Fluor 700-conjugated CD11b mAb (clone N418; eBioscience), and Alexa Fluor 750-conjugated CD11c mAb (clone MI70; Biolegend). For intracellular staining for IL-17A, cells were permeabilized (Cytoperm/Cytoperm; BD Biosciences) and incubated with Alexa Fluor 488-conjugated IL-17 Ab or isotype control (Alexa Fluor 647-conjugated rat IgG2a eBioscience). To confirm that the IL-17A observed in macrophages was localized intracellularly rather than on the cell surface, control experiments were conducted in which we did not perform the

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permeabilization step. To examine IL-17A expression in Th17 and γδ T cells, aliquots of cells were incubated either with or without PMA (100 ng/ml) and ionomycin (500 ng/ml) for 5 h prior to staining for flow cytometry. All labeled cells were passed through a Becton flow cytometry (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Statistical analysis

Data were analyzed by factorial ANOVA with Fisher-least significant difference as post hoc (Statistica; StatSoft, Tulsa, OK). BAL cells were log transformed to normalize the data. A p value <0.05 was considered statistically significant.

Results

Ozone increases lung adiponectin

In wild-type mice, exposure to ozone (0.3 ppm for 72 h) had no effect on serum adiponectin (Fig. 1A). In contrast, ozone increased BAL adiponectin (Fig. 1B), likely as a result of increased permeability of the lung epithelial barrier (see below). The increase in BAL adiponectin was time dependent, being absent 12 h after initiation of exposure and increasing to maximum at ~48 h after the onset of exposure (Fig. 1C). Ozone exposure for 12, 24, or 48 h had no effect on serum adiponectin similar to the results obtained at 72 h (data not shown). There were also changes in the adiponectin isoform distribution in the BAL fluid. In air-exposed mice, HMW adiponectin dominated BAL fluid, with slightly lesser amounts of hexameric adiponectin, and no detectable trimeric isoform. Ozone exposure for 72 h caused marked increases in both hexamer and trimeric adiponectin, with only comparably small increases in HMW adiponectin (Fig. 1D).

Ozone-induced inflammation is increased in Adipo−/− mice

Ozone caused a significant increase in the number of neutrophils and macrophages in BAL fluid (Fig. 2). In wild-type mice, neutrophils were increased as early as 24 h after initiation of exposure, peaking at 48 h (Fig. 2A). BAL neutrophils were increased in Adipo−/− versus wild-type mice at all three time points examined. In wild-type mice, BAL macrophages increased by 48 h of exposure and remained elevated thereafter (Fig. 2B). In Adipo−/− mice, 72 h of ozone exposure were required for macrophages to increase, ultimately reaching the same level as in wild-type mice. Ozone causes damage to the pulmonary epithelial barrier resulting in increased permeability and an increase in total BAL protein as serum proteins leak into the airspaces (55). In wild-type mice, BAL protein increased by 24 h of exposure, peaked by 48 h and remained elevated thereafter (Fig. 2C). BAL protein was greater in Adipo−/− versus wild-type mice.

BAL cytokines and chemokines implicated in neutrophil recruitment were measured in mice exposed to air or to ozone for 72 h (Fig. 3). In wild-type mice, BAL IL-6, TNF-α, and KC were significantly increased in ozone- versus air-exposed mice (p < 0.05), whereas MIP-2, LIX, and G-CSF were not. In Adipo−/− mice, ozone caused significant increases in BAL LIX, and G-CSF, as well as IL-6, TNF-α, and KC. Except for BAL TNF-α, each of these moieties was significantly greater in ozone-exposed Adipo−/− versus wild-type mice (p < 0.05).

Because others have reported that adiponectin can augment expression of the anti-inflammatory cytokine, IL-10 (27), and because Backus et al. (56) have reported increased pulmonary inflammation in IL-10−/− mice exposed to the same regimen of ozone exposure as we used, we also measured lung IL-10 mRNA expression. We did not observe any significant effect of ozone exposure on IL-10 mRNA (data not shown), and there was no effect of genotype, suggesting that the increased inflammation observed in Adipo−/− mice was not the result of loss of adiponectin-induced IL-10 expression.

We measured the endogenous IL-1Ra because it is known to be induced by subacatec oxygen (57), and it antagonizes the effects of IL-1 (58), which is required for the neutrophil influx induced by subacute ozone (47). Furthermore, IL-1Ra expression in macrophages is induced by adiponectin (27). We reasoned, therefore, that ozone-induced expression of IL-1Ra might be impaired in Adipo−/− mice, leading to greater IL-1 signaling, which may account for the increase in neutrophils (Fig. 2A). Our results do not support this hypothesis, because IL-1Ra was actually elevated to a greater degree in Adipo−/− versus wild-type mice exposed to ozone (Fig. 3G).

Role of IL-17A in the augmented neutrophil influx observed in Adipo−/− mice

Compared with air, ozone increased IL-17A mRNA expression in Adipo−/− mice (Fig. 4A). A similar trend was observed in wild-type mice but did not reach statistical significance. Moreover, after 48 h of ozone exposure, IL-17A mRNA expression was significantly greater in Adipo−/− than in wild-type mice.

To determine whether the greater induction of IL-17A in the Adipo−/− mice contributed to their enhanced neutrophil recruitment, both Adipo−/− and wild-type mice were treated with anti–IL-17A neutralizing Ab or isotype IgG control Ab and exposed to ozone for 48 h. After control Ab treatment, BAL neutrophils were higher in Adipo−/− than in wild-type mice, consistent with our observations in Fig. 2. In Adipo−/− mice, BAL neutrophils were significantly reduced after anti–IL-17A Ab compared with isotype control Ab (Fig. 4B). However, neutralization of IL-17A did not significantly affect neutrophil recruitment in wild-type mice. In contrast to its effects on neutrophil recruitment, anti–IL-17A Ab treatment did not affect ozone-induced increases in BAL macrophages (Fig. 4D) or protein (Fig. 4C), IL-6, or LIX in either genotype studied (Fig. 4E, 4G). BAL KC was paradoxically increased in Adipo−/− treated with anti–IL-17A Ab (Fig. 4F), whereas BAL G-CSF was significantly reduced in ozone-exposed Adipo−/− mice treated with anti–IL-17A Ab (Fig. 4H).

FIGURE 1. Adiponectin levels in serum and BAL fluid and adiponectin isoform distribution in BAL. Serum (A) or BAL adiponectin (B) from air- and subacute ozone (0.3 ppm for 72 h)-exposed wild-type mice quantified by ELISA. (C) Time course of adiponectin measured in the BAL of wild-type mice exposed to air and ozone at 12, 24, and 48 h. (D) Adiponectin isoform distribution in BAL from air- and subacute ozone-exposed wild-type mice. Equal amounts of BAL fluid were loaded in each lane. Data expressed in mean ± SEM. n = 3/group.
Flow cytometry was performed to determine which cell types in the lung were the source of the IL-17A that was induced following ozone exposure. Ozone exposure (0.3 ppm for 72 h) increased the number of IL-17A–expressing CD45\(^+\) cells in the lung (Fig. 5A) consistent with the increase in IL-17A mRNA (Fig. 4A). Moreover, the number of IL-17A–expressing cells was significantly higher in Adipo\(^{-/-}\) than in wild-type mice (Fig. 5A).

Staining for F4/80 Ag, a macrophage marker, indicated the presence of IL-17A–positive macrophages in the lung tissue (Fig. 5B). In contrast, BAL macrophages from ozone-exposed mice were not IL-17A positive (see Supplemental Fig. 1B). BAL macrophages from ozone-exposed mice were almost exclusively F4/80\(^+/\)CD11c\(^-\), whereas the lung tissue had both F4/80\(^+/\)CD11c\(^+\) “alveolar” macrophages and F4/80\(^+/\)CD11c\(^-\) “interstitial” macrophages, and it was the latter that expressed IL-17A in air-exposed wild-type and Adipo\(^{-/-}\) was significantly different (1.0 \(\pm\) 0.1 and 2.1 \(\pm\) 0.1%, respectively; \(p < 0.05\)). Ozone induced a significant increase in IL-17A\(^+\) macrophages in both wild-type and Adipo\(^{-/-}\) mice to 2.5 \(\pm\) 0.3 and 3.2 \(\pm\) 0.3\%, respectively (\(p < 0.05\) in each case). There was a significant increase in the total number of CD45\(^+/\)F4/80\(^+/\)IL-17A\(^+\) cells in ozone- versus air-exposed lungs and significantly higher numbers of CD45\(^+/\)F4/80\(^+/\)IL-17A\(^+\) cells were observed, confirming the intracellular source of this IL-17A (Supplemental Fig. 2). The observation that surface labeling for IL17R1 was observed on CD11c\(^+\) but not CD11c\(^-\) macrophages also suggests that the IL-17 expression observed in CD45\(^+/\)F4/80\(^+/\)CD11c\(^+\) cells was intracellular and not surface associated (data not shown).

To examine the impact of ozone and adiponectin deficiency on IL-17A expression in T lymphocytes, cells were incubated with PMA and ionomycin for 5 h before IL-17A staining and permeabilization. Virtually no IL-17A was observed in either CD4 T cells (CD45\(^+/\)CD3\(^+/\)CD4\(^+\)) or γδ T cells (CD45\(^+/\)CD3\(^+/\)TCR\(^{\delta/\delta}\)) in the absence of stimulation (Supplemental Fig. 1A, Fig. 5C, respectively). IL-17A was observed in some CD45\(^+/\)CD3\(^+/\)CD4\(^+\) cells after stimulation with PMA and ionomycin. However, these Th17 cells accounted for only a small percentage of CD4\(^+\) cells even after ozone exposure (Supplemental Fig. 1A). In contrast, a much larger percentage of γδ T cells expressed IL-17A after stimulation with PMA and ionomycin (Fig. 5C). Ozone increased total number of γδ T cells (Fig. 6B) in lungs from wild-type and, to a greater extent, Adipo\(^{-/-}\) mice. IL-17A\(^+\) γδ T cells were also significantly greater in ozone-exposed Adipo\(^{-/-}\) versus wild-type mice (Fig. 6C). However, ozone had no effect on the number of IL-17A–positive invariant NKT cells (iNKT) or neutrophils (data not shown).

**Regulation of IkB\(\alpha\) expression by adiponectin**

IkB\(\alpha\) or molecule possessing ankyrin repeats induced by LPS is a transcription factor that is required for the induction of IL-17A in CD4\(^+\) T cells (59) and is also required for mediating IL-17A–induced expression of certain genes (60–62). Factorial ANOVA indicated an increase in IkB\(\alpha\) mRNA expression in Adipo\(^{-/-}\) versus wild-type mice (\(p = 0.004\); Fig. 7) regardless of exposure.

**Discussion**

Our data indicate that adiponectin is present in the BAL fluid and is markedly increased following subacute ozone exposure (Fig. 1B, 1C). Furthermore, adiponectin is functionally important, because ozone-induced inflammation and injury were augmented in mice deficient in adiponectin (Figs. 2, 3). Importantly, ozone increased the number of IL-17A–expressing CD11c\(^+\) macrophages in the lung tissue (Figs. 5B, 6A). Greater numbers of IL-17A\(^+\) macrophages occurred in Adipo\(^{-/-}\) versus wild-type mice. Ozone also caused a greater increase in the number of PMA and ionomycin-stimulated IL-17A\(^+\) γδ T cells in lungs of Adipo\(^{-/-}\) versus wild-type mice (Fig. 6C). Importantly, neutralization of IL-17A reduced ozone-induced neutrophilic inflammation, but not injury, in Adipo\(^{-/-}\) mice (Fig. 4). Taken together, the data are consistent with the hypothesis that during extended ozone exposure, adiponectin exerts anti-inflammatory effects that inhibit the expression of IL-17A in the lung, thus limiting the influx of neutrophils. The
data also suggest that interstitial macrophages and/or γδ T cells are the likely source(s) of this IL-17.

We observed a marked increase in the amount of adiponectin present in the lung following ozone exposure as well as differences in the adiponectin isoform distribution (Fig. 1). Adiponectin is expressed almost exclusively in adipocytes and has to enter the lung from the circulation. The increase in BAL adiponectin was not the result of increased serum adiponectin, which did not change with ozone (Fig. 1A). Ozone impairs both epithelial and endothelial integrity (63), and it is likely that both the increased levels of BAL adiponectin (Fig. 1B, 1C) and the altered adiponectin isoform distribution (Fig. 1D) are the result of the marked increase in lung permeability that occurs following subacute ozone exposure (Fig. 2B). Given the very high m.w., especially of the hexameric and HMW adiponectin isoforms, movement of adiponectin from the blood into a normal air-exposed lung is unlikely to be via simple diffusion through gaps between endothelial cells: such a diffusive process would result in the greatest influx of trimeric adiponectin, because it is smallest and, hence, should diffuse most easily. Instead, HMW adiponectin was the isoform most plentiful in BAL fluid, with slightly lesser amounts of hexameric adiponectin and virtually undetectable amounts of the trimeric isoform (Fig. 1D), consistent with a previous report from our laboratory (40). Indeed, in the normal lung, the adiponectin binding protein, T-cadherin, appears to be required for adiponectin transit into the lung (40). In contrast, under conditions of the marked increase in permeability that characterizes the ozone-exposed lung, adiponectin may be able to diffuse. The observation that it is the smallest isoform, trimeric adiponectin, whose concentration undergoes the most marked increase in BAL fluid following subacute ozone exposure (Fig. 1D), suggests that this is indeed the case.

These ozone-induced changes in adiponectin concentration and isoform distribution appear to be functionally important, because exposing mice deficient in adiponectin to ozone resulted in a significant increase in BAL neutrophils (Fig. 2) and in IL-6, KC, LIX, G-CSF, and IL-17A (Figs. 3, 4), chemotactic factors that can contribute to neutrophil recruitment. The data suggest that under conditions of subacute ozone exposure, adiponectin has anti-inflammatory effects, decreasing the induction of proinflammatory mediators and subsequent neutrophilic inflammation. In contrast, our previous data indicate that following an acute 3-h exposure to a much higher concentration of ozone (2 ppm), adiponectin deficiency does not augment but rather attenuates both the neutrophil recruitment and the cytokine and chemokine induction that occur (40). Consistent with these observations, adiponectin deficiency causes a reduction in IL-17A mRNA expression induced by this acute ozone exposure (data not shown). Other differences in the
factors that modify responses to acute versus subacute ozone have been reported. For example, Kleeberger et al. (7, 24, 48) have demonstrated, using mice backcrossed from ozone-susceptible and -resistant strains, that the genetic factors that control neutrophil influx induced by subacute versus acute ozone are not the same. Furthermore, deficiency in IL-1R1, the main signaling receptor for both IL-1β and IL-1α or in TNF-α signaling causes a marked reduction in the neutrophil influx that occurs with subacute ozone but does not alter responses to acute ozone (3, 8, 24, 47). The mechanistic basis for differences in the role of adiponectin under conditions of acute versus subacute ozone is not clear. However, both pro- and anti-inflammatory effects of adiponectin have been reported by others [see Fantuzzi et al. (64) for review]. It is possible that differences in the amount or isoform distribution of adiponectin in the lung under the two ozone exposure conditions may play a role. We did not observe any increase in BAL adiponectin in wild-type mice following acute ozone exposure (40), whereas subacute ozone caused profound increases in BAL adiponectin (Fig. 1). We have not measured the adiponectin isoform distribution in BAL of mice exposed to acute ozone, but the fact that the increase in lung permeability was much more limited in those mice [about a 2-fold increase in BAL protein (40)] versus mice exposed subacutely (~5 fold; Fig. 2C) suggests that the ratio of the trimeric to HMW isoforms following the two different ozone exposures is likely to be quite different. The HMW isoform of adiponectin has been shown to cause NF-κB activation, whereas in the same cell type, trimeric adiponectin does not (65, 66).

IL-6, KC, LIX, G-CSF, and IL-17A were each induced by ozone exposure to a greater extent in Adipo−/− versus wild-type mice (Figs. 3, 4). Each of these cytokines and chemokines has been shown to play a role in neutrophil recruitment by various factors, in some cases including ozone exposure (6, 41, 50, 51, 67), albeit with different ozone exposure regimens. We chose to focus on IL-17A because it is known to play a role in neutrophil recruitment in response to diverse stimuli (infection, treatment with LPS, and allergen challenge) (50, 67–74). In addition, previous data from our group indicate that IL-17A is required for the neutrophil influx that occurs after three repeated exposures to 1 ppm ozone (each for 3 h) over the course of 5 d (51). Indeed, our data indicate that inhibiting IL-17A with neutralizing Abs attenuates the neutrophil influx that occurs with ozone exposure in Adipo−/− mice and normalizes differences in neutrophil recruitment between Adipo−/− and wild-type mice.
and wild-type mice (Fig. 4). The neutrophil recruitment induced by IL-17A is not direct but is typically mediated by induction of other factors that recruit neutrophils such as IL-6, KC, LIX, and G-CSF (6, 41, 67). Our results indicate that IL-17A induction of G-CSF is likely to account for the increased neutrophil recruitment induced by adiponectin deficiency, because anti–IL-17A Abs inhibited G-CSF but not LIX, IL-6, or KC expression (Fig. 4).

We were somewhat surprised to find that CD11c<sup>+</sup> macrophages and γδ T cells were the sources of the IL-17A in the lung after subacute ozone exposure (Fig. 5). Previous data with ozone-exposed mice had indicated that iNKT cells were involved (51), whereas in the current study, exposure to 0.3 ppm ozone for 72 h did not induce recruitment of iNKT cells to lung. The primary difference between the studies is the ozone exposure regimen: ozone exposure in this study was to lower concentrations (0.3 ppm) versus 1 ppm in the study by Pichavant et al. (51), although it is also possible that the continuous nature of the 72-h exposure used in this study results in adaptive mechanisms that do not occur.
with the briefer intermittent (3 h × 3) exposures used in the Pichavant study (51). IL-17A expression in macrophages is not without precedent. IL-10–deficient macrophages stimulated with LPS produce IL-17A (75), and exposure to chitin can also induce IL-17A in macrophages (76). IL-17–positive macrophages are also observed in the lungs of mice with allergic airways inflammation (77) and after respiratory syncytial virus infection (78).

The IL-17A+ macrophages that were elicited in the lung following subacute ozone exposure were CD11c+. Indeed, IL-17A+ macrophages were observed in the lung tissue but not in BAL fluid, which contains mostly CD11c+, alveolar macrophages. CD11c+ macrophages are considered “inflammatory” or “interstitial” macrophages (79). The observation that these IL-17A+ macrophages were increased to a greater extent in ozone-exposed Adipo−/− versus wild-type mice suggests either that adiponectin inhibits the expression of IL-17A in these macrophages, or that in the lung, adiponectin reduces the recruitment or proliferation of IL-17A–expressing macrophages.

The observation that IL-17A+ T cells were increased to a greater extent in ozone-exposed Adipo−/− versus wild-type mice suggests either that adiponectin inhibits the expression of IL-17A in these cells, or that in the lung, adiponectin reduces the recruitment or proliferation of IL-17A–expressing macrophages. Although the precise stimulus that induces the increased numbers of IL-17+ γδ T cells in the lungs following ozone exposure (Fig. 6A) is not established, it is important to note that IL-17+ γδ T cells do express TLR2 (83). Others have reported that TLR2 is required for other aspects of the pulmonary response to ozone (84).

In conclusion, adiponectin plays an important role in the pulmonary inflammatory response to subacute ozone exposure. Adiponectin exerts anti-inflammatory effects, leading to decreased neutrophil recruitment and decreased expression of proinflammatory cytokines and chemokines. There was a marked increase in IL-17 mRNA expression in lungs of Adipo−/− mice following subacute ozone exposure. This IL-17 was required for the augmented ozone-induced neutrophil influx that occurred in Adipo−/− mice. Interstitial macrophages and/or γδ T cells were the main source of this cytokine. IL-17 was also required for ozone-induced G-CSF release into BAL fluid, suggesting that IL-17–induced increases in G-CSF may participate in the augmented recruitment and/or survival of neutrophils in Adipo−/− mice.

Disclosures

The authors have no financial conflicts of interest.


Legend for Supplementary Figures

Supplementary Figure 1

S1. (A) IL-17A expression was evaluated in CD4\(^+\) T cells that were incubated for 5 hours with or without PMA and ionomycin and stained for IL-17A. (B) IL-17A was not detected in alveolar macrophages from BAL of WT and Adipo\(^{-/-}\) mice. Shaded area is the isotype control, blue line is air exposure and red line is ozone exposure.

Supplementary Figure 2

S2. IL-17A expression in F4/80\(^+\) macrophages was evaluated in cells with or without permeabilization and fixation. IL-17A was detected only after permeabilization and fixation.
Supplementary Fig. 1

A

CD45+ cells

T cells

IL-17A

Air

(-) P+I stimulation

Ozone

(-) P+I stimulation

WT

Adipo⁻/⁻

B

F4/80

Air

Ozone

CD11c

CD45+

% of MAX

WT

Adipo⁻/⁻
Supplementary Fig. 2

WT

Adipo^−^