Ozone Enhances Pulmonary Innate Immune Response to a Toll-Like Receptor–2 Agonist

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Previous work demonstrated that pre-exposure to ozone primes innate immunity and increases Toll-like receptor–4 (TLR4)–mediated responses to subsequent stimulation with LPS. To explore the pulmonary innate immune response to ozone exposure further, we investigated the effects of ozone in combination with Pam3CYS, a synthetic TLR2/TLR1 agonist. Whole-lung lavage (WLL) and lung tissue were harvested from C57BL/6 mice after exposure to ozone or filtered air, followed by saline or Pam3CYS 24 hours later. Cells and cytokines in the WLL, the surface expression of TLRs on macrophages, and lung RNA genomic expression profiles were examined. We demonstrated an increased WLL cell influx, increased IL-6 and chemokine KC (Cxcl1), and decreased macrophage inflammatory protein (MIP)-1α and TNF-α in response to Pam3CYS as a result of ozone pre-exposure. We also observed the increased cell surface expression of TLR4, TLR2, and TLR1 on macrophages as a result of ozone alone or in combination with Pam3CYS. Gene expression analysis of lung tissue revealed a significant increase in the expression of genes related to injury repair and the cell cycle as a result of ozone alone or in combination with Pam3CYS. Our results extend previous findings with ozone/LPS to other TLR ligands, and suggest that the ozone priming of innate immunity is a general mechanism. Gene expression profiling of lung tissue identified transcriptional networks and genes that contribute to the priming of innate immunity at the molecular level.

Keywords: ozone; Toll-like receptors; air pollution; gene expression profiles; macrophages

Air pollution accounts for substantial morbidity and mortality in children and adults (1). Air pollutants are known to exacerbate the symptoms of asthma, and may be involved in the initiation of this disease. In most urban areas, and increasingly in suburban areas, traffic-related emissions, including ozone precursors, comprise a major source of air pollution. Several studies reported increased emergency room visits by school-age children in response to ozone levels at or below the current standards (2–5). Recent evidence also suggests that ozone exposure may promote the development of asthma (6). Controlled ozone exposures in healthy volunteers have consistently demonstrated a decrease in forced expiratory volume in 1 second (FEV_1), an increase in nonspecific airway hyperresponsiveness, and neutrophilic inflammation as early as 1 hours after exposure and persisting up to 24 hours (7, 8). Ozone exposure was also shown to cause an enhanced response to inhaled allergens, both in normal volunteers and in patients with allergic asthma (9–11). Animal models of allergic asthma have been used to study the complex interaction between ozone and allergen (12–14).

In addition to the role it plays in the development of allergic airway disease, ozone may also predispose individuals, particularly children who are more susceptible, to develop respiratory infections. A number of studies in animals suggested that pre-exposure to ozone exerts an influence on pulmonary host defense. Exposure to ozone was associated with the impaired clearance of several bacteria, including *Klebsiella pneumoniae* (15), *Listeria monocytogenes* (16), *Mycobacterium tuberculosis* (17), *Staphylococcus aureus* (18), *Streptococcus pyogenes* (19), and *Streptococcus zooepidemicus* (20). This impairment in antibacterial host defense is at least in part explained by the disruption of the epithelial barrier and the inefficiency of phagocytosis. Studies in humans have shown that exposure to ambient levels of ozone can impair selective epithelial permeability (21), and that this loss of epithelial integrity is parallel to but not necessarily coupled with increased inflammation in the lower respiratory tract (7, 22). Moreover, both human and murine alveolar macrophages *in vitro* demonstrated impaired phagocytosis and superoxide production in response to ozone exposure (23, 24).

We have hypothesized that the expression of Toll-like receptors (TLRs) in the lung are influenced by exposure to ozone, and that the dynamic expression of TLRs exerts profound effects on lung host defense. An earlier study from our laboratory demonstrated that ozone pre-exposure altered the innate immune response to inhaled LPS in mice. Pre-exposure to ozone resulted in enhanced airway hyperresponsiveness (AHR), increased concentrations of total protein and proinflammatory cytokines in whole-lung lavage (WLL), and reduced inflammatory cell recruitment to the lower airways (25). That study also showed how ozone exposure results in the increased surface expression of TLR4 and enhanced LPS-mediated signaling in lung tissue. To test whether priming of the innate immune system by ozone constitutes a more general mechanism, we investigated the effects of ozone in combination with Pam3CYS, a synthetic TLR2/TLR1 agonist. We demonstrated changes in the inflammatory response to Pam3CYS as a result of ozone pre-exposure that appears to be caused by the enhanced expression of cell-surface TLRs on macrophages. In addition, we identified molecular processes and transcriptional networks associated with these phenotypes by performing gene expression analyses of mRNA from lung tissue.

MATERIALS AND METHODS

Animals

Male 7- to 8-week-old C57B/L6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All experiments were performed according to National Institutes of Health guidelines, and were approved by the

(*Received in original form May 22, 2012 and in final form August 25, 2012*)

This work was supported by National Institute of Environmental Health Sciences grant P01-ES18181 (J.L.O.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 48, Iss. 1, pp 27–34, Jan 2013

Originally Published in Press as DOI: 10.1165/rcmb.2012-0187OC on September 20, 2012
Internet address: www.atsjournals.org

Institutional Animal Care and Use Committee at National Jewish Health.

Exposure Protocol

Mice were exposed to either 2 ppm ozone ($n = 10$) or filtered air (FA) ($n = 10$) for 3 hours, according to a published protocol (25) described in detail in the online supplement. Twenty-four hours after ozone exposure, mice from the ozone or FA groups were treated intratracheally with 100 μg of Pam3CYS in saline ($n = 5$) or saline alone ($n = 5$). Animals were killed 4 or 24 hours after Pam3CYS exposure. WLL, total and differential cell counts, ELISAs, and total protein assays were performed according to published protocols (25, 26), as described in detail in the online supplement.

Flow Cytometry

Fixed WLL cells were washed and resuspended in CD16/CD32 Mouse BD FC Block (BD Biosciences, San Jose, CA) for 15 minutes. Cells were then stained with several fluorochrome-labeled antibodies: F4/80:FITC (AbD Serotec, Oxford, United Kingdom), TLR-1:PE, TLR 2:Alexa Fluor647, and CD11c:APC-e780 (eBioscience, San Diego, CA). Flow cytometry was performed using a FacScan from BD Biosciences, and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical Analysis of Cell, Cytokine, and Flow Cytometry Data

Data were analyzed using GraphPad Prism statistical software (GraphPad, San Diego, CA), and are expressed as means ± SEMs ($n = 10$–15 from three independent experiments). Comparisons between groups were performed with the two-tailed Mann-Whitney U test.

Western Blots

Lung tissues were homogenized and lysed in ice-cold lysis buffer, as described in the online supplement. After centrifugation, tissue extracts were resolved by SDS-PAGE and analyzed by immunoblotting. The membranes were probed with antibodies to phospho-JUN N-terminal kinase (JNK), phospho-p44/42 mitogen-activated protein kinase (MAPK; Erk1/2), and p44/42 MAPK (Cell Signaling Technology, Danvers, MA). Blots were developed with SuperSignal West Dura (Thermo Scientific, Rockford, IL).

RNA Extractions, Microarray, and Quantitative RT-PCR

RNA from the right lung was extracted using an Ambion mirVana RNA extraction kit, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Gene expression profiling was performed on Agilent Whole Mouse Genome arrays (Agilent Technologies, Santa Clara, CA), according to the manufacturer's protocols, as briefly described in the online supplement. All primary data were deposited at the Gene Expression Omnibus database (accession number GSE38014). Quantitative RT-PCR with SYBRGreen (Life Technologies) was performed using standard protocols, as described in the online supplement, and primers were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD) (Table E1 in the online supplement).

Statistical Analysis of Microarray and Quantitative RT-PCR Data

Expression data from 32 mRNA arrays (four exposure groups, two time points, and four animals/group) were analyzed using Partek software (Partek, Inc., St. Louis, MO). Intensity data were imported, $\log_2$-transformed, and quantile-normalized, using the robust multi-array average (RMA) algorithm (27). The differential expression of individual transcripts was identified using an ANOVA model incorporating exposure groups and time points. Differentially expressed genes with a false discovery rate (FDR) of 5% and 2-fold change between O_3/saline versus FA/saline, O_3/Pam3CYS versus O_3/saline, and O_3/Pam3CYS versus FA/Pam3CYS at two time points after Pam3CYS exposure were identified and analyzed for enriched pathways and transcriptional networks, using the Ingenuity Pathway Analysis (IPA) database (Ingenuity Systems, Redwood City, CA). Quantitative RT-PCR data were analyzed according to the ΔΔCt method (28).

RESULTS

Ozone Pre-Exposure Alters the Inflammatory Response to Pam3CYS in WLL

To study the effects of ozone pre-exposure on the innate immune response to Pam3CYS in the lung, we first examined the inflammatory response in the lavage fluid of C57BL/6 mice that were exposed to 2 ppm O_3 (2 ppm) or FA, followed by an intratracheal instillation of Pam3CYS or saline 24 hours after exposure to ozone. The WLL was examined either 4 hours or 24 hours after Pam3CYS instillation (Figure 1 and Figure E1 in the online supplement). The effect of ozone exposure (ozone/saline group) compared with FA (FA/saline group) was characterized by a significant decrease in total cell macrophages and neutrophils at 4 hours and neutrophils at 24 hours (Figure 1A). Not surprisingly, a significant increase of neutrophil influx was observed in response to Pam3CYS (mice exposed to FA/Pam3CYS or O_3/Pam3CYS, compared with FA/saline and O_3/saline). Importantly, ozone pre-exposure resulted in a significant increase of, neutrophil influx in response to Pam3CYS at both time points (O_3/Pam3CYS versus FA/Pam3CYS) with a greater increase at 24 hours. Macrophage influx was also significantly increased in O_3/Pam3CYS compared with O_3/saline mice. These data demonstrate that O_3 pre-exposure enhances the influx of both macrophages and neutrophils to Pam3CYS.

We next investigated the inflammatory cytokine and chemokine response in our model by measuring WLL total protein, IL-6, TNF-α, KC, MIP-1α, and MIP-2. As in previous studies (25, 29), O_3/saline exposure increased total protein in WLL (compared with FA/saline) at both time points (Figure E1A). At 24 hours, O_3/Pam3CYS exposure resulted in a significant increase of WLL protein compared with FA/Pam3CYS, and was higher than at 4 hours, reflecting an increase in lung permeability over time. As in previous reports, IL-6 in the WLL of O_3 exposed mice was increased compared with FA at both 4 hours and 24 hours, but the difference did not reach statistical significance (Figure 1B). Pam3CYS exposure (FA/Pam3CYS and O_3/Pam3CYS, compared with FA/saline and O_3/saline) led to significant increases in IL-6 concentration. Similar to previous exposure studies with O_3 and LPS (25), we observed a fivefold increase in IL-6 in the O_3/Pam3CYS group compared with the FA/Pam3CYS group at 4 hours, suggesting an enhanced IL-6 response with O_3 priming, followed by Pam3CYS. However, in contrast to our previous ozone/LPS studies (25), this response diminished over time. KC, a neutrophil chemokine, follows a similar pattern as IL-6, with the only difference involving a significant decrease in KC in the O_3/Pam3CYS group compared with the FA/Pam3CYS group. A second well-characterized neutrophil chemokine, MIP-2, was increased in the WLL from animals exposed to FA/Pam3CYS and O_3/Pam3CYS compared with their saline counterparts, but not significant compared with each other. Similar to IL-6 and KC, the 24-hour time point was significantly diminished compared with 4 hours, but with no differences between treatment groups (Figure E1B). In contrast to increased concentrations of IL-6, KC, and MIP-2, ozone pre-exposure before exposure to Pam3CYS did not affect concentrations of TNFα and MIP-1α. Taken together, these data demonstrate an enhanced, but not consistent, pulmonary inflammatory response to Pam3CYS as a result of ozone pre-exposure, providing a rationale for the further characterization of this response.

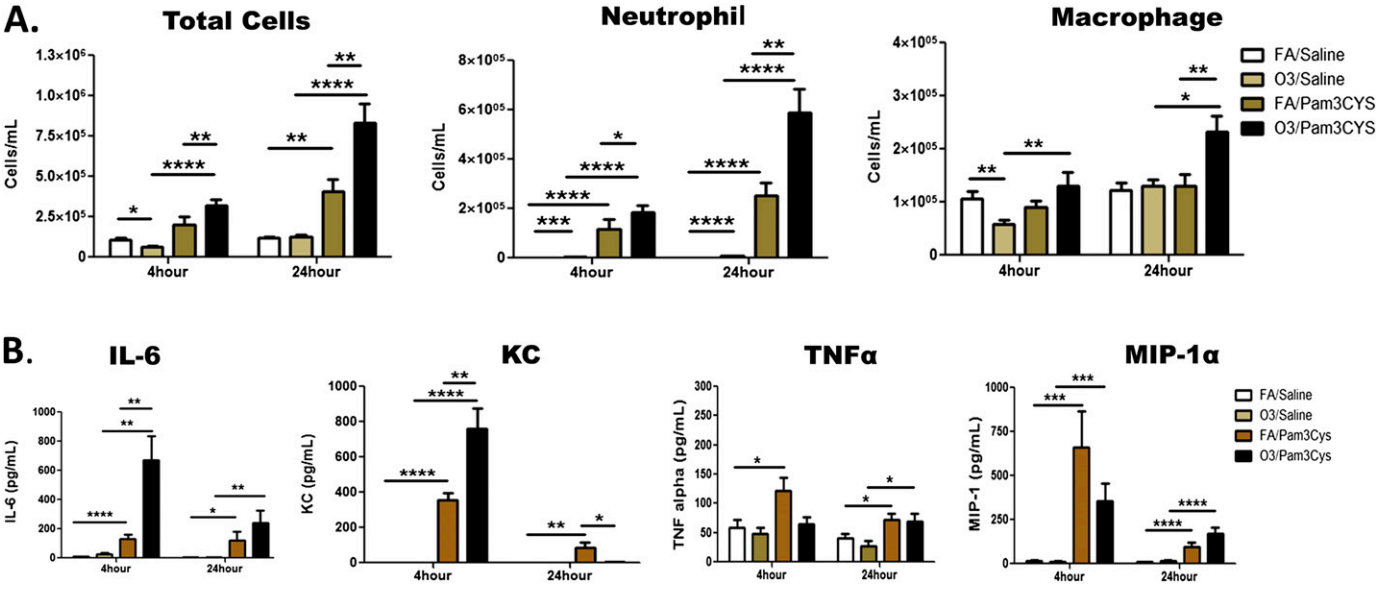

Figure 1. Ozone alters the inflammatory response to the synthetic Toll-like receptor–2 (TLR2)/TLR1 agonist Pam3CYS in murine whole-lung lavage (WLL). (*A*) Cell counts and (*B*) selected cytokines and chemokines in the WLL of C57BL/6 mice in response to intratracheal instillation of saline or Pam3CYS 24 hours after ozone (2 ppm, 3 h) or filtered air (FA) exposure. Measurements were performed at two time points, 4 hours and 24 hours after Pam3CYS instillation (28 h and 48 h after ozone exposure, respectively). Data are expressed as mean ± SEM ($n = 10$–15 C57BL/6 mice from three independent experiments). Group comparisons included O_3/saline versus FA/saline, O_3/saline versus O_3/Pam3CYS, FA/Pam3CYS versus FA/saline, and O_3/Pam3CYS versus FA/Pam3CYS. Statistical significance was determined using the Mann-Whitney *U* test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P < 0.0001$).

Ozone Exposure Leads to Increased Surface Expression of TLR1, TLR2, and TLR4 in Alveolar Macrophages

We next investigated the cell-surface expression of TLR1, TLR2, and TLR4 in alveolar macrophages. We hypothesized that the synergistic response of ozone and Pam3Cys in WLL cytokines may be at least partly attributable to an increase in cell-surface TLRs, in keeping with previous findings of an increase in TLR4 with O_3 (25). We identified WLL macrophages from each treatment group by forward scatter and side scatter and by positivity for F4/80 and CD11b (Figure E2). Mice exposed to O_3/saline demonstrated a significant increase in TLR1, TLR2, and TLR4 compared with FA/saline at 4 hours after saline. At 24 hours, a significant increase in TLR2 and TLR4 was still evident, but no difference in TLR1 was observed (Figure 2). Ozone pre-exposure resulted in a significant increase in the expression of TLR2, but not of TLR1 and TLR4, in response to Pam3CYS at 4 hours, with a significant increase in both TLR1 and TLR2, but not TLR4, at 24 hours (O_3/Pam3CYS versus FA/Pam3CYS groups). Although no increase in TLR4 surface expression as a result of ozone pre-exposure was evident in response to Pam3CYS (O_3/Pam3CYS versus FA/Pam3CYS), treatment with Pam3CYS (FA/Pam3CYS versus FA/saline, and O_3/Pam3CYS versus O_3/saline) surprisingly led to an increase in surface TLR4. These data confirm our earlier findings of an increase in surface TLR4 as a result of ozone exposure, and extend these findings to other TLRs, but also demonstrate the dynamic nature of TLR expression as a result of both ozone and TLR ligand exposure.

We also investigated the expression of TLR1, TLR2, and TLR4 in lung tissue via quantitative RT-PCR (Figure E3). TLR1, TLR2, and TLR4 mRNA expression in whole lung exhibited a similar pattern to that identified by flow cytometry analysis on the cell surface of WLL macrophages. The most prominent change involved a greater than twofold increase in TLR2 mRNA expression in the O_3/Pam3CYS group compared with FA/Pam3CYS at the 4-hour time point (Figure E3A). TLR expression in cell types other than alveolar macrophages and intracellular expression, at least in the case of TLR4, provides the likely explanation for any differences observed between surface expression measured by flow cytometry and expression in whole-lung tissue measured by quantitative RT-PCR.

Ozone Exposure Leads to Increased TLR Signaling in the Lung

To determine whether the ozone priming of innate immunity leads to enhanced downstream signaling in the TLR pathway, we investigated the effects of ozone alone or followed by Pam3CYS on downstream signaling in whole-lung homogenates. At 4 hours, ozone alone significantly increased the phosphorylation of p44/42 (Erk1/2) MAPK but not JNK, whereas Pam3CYS did not increase the phosphorylation of either kinase (Figure 3). Strikingly, ozone followed by Pam3CYS resulted in a large increase of phosphorylation for both kinases and a significant reduction in nonphosphorylated p44/42 MAPK. This enhanced signal associated with ozone/Pam3CYS treatment was not present at 24 hours (data not shown). These data, analogous to findings in our earlier study (25), demonstrate an enhancement in TLR signaling as a result of the ozone priming of innate immunity.

Gene Expression Analysis in the Lung Reveals Novel Candidate Genes for the Ozone Priming of Innate Immunity

To characterize the priming effect of ozone on innate immunity at the molecular level in an unbiased manner, we performed gene expression profiling on lung tissue of mice from the four exposure groups. Differentially expressed genes were identified as those with an FDR of 5% in an ANOVA model and a two

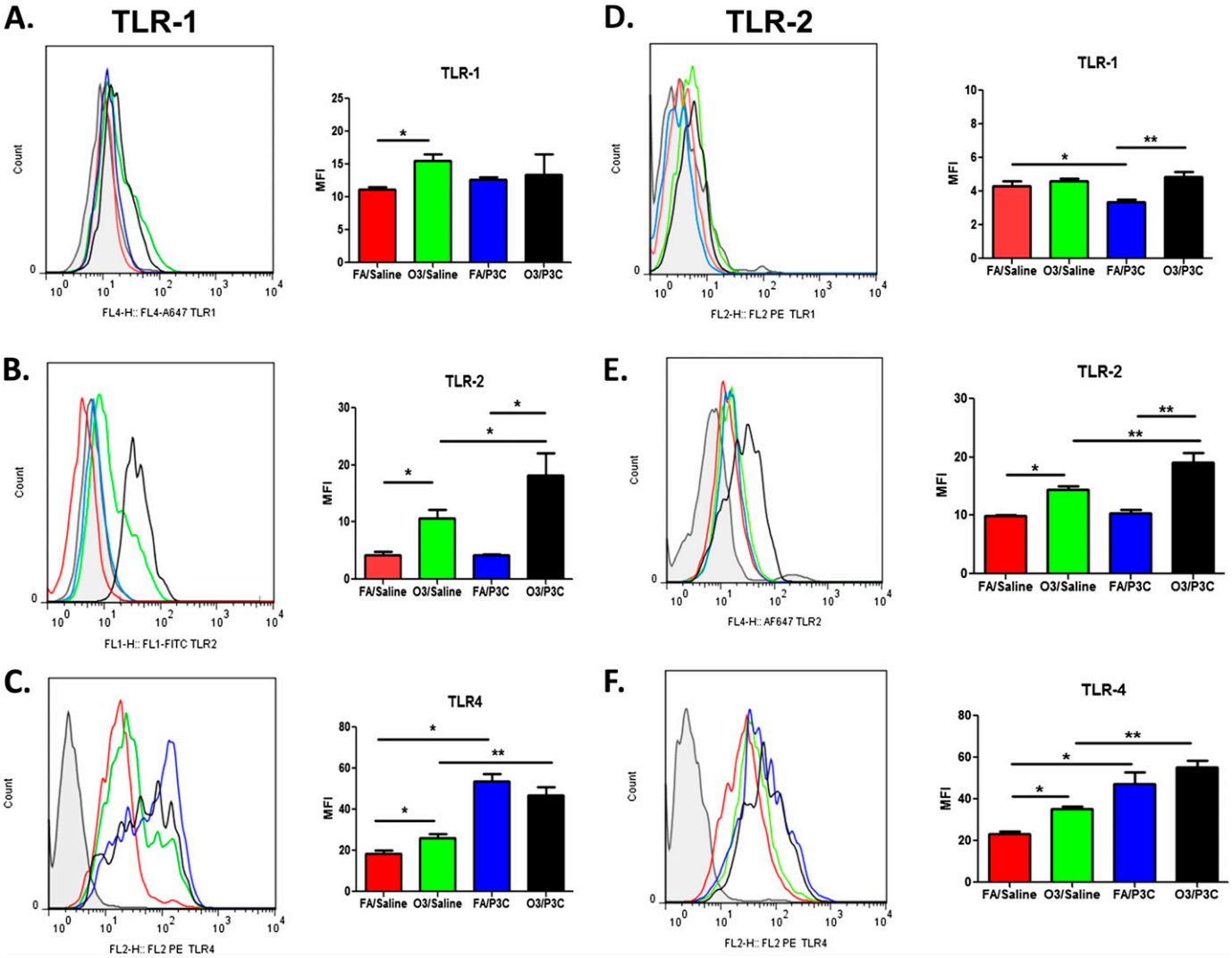

Figure 2. Surface expression of TLR1, TLR2, and TLR4 on alveolar macrophages after ozone and Pam3CYS exposure (*A–C*) at 4 hours and (*D–F*) at 24 hours. Cell-surface expression of TLR1, TLR2, and TLR4 on alveolar macrophages collected from WLL after ozone (2 ppm, 3 h) or FA pre-exposure was followed by intratracheal instillation of saline or Pam3CYS. WLL macrophages were identified by forward scatter and side scatter and by positivity for F4/80 and CD11c, and were then analyzed for TLR expression. Group comparisons involved O_3/saline versus FA/Saline, O_3/saline versus O3/Pam3CYS, FA/Pam3CYS versus FA/Saline, and O_3/Pam3CYS versus FA/Pam3CYS. Data are expressed as mean ± SEM ($n = 10$–15 C57 mice from three independent experiments). Statistical significance was determined using the Mann-Whitney *U* test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, and ****$P < 0.0001$).

fold change in O_3/saline versus FA/Saline (effect of ozone), O_3/Pam3CYS versus O_3/saline (effect of Pam3CYS), and O_3/Pam3CYS versus FA/Pam3CYS (effect of ozone priming on Pam3CYS response) at two time points (4 h and 24 h). Venn diagrams for the three sets of genes (Figure 4A and Tables E2–E7) reveal that ozone exposure exerts the strongest effect on gene expression at the 4-hour time point, with the effect diminishing by the 24-hour time point. Pam3CYS alone or when preceded by ozone results in less pronounced transcriptional changes.

We focused the remainder of our analyses on the effect of ozone priming on Pam3CYS response as the primary goal of a gene expression study, to identify molecular processes that mediate this effect. To examine the most prominent changes in gene expression, we focused on 50 genes at each time point with the highest mean fold change in the O_3/Pam3CYS versus FA/Pam3CYS comparison (Figure 4B). Ozone pre-exposure before Pam3CYS treatment (O_3/Pam3CYS versus FA/Pam3CYS) enhanced the induction of transfer RNA (tRNA) methyltransferase 5 (Trmt5) at 4 hours and cholecystokinin (Cck) at 24 hours. The expression of Ttk protein kinase (4-h time point), phosphatidylethanolamine binding protein 2 (Pbp2), gap junction protein, beta 4 (Gjb4), non-SMC condensin I complex, subunit G (Ncapg), and PDZ binding kinase (Pbk) (24-h time point) were also increased in O_3/Pam3CYS versus FA/Pam3CYS, but to a significantly lesser extent than in the O_3/saline versus FA/saline comparison. The most significant result among down-regulated genes involved the down-regulation at 24 hours of killer cell lectin-like receptors (Klra3, Klra8, Klra9, Klra10, Klra15, Klra21, Klra22, Klra23, Klrb1a, and Klrk1.

We also used ingenuity pathway analysis (IPA) to identify significantly overrepresented biological functions in the gene expression data. Biological function analysis revealed significant changes in cellular (Figure E4A) and immune (Figure E4B) function. Ozone exposure followed by exposure to Pam3Cys resulted in significant changes in cell cycle, DNA replication/recombination/repair, and cellular assembly and organization. These same gene categories

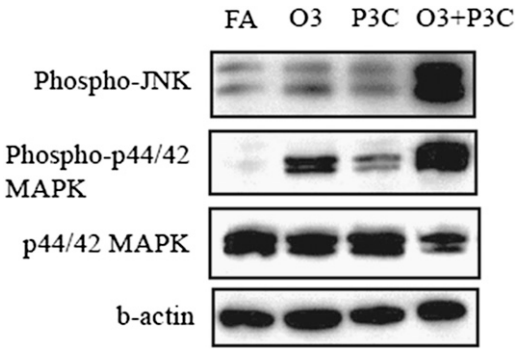

Figure 3. Ozone pre-exposure resulted in significantly enhanced TLR signaling in response to Pam3CYS, as assessed by mitogen-activated protein kinase (MAPK) phosphorylation at 4 hours (28 h after ozone). Western blot analysis of lung homogenates was performed on animals exposed to FA/saline (FA), FA/Pam3CYS (P3C), ozone/saline (O_3), and ozone/Pam3CYS (O_3 + P3C).

were enhanced in response to ozone alone, but were different from Pam3CYS-induced genes, which were enhanced in cell signaling, immune cell trafficking, inflammatory response/disease, and infection. We also used IPA to identify transcriptional regulators whose binding sites are overrepresented in promoters of differentially expressed genes (Table E8). Among the most significant transcriptional regulators whose target gene expression changed as a result of ozone followed by Pam3CYS exposure were those involved in the control of the cell cycle (E2F and Rb families), cancer (Myc, Rb, and TP53), and chromatin structure (the SMARC family and HDAC1). These same transcription factors were overrepresented in response to ozone alone, but produced gene expression changes different from those induced by Pam3CYS, which are regulated by transcription factors involved in innate immunity and inflammation (the NF-κB, Sp1, AP1, HMGB1, CEBP, IRF, and STAT families).

Using quantitative PCR, we confirmed gene expression changes in six selected genes identified in the array analysis: Cck, Retnla (RELMα), Retnlb (RELMβ), Hhmr, Il24, and Tnf (Table E9).

DISCUSSION

Our results extend previous findings in regard to ozone/LPS to other TLR ligands, and suggest that the ozone priming of innate immunity is likely a general mechanism. We demonstrate an increased inflammatory response to Pam3CYS as a result of ozone pre-exposure. We also observed an increased cell surface expression of TLRs in macrophages as a result of ozone alone or in combination with Pam3CYS. The gene expression profiling of lung tissue identified transcriptional networks and genes that may contribute to the priming of innate immunity at the molecular level.

Our study demonstrates that ozone priming of the innate immune system is likely a general mechanism. Components of air pollution such as ozone and endotoxin are well-documented to exacerbate the symptoms of asthma, and may also play a role in the initiation of this disease. Although our study used a high concentration of ozone, exposure to 2 ppm of ozone in a rodent was shown to be comparable to 0.2 ppm of ozone in a susceptible human subject (30). Ozone and LPS pre-exposures have been shown to alter the innate immune response to pathogens in

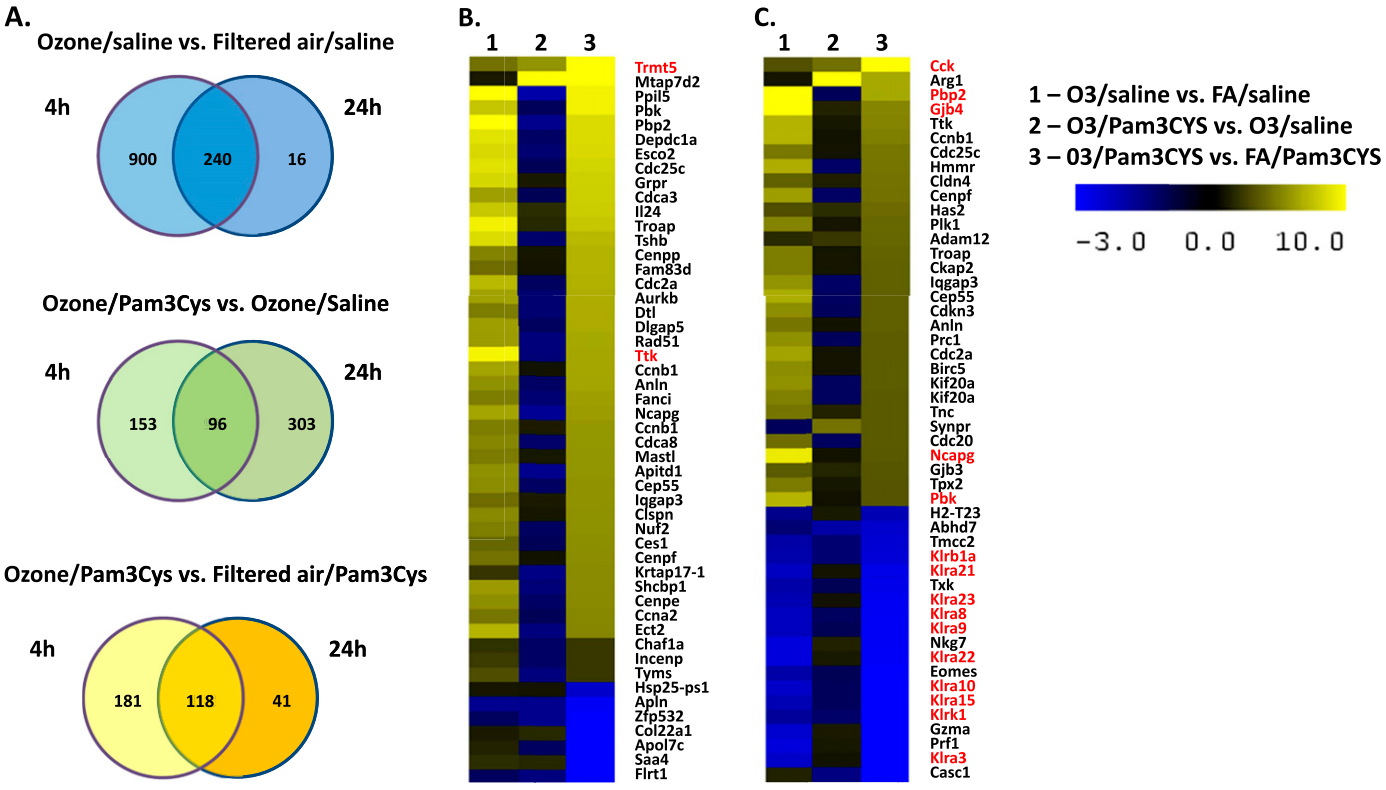

Figure 4. Gene expression in lung tissue after ozone pre-exposure and intratracheal instillation of Pam3CYS. (*A*) Venn diagrams depict overlaps in expression profiles at 4 hours (28 h after ozone) and 24 hours (48 h after ozone) after Pam3CYS instillation. Differentially expressed genes were defined as those with a false discovery rate (FDR) of 5% and 2-fold change between the comparison groups at two time points. (*B* and *C*) Heat maps of the most differentially expressed genes as a result of ozone pre-exposure, followed by Pam3CYS treatment at the (*B*) 4-hour time point and (*C*) 24-hour time point. The heat maps depict the 50 most up-regulated and down-regulated genes in the O_3/Pam3CYS versus FA/Pam3CYS comparison.

mice, as well as in murine and human macrophages. However, the mechanisms underlying the host-defense response to the components of air pollution are poorly understood. Taken together, our previous and current work provides evidence for the role of ozone exposure in altering the basic innate immune signaling that renders the pulmonary environment more responsive to pathogens and possibly allergens.

Although the ozone priming of innate immunity appears to be a general mechanism and our findings are largely parallel to those in an earlier study by Hollingsworth and colleagues (25), a few differences between the two studies are evident. In regard to ozone alone exposure, Hollingsworth and colleagues (25) did not observe a difference in total protein in the BAL, whereas we observed a small but statistically significant increase in the group with ozone alone compared with the free air group. Given that the ozone exposure and the strain of mice in the two studies were the same, and that the timings of exposure were similar, the most likely explanation involves the larger number of animals included in each group in our study. In terms of the enhanced response to TLR stimulation, Hollingsworth and colleagues (25) observed a decrease in neutrophils as a result of the combined ozone/LPS exposure compared with LPS alone, whereas we observed a significant increase in neutrophils in the ozone/Pam3CYS group compared with the Pam3CYS-alone group. Moreover, a few subtle differences in cytokine production were detected in the ozone/Pam3CYS study compared with the earlier ozone/LPS study. These differences are most likely a result of differences in the TLR ligands used in these studies. In addition, the route of administration of the TLR ligand (intratracheal versus aerosolized) may also explain some of the observed differences.

The mechanisms by which ozone increases the expression of TLRs on the surface of alveolar macrophages are not understood. Previously, the increase in the response of TLR4 to ozone alone was determined to be a result of the ozone-induced release of endogenous ligands (hyaluronan and heat-shock proteins) that stimulate TLR4, resulting in the migration of TLR4 to the surface of the cell (31). A subsequent gene expression profiling study by another group showed that heat-shock protein–70 is an effector molecule downstream of TLR4, and is involved in the regulation of ozone-induced lung inflammation by triggering pathways similar to those of TLR4 (32). This is likely a general mechanism that acts on all TLRs and accounts for the increases of TLR1 and TLR2 in response to ozone alone in the present study. In addition, we observed a further enhancement in the expression of TLR2 and TLR4 as a result of Pam3CYS treatment after ozone pre-exposure. Potentially, endogenous ligands such as hyaluronan and heat shock proteins also contribute to this priming effect. Alternately, a recent study demonstrated that the recruitment of TLR4 to the cell membrane can be activated by mechanisms involving the generation of nicotinamide adenine dinucleotide phosphate–reduced oxidase–dependent reactive oxygen species (33). Both Pam3CYS and pulmonary ozone exposure can initiate a cascade of reactive oxygen species, thus establishing an environment conducive to TLR2 and TLR4 recruitment to the cell surface (34, 35).

Gene expression analysis identified novel molecular targets associated with the priming effect of ozone on the innate immune response. These include changes in cell cycle, DNA replication/recombination/repair, cellular assembly and organization, and chromatin structure. The expression of the Trmt5 and Cck genes was induced by ozone alone, and was further enhanced by subsequent Pam3Cys stimulation. Trmt5 is a DNA methyltransferase that methylates the N1 position of guanosine-37 in selected tRNAs. It is difficult to speculate how this gene may be involved in the priming of innate immunity, but recently, oxidative stress induced by ozone and other environmental toxins was postulated to regulate 5-methyl-cytosine and 5-hydroxymethyl-cytosine (36). On the other hand, the role of Cck in the priming of innate immunity may be more straightforward. Cck is a neuropeptide that was shown to inhibit inflammation by down-regulating the expression of proinflammatory cytokines during endotoxin shock (37), and it inhibits LPS-induced IL-1β production in pulmonary interstitial macrophages by modulating cAMP-dependent protein kinase (PKA), p38, and NF-κB activity (38).

Among genes highly induced by ozone alone, but whose expression was dampened in response to Pam3Cys after exposure to ozone, are Ttk, Pbp2, Gjb4, Ncapg, and Pbk. Three of these genes are related to kinase activity. Phosphatidylethanolamine-binding protein–2 is a member of the evolutionarily conserved family of proteins (i.e., RAF kinase inhibitory proteins) implicated in MAPK pathway regulation (39). Pbk is a member of the dual specific MAPKK family. Ttk, also known as Mps1, is a mitotic protein kinase with a well-established role in the cell cycle and in carcinogenesis (40). Finally, seven members of the killer cell lectin–like receptor family are down-regulated by ozone, and their expression is further dampened by Pam3CYS. Killer cell lectin–like receptors are preferentially expressed on natural killer T (NKT) cells, which are innate immune lymphocytes that play a major role in the host rejection of virally infected cells (41), and which have also been implicated in the pathogenesis of asthma (42). A previous study demonstrated that NKT cells are required for the development of both ozone-induced and allergen-induced AHR (43). Together with our findings regarding the potential role of NKT cells on the priming of innate immunity, these results strongly suggest that NKT cells mediate a unifying pathogenic mechanism for several distinct forms of airway inflammation, and may represent a therapeutic target.

Four published studies assessed the genomic transcription profiles of murine lung tissue after ozone exposure (26, 32, 44). Park and colleagues profiled lung tissue from C57BL/6 mice exposed to 2 ppm ozone for 3 hours, either 4 or 24 hours after exposure, but focused their analysis primarily on inflammatory markers (26). Gohil and colleagues used an early genomic array to identify 260 differentially expressed transcripts (out of 4,000 represented on the array) in C57BL/6 mice exposed to 1 ppm O_3 for 3 consecutive nights (8 hours per night) (45). Among the differentially expressed transcripts were genes related to the immune response, DNA synthesis, cell-cycle progression, xenobiotic metabolism, and cytoskeletal functions. Williams and colleagues identified 400 differentially expressed genes (greater than twofold) in BALB/c mice 3 hours after ozone exposure, and also identified a number of markers of inflammation as the most affected genes (44). Bauer and colleagues profiled C3H/HeJ and C3H/HeOuJ mice to identify ozone candidate genes downstream of TLR4, and demonstrated a role for heat shock protein–70 (32). Our analysis of gene expression in the lung after ozone exposure differs from these previously published studies because it focused on the priming effect of ozone on innate immunity. As such, our study identified a number of novel targets for further exploration, both mechanistically and as therapeutic targets.

In conclusion, we have shown that the priming of innate immunity is a general mechanism, and we have identified novel molecular targets in this important process. Our study demonstrates that the expression of TLRs on macrophage surfaces is a dynamic process that is influenced by ozone, and that this process is associated with the differential expression of numerous previously unexplored genes. This dynamic nature of TLR expression is likely more general, and could be influenced by other components of air pollution and in cell types other than macrophages. This priming effect of air pollution and genes that are associated

with the process may also lead to potential therapeutic targets for air pollutant exposure in the context of pulmonary infection or allergic airway inflammation.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank David MCKean for his assistance with the GEO submission.

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