Role of TLR2, TLR4, and MyD88 in murine ozone-induced airway hyperresponsiveness and neutrophilia

Alison S. Williams,1 Sum-Yee Leung,1 Puneeta Nath,1 Nadia M. Khorasani,1 Pankaj Bhavsar,1 Razao Issa,1 Jane A. Mitchell,2 Ian M. Adcock,1 and Kian Fan Chung1

1Experimental Studies and Airway Disease Section and 2Cardiothoracic Medicine Section, National Heart and Lung Institute, Imperial College, London, United Kingdom

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Ozone is a potent oxidizing pollutant. High levels of ambient ozone are a significant threat to respiratory health and are linked to the worsening of symptoms and increased hospitalizations of patients with obstructive lung disease, such as asthma and chronic obstructive pulmonary disease (COPD) (2, 8, 10, 23, 27, 33, 38). Experimental ozone exposure induces airway hyperresponsiveness (AHR) to bronchoconstrictor agents and lung neutrophilia (6, 9, 21, 30, 31, 39). The mechanisms underlying ozone-induced release of AHR and inflammation are unclear; the influx of neutrophils may be due to their recruitment by ozone-induced proinflammatory cytokines and chemokines, such as cytokine-induced neutrophil chemoattractant (CINC), macrophage inflammatory protein-2 (MIP-2), TNF-α, and IL-1β (4, 5, 12, 15, 17, 21, 28).

Toll-like receptors sense pathogens and exhibit sequence and functional homology with the interleukin-1 receptor (Toll/IL-1R or TIR domain). The TIR domain is common to all TLRs and is required to initiate intracellular signaling. Following ligand binding, one of four common adapter proteins containing the TIR domain, such as myeloid differentiation factor-88 (MyD88), is recruited to the TLR. TLR and MyD88 combine to signal through IL-1 receptor-associated kinase (IRAK)-1 and -4 and tumor necrosis factor receptor-associated factor 6 (TRAF-6). IRAK and TRAF-6 initiate a series of signaling cascades that terminate in activation of the transcription factors AP-1 and NF-κB via IκB kinase (IKK)-mediated and mitogen-activated protein kinase (MAPK) pathways, respectively (36). Over 10 TLRs have been cloned in mammals, and each member recognizes individual groups of pathogens. For example, TLR2 recognizes ligands including bacterial lipoproteins and peptidoglycan from gram-positive bacteria, while TLR4 recognizes ligands including gram-negative bacteria (25). TLR2 can form heterodimers with TLR1 or TLR6 and signals through MyD88, while TLR4 can act dependently or independently of MyD88. Each combination of receptor and signaling response leads to the transcription of distinct profiles of genes.

In addition to a role for TLRs in immune responses to infection, there may be a role for TLRs in the pathogenesis of noninfectious events. Using inbred strains of mice derived from ozone-susceptible and ozone-resistant progenitors, Kleeberger et al. (20) found that TLR4 was differentially expressed between these mice after ozone exposure. Hollingsworth et al. (13) found that TLR4+/− mice did not develop AHR after ozone exposure but still exhibited a neutrophilic inflammatory response to ozone. On the other hand, in airway epithelial cells, particulates stimulated IL-8 release through oxidative stress pathways, dependent on TLR2 pathways (3). Therefore, both TLR2 and TLR4 may mediate effects of oxidative stress.

We therefore investigated the sensing of oxidative stress by TLR2, TLR4, and MyD88 in a model of ozone exposure using TLR2, TLR4, and MyD88 knockout mice.

METHODS

The protocols were approved by the Imperial College BioSciences Group and performed under a Project License from the British Home Office, UK, under the Animals (Scientific Procedures) Act 1986.
Mice. Genetically modified male mice (20–25 g) deficient in TLR2, TLR4, or the common adapter protein MyD88 were genotyped during an initial breeding program. Breeding pairs of mice deficient in TLR2 or TLR4 receptors were originally kindly supplied by Dr. O. Takeuchi (34), and mice deficient in MyD88 were originally kindly supplied by Dr. S. Akira (1). The mice used in this study were homozygotes of either TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, or MyD88<sup>−/−</sup> genotype and were back-bred for more than seven generations with C57BL6 mice; thus C57BL6 were used as wild-type (WT) controls.

Ozone exposure. TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, MyD88<sup>−/−</sup>, or WT C57BL6 mice were exposed to ozone generated from an ozonizer (model 500 Sander Ozonizer) mixed with air to provide a concentration of 3 parts per million (ppm) in a sealed Perspex container for 3 h. We used 3 ppm concentration because, in preliminary ozone concentration-response studies in C57BL6 mice, we found the 3-ppm exposure for 3 h to induce the maximal degree of AHR at 20–24 h associated with significant neutrophilic response. Ozone concentration was continually monitored with a probe from ATI, Oldham, UK. Control animals received air only. Two groups of 12 mice/train were studied (one exposed to ozone and the other to air) at two time points, 3 h and 20–24 h following exposure, except for MyD88<sup>−/−</sup> mice, which were studied at 20–24 h only.

Assessment of AHR. Twenty-four hours following exposure, mice were anesthetized with an intraperitoneal injection of anesthetic solution containing midazolam (Roche Products, Welwyn Garden City, UK) and Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml flunisolide; Janssen Animal Health, Wantage, UK). Mice were tracheostomized and ventilated (MiniVent type 845, Hugo Sach Electronic; rate 250 breaths/min and tidal volume 250 μl). Mice were monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS, Hants, UK). Transpulmonary pressure was assessed via an esophageal catheter (EMMS). Instantaneous calculation of pulmonary resistance (R<sub>t</sub>) was obtained. Increasing concentrations of acetylcholine chloride (ACh) (Sigma, Dorset, UK) (4–256 mg/ml) were administered with an ultrasonic nebulizer, and R<sub>t</sub> was recorded for a 5-min period following each concentration. R<sub>t</sub> after each concentration was expressed as percent change from baseline R<sub>t</sub>. The concentration of acetylcholine required to increase R<sub>t</sub> by 250% from baseline was calculated (PC<sub>250</sub>).

Bronchoalveolar lavage. We used a previously published method (24). Briefly, following an overdose of anesthetic, mice were lavaged with six aliquots of 0.5 ml PBS via the endotracheal tube (24). Briefly, following an overdose of anesthetic, mice were ventilated (MiniVent type 845, Hugo Sach Electronic; rate 250 breaths/min and tidal volume 250 μl). Mice were monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (EMMS, Hants, UK). Transpulmonary pressure was assessed via an esophageal catheter (EMMS). Instantaneous calculation of pulmonary resistance (R<sub>t</sub>) was obtained. Increasing concentrations of acetylcholine chloride (ACh) (Sigma, Dorset, UK) (4–256 mg/ml) were administered with an ultrasonic nebulizer, and R<sub>t</sub> was recorded for a 5-min period following each concentration. R<sub>t</sub> after each concentration was expressed as percent change from baseline R<sub>t</sub>. The concentration of acetylcholine required to increase R<sub>t</sub> by 250% from baseline was calculated (PC<sub>250</sub>).

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Measurement of BAL protein and IL-6 levels. Protein concentration for each BAL sample was determined using a protein assay reagent kit (Pierce Chemical, Rockford, IL) according to the manufacturers’ instructions. Levels of IL-6 were measured in the same sample using a DuoSet ELISA kit (R & D Systems) according to the manufacturers’ instructions.

cDNA synthesis, reverse transcription, and real-time PCR. RNA extracted from frozen lung tissue samples was amplified using PCR. RNA was extracted from stored lung tissue using an RNeasy Mini kit (Qiagen). RNA yield was then amplified via PCR using an Omniscript Reverse Transcriptase kit (Qiagen) and stored at −80°C until required. Five micrograms per sample of RNA was used to synthesize single-stranded complementary DNA (cDNA) using random hexamers and an avian myeloblastosis virus reverse transcriptase (Promega). The cDNA generated was used as a template in subsequent real-time PCR analyses. Transcript levels were determined by real-time PCR (Rotor Gene 3000, Corbett Research) using SYBR Green PCR Master Mix Reagent (Qiagen). The murine forward and reverse primers (0.5 μM) used are specified in Table 1. Cycling conditions were as follows: step 1, 15 min at 95°C; step 2, 20 s at 94°C; step 3, 20 s at 60°C; step 4, 20 s at 72°C, with step 2 to step 4 repeated 45 times. The standard curves used to determine relative expression for each primer were obtained by running real-time PCR for a diluted sample, for example, to 1:1, 1:10, 1:100, and 1:1,000. mRNA gene expression was expressed as a ratio of gene of interest mRNA to GAPDH mRNA.

Data analysis. Data are presented as means ± SE. For multiple comparisons of different groups, we used the Kruskal-Wallis test for ANOVA. If the Kruskal-Wallis test for ANOVA was significant, we performed the Dunn test for comparison between two individual groups or Mann-Whitney test. A P value of <0.05 was accepted as significant.

RESULTS

AHR. There were no significant differences in the baseline lung resistance values following PBS challenge in the six experimental groups. Twenty to twenty-four hours following ozone exposure, AHR to ACh in WT ozone-exposed mice was increased (ozone: −log PC<sub>250</sub> = 1.384 ± 0.124, n = 8) compared with WT air-exposed mice (−1.788 ± 0.119; n = 6, P < 0.05). TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice exposed to ozone did not exhibit AHR compared with air-exposed mice. Mean −log PC<sub>250</sub> of TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, or MyD88<sup>−/−</sup> mice exposed to ozone was less than that of WT mice exposed to ozone. TLR2<sup>−/−</sup> mice exposed to ozone had a reduced AHR compared with TLR4<sup>−/−</sup> mice (ozone: −log PC<sub>250</sub> = 2.178 ± 0.129, n = 6; air: −1.775 ± 0.107, n = 6; P = <0.05) (Fig. 1).

BAL fluid. Total BAL cells were increased time dependently at 3 h and at 20–24 h following ozone exposure compared with air exposure in all mice (Fig. 2A). The total cell increase observed in ozone-exposed MyD88<sup>−/−</sup> mice was less than that of ozone-exposed WT mice, 20–24 h following exposure (P < 0.001, n = 6). At 3 h and 20–24 h following exposure, macrophage numbers were increased by ozone in all mouse strains, except in MyD88<sup>−/−</sup> mice (Fig. 2B). The number of macrophages in MyD88<sup>−/−</sup> mice after ozone was less than in
WT ozone-exposed mice ($P < 0.001, n = 6$). At 3 h following ozone, the number of neutrophils after ozone exposure in TLR2$^{-/-}$ and TLR4$^{-/-}$ was less compared with ozone-exposed WT mice ($P < 0.001$). However, at 20–24 h following exposure, there was no difference between the numbers of neutrophils recovered from ozone-exposed WT compared with those in TLR2$^{-/-}$ or TLR4$^{-/-}$ mice. Neutrophil counts from ozone-exposed MyD88$^{-/-}$ mice were less compared with controls ($P < 0.001, n = 6$).

**Protein and IL-6 levels in bronchoalveolar fluid.** Protein concentrations in the supernatant collected at 20–24 h were significantly increased in all ozone-exposed mice compared with their respective air-exposed controls. Protein levels in ozone-exposed MyD88$^{-/-}$ were the least elevated compared with ozone-exposed WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice (all $n = 6$) (Fig. 3A). Ozone increased the levels of IL-6 at 20–24 h in WT mice, but the increase in IL-6 levels did not achieve significance in TLR4$^{-/-}$ and TLR2$^{-/-}$ mice ($n = 6$ in each group; Fig. 3B). There was no increase in IL-6 levels in ozone-exposed MyD88$^{-/-}$ mice; these levels were significantly less compared with ozone-exposed WT mice ($n = 6, P < 0.05$).

**Real-time PCR.** Ozone induced the expression of IL-6 and keratinocyte-derived chemokine (KC) at 3 h and 20–24 h compared with air-exposed WT mice, although the increase for IL-6 at 20–24 h was not significant (Fig. 4A). Expression of IL-6 was less in ozone-exposed TLR2$^{-/-}$ mice at 3 h and in MyD88$^{-/-}$ mice at 20–24 h compared with WT ozone-exposed mice (both $P < 0.05, n = 6$). The increased expression of IL-6 in TLR4$^{-/-}$ exposed mice was not significantly affected. Ozone-induced KC expression was blunted in TLR2$^{-/-}$ ($P < 0.05, n = 6$), TLR4$^{-/-}$ ($P < 0.05, n = 6$), and MyD88$^{-/-}$ mice compared with WT at 3 h (Fig. 4B). mRNA expression for TLR2, TLR4, and MyD88 was not increased following ozone in their respective knockout mice, and these expression levels were less than ozone-exposed WT mice (Fig. 5). At 3 h following ozone, TLR2 expression was increased in TLR4$^{-/-}$ mice, and TLR4 expression was increased in TLR2$^{-/-}$ mice, although these increases were not significant. MyD88 expression was elevated at 3 h and 20–24 h in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice compared with air-exposed controls ($P < 0.05, n = 6$).

**DISCUSSION**

We have investigated the role of TLR2, TLR4, and the common adapter molecule MyD88 in the lung response to the oxidant ozone. AHR induced by ozone exposure in WT mice was absent in TLR2$^{-/-}$ and MyD88$^{-/-}$ mice, while it was significantly reduced in TLR4$^{-/-}$ mice. BAL neutrophil counts from TLR2$^{-/-}$ or TLR4$^{-/-}$ mice exposed to ozone were significantly less compared with WT mice at 3 h but not at 20–24 h, while in MyD88$^{-/-}$ mice, they were reduced at 20–24 h. Ozone-induced expression of IL-6 was reduced in TLR2$^{-/-}$ and MyD88$^{-/-}$ mice but was intact in TLR4$^{-/-}$ mice, and the larger increase in KC expression in WT exposed mice at 3 h was blunted in all knockout ozone-exposed species. TLR2, TLR4, and MyD88 expression were increased in WT mice exposed to ozone and reduced in their respective knockout strains. It is of interest that expression of IL-6, KC, TLR2, TLR4, and MyD88 was not significantly increased following ozone in MyD88$^{-/-}$ mice. Together, these data show TLR2, TLR4, and MyD88 are important in the development of ozone-induced AHR, with TLR4 being less dependent. MyD88 appears to be the most important in regulating ozone-induced neutrophil recruitment, with TLR2 and TLR4 playing a role in
hastening neutrophilic influx. We also observed a decrease in macrophage numbers in BAL fluid of TLR4−/− and MyD88−/− mice at 20–24 h after ozone exposure, indicating that TLR4 and MyD88 may be involved in the recruitment of macrophages to the lungs.

Our data regarding the role of TLR4 in ozone-induced AHR and neutrophilic inflammation is similar to that reported by Hollingsworth et al. (13). A mutant TLR4 allele in a C3H mouse strain has been shown to confer resistance to ozone-induced hyperpermeability (14). Our data indicate that in the C57/Bl6, the WT TLR2 or TLR4 gene does not modulate ozone-induced hyperpermeability. However, these toll receptors play a role in ozone-induced lung effects. Thus TLR2 appears to be more important than TLR4 in AHR, and TLR2 and TLR4 may also have a role in hastening neutrophilic inflammation. Additional information from our studies places a most important role for MyD88 in ozone-induced AHR and neutrophilic inflammation. In addition, although we did not examine MyD88−/− mice at the 3-h time point after ozone, our data indicate that MyD88 is also important for the induced expression of KC and IL-6 at 24 h. Interestingly, in TLR2−/− and TLR4−/− mice, the induced expression of IL-6 and KC was reduced at 3 h (more so in TLR2−/− mice) but increased at 24 h toward levels seen in WT mice. This temporal pattern is reminiscent of that observed with lavage neutrophilia. Because KC and IL-6 are cytokines that have been implicated in ozone-induced neutrophilia (18, 19), it would be reasonable to hypothesize that TLR2 and TLR4 are important in accelerating the induction of these cytokines. The measurement of IL-6 protein in BAL fluid at 20–24 h corroborates well with the gene expression in the lungs. These studies confirm the essential role of MyD88 in regulating IL-6 and KC production induced by ozone.

Our data therefore indicate that MyD88 is most important for ozone-induced AHR, neutrophilic inflammation, and KC and IL-6 expression. Thus, following activation of TLR2 or TLR4, MyD88 is recruited to these receptor complexes, and

Fig. 2. Time-dependent effect of ozone exposure on bronchoalveolar lavage (BAL) cells. A: total BAL cells. B: BAL macrophages. C: BAL neutrophils. At 3 h and 20–24 h, increases in total cells and macrophages were not different in TLR2−/− or TLR4−/− mice compared with WT mice. Ozone-induced neutrophilic numbers were blunted in TLR2−/− (P < 0.05, n = 6) and TLR4−/− (P < 0.05, n = 6) mice compared with WT mice but were comparable to WT 20–24 h following exposure. Total cells, macrophages, and neutrophils were blunted in MyD88−/− mice (P < 0.05, n = 6). Data are expressed as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 compared with air-exposed mice of corresponding strain. †P < 0.05, ††P < 0.01 compared with ozone-exposed WT at 3 h. ##P < 0.01 compared with ozone-exposed WT mice at 20–24 h.

Fig. 3. A: protein levels in bronchoalveolar lavage (BAL) fluid, 20–24 h following exposure to ozone, was increased in all 4 strains of mice. B: IL-6 levels were increased in WT, TLR4−/−, and TLR3−/− mice after ozone exposure but not in MyD88−/− mice. Data are expressed as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 compared with air-exposed mice of corresponding strain. #P < 0.05 compared with ozone-exposed WT mice.
 ROLE OF TLRs AND MyD88 AFTER OZONE EXPOSURE

It is interesting that ozone exposure itself importantly modulates the expression of TLR2, TLR4, and MyD88, effects that need to be taken into account in the interpretation of the results we have obtained. Thus, in TLR2<sup>−/−</sup> mice, as expected, the expression of baseline and of ozone-induced TLR-2 was abrogated, while in TLR4<sup>−/−</sup> mice, the expression of TLR2 was significantly increased at 3 h with a pattern similar to that found in WT mice. Conversely, in TLR4<sup>−/−</sup> mice, TLR4 expression was ablated at baseline and after ozone exposure but increased after ozone exposure in TLR2<sup>−/−</sup> mice, similar to WT mice, albeit nonsignificantly. Of greater significance is the significant expression of MyD88 in WT mice exposed to ozone particularly at 24 h, an effect that is preserved in TLR2<sup>−/−</sup> and TLR4<sup>−/−</sup> mice; as expected, the expression of MyD88 was virtually ablated in MyD88 mice following ozone exposure at 24 h. It is of interest that the high levels of MyD88 expression in the lungs of ozone-exposed TLR2<sup>−/−</sup> and TLR4<sup>−/−</sup> mice were associated with lack of AHR, likely to be due to the suppressed levels of upstream TLR2 and TLR4 receptors, respectively. On the other hand, in terms of the neutrophilic response, it is possible that its persistence in the lungs of ozone-exposed TLR2<sup>−/−</sup> and TLR4<sup>−/−</sup> mice may be related to the upregulation of MyD88. This is the first time that an oxidant stress has been shown to induce the expression of three components of the TLR pathway, namely, TLR2, TLR4, and MyD88. However, what has not been determined is whether the gene expression is accompanied by a functional increase of these components. The molecular mechanisms by which oxidative stress regulates these components of the innate immune system are unknown.

Because ozone itself is not a receptor ligand, we considered the nature of TLR activation in this model. This model of ozone exposure is an oxidant model since we have shown that N-acetylcysteine can prevent ozone-induced AHR and neutrophilia in mice (Leung SY, Blanc FX, Williams A, and ChungKF, personal communication). Activation of TLR4 particularly by endogenous ligands other than bacterial components such as hyaluronic, heat shock proteins, fibronectin, fibrinogen, and surfactant protein A has been reported (11, 16, 26, 32, 35, 37). Increased levels of hyaluronic and fibronectin have been reported following ozone exposure (7, 22) and could therefore activate TLR4 and to a lesser extent TLR2. Recently, oxidative stress in the form of hydrogen peroxide has been shown to cause the recruitment of TLR4 from cytoplasmic compartments to the cell surface into lipid rafts, conferring increased responsiveness to stimulation with TLR4 ligands such as lipopolysaccharide (29). This mechanism may also interact with...
the increased gene expression of TLR2, TLR4, and MyD88 observed in our study following oxidant stress induced by ozone. It is possible ozone itself or secondary oxidizing species generated by ozone may oxidize one or all TLRs, in turn altering the affinity of the receptor(s) for adapter proteins, such as MyD88, thereby facilitating and exaggerating TLR signaling. So far, no evidence is available to support this.

In conclusion, we report that TLR2, TLR4, and MyD88 are involved in the development of ozone-induced AHR; in particular, the common adapter protein MyD88 is most crucial for not only ozone-induced AHR but also for the induction of neutrophilia and the cytokines IL-6 and KC.

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REFERENCES


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