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Ozone Inhalation Promotes CX3CR1-Dependent Maturation of Resident Lung Macrophages That Limit Oxidative Stress and Inflammation

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Inhalation of ambient ozone alters populations of lung macrophages. However, the impact of altered lung macrophage populations on the pathobiology of ozone is poorly understood. We hypothesized that subpopulations of macrophages modulate the response to ozone. We exposed C57BL/6 mice to ozone (2 ppm × 3 h) or filtered air. At 24 h after exposure, the lungs were harvested and digested and the cells underwent flow cytometry. Analysis revealed a novel macrophage subset present in ozone-exposed mice, which were distinct from resident alveolar macrophages and identified by enhanced Gr-1+ expression [Gr-1 macrophages (Gr-1 Macs)]. Further analysis showed that Gr-1+ Macs exhibited high expression of MARCO, CX3CR1, and NAD(P)H:quinone oxidoreductase 1. Gr-1+ Macs were present in the absence of CCR2, suggesting that they were not derived from a CCR2-dependent circulating intermediate. Using PKH26-PCL to label resident phagocytic cells, we demonstrated that Gr-1 Macs were derived from resident lung cells. This new subset was diminished in the absence of CX3CR1. Interestingly, CX3CR1-null mice exhibited enhanced responses to ozone, including increased airway hyperresponsiveness, exacerbated neutrophil influx, accumulation of 8-isoprostanes and protein carbonyls, and increased expression of cytokines (CXCL2, IL-1β, IL-6, CCL2, and TNF-α). Our results identify a novel subset of lung macrophages, which are derived from a resident intermediate, are dependent upon CX3CR1, and appear to protect the host from the biological response to ozone.

Ozone, a gas formed via a chemical reaction between oxides of nitrogen and volatile organic compounds in the presence of sunlight, is a source and cause of environmental lung injury. Inhaled oxidants, such as ozone, are important contributors to a variety of respiratory diseases, including chronic obstructive pulmonary disease, asthma, cystic fibrosis, and adult respiratory distress syndrome (1). The mechanism of action for the response to ozone appears to be through a combination of several mechanisms, including the alteration of barrier functions in the epithelium (2, 3), the generation of oxidized species such as 5β, 6β-epoxycholesterol found in the epithelial lining fluid (4), and the fragmentation of matrix proteins such as hyaluronan to fragments of low m.w. (5). Inhalation of ozone results in the activation and recruitment of many cell types within the lung (6).

Macrophages are increased in the airspace after inhalation of ambient ozone and appear central to the host response to ozone (7, 8). Macrophages can produce proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8, which contribute to the biological response to ozone (7, 9). Despite the evidence indicating a proinflammatory role for macrophages, additional evidence suggests that macrophages can be protective during ozone-induced lung injury (10). Macrophages are important for clearance of both apoptotic cells and oxidized species generated by ozone, which, if not cleared, can exacerbate inflammation (10, 11). Just how macrophages can cause both pro- and anti-inflammatory outcomes remains poorly understood. We hypothesized that individual macrophage subpopulations within the lung account for the divergent reported functions of macrophages in the setting of ozone inhalation.

Interest has grown in the identification of macrophage subpopulations in the lung and in other organ systems as a means to understand their diverse function during injury and repair. Given the diverse functional roles of macrophages, it is not surprising that subpopulations of macrophages exist that are either recruited from or mature in resident cells in response to environmental challenge. Recent studies have identified distinct macrophage subpopulations with unique functions in multiple tissues (12). These data also support plasticity of macrophages in a manner dependent on microenvironment. In the lung, distinct macrophage subpopulations have been characterized in murine models of infectious disease and asthma (13–16). Work from our laboratory identified a lung macrophage subpopulation, exudative macrophages (ExMacs), that are recruited following noninfectious lung injury (17). Separable macrophage lineages result from the heterogeneity in macrophage precursor cells (i.e., monocytes) and the specific developmental pathways that are initiated by stimulatory local conditions at the time of monocyte differentiation (12). This work has largely focused on macrophage recruitment and development from circulating monocytes during pulmonary injury. However, it is unclear whether resident lung macrophages...
can extend beyond their primary alveolar defense phenotype, adapt, and undergo development into unique populations in the setting of acute lung injury.

Macrophages are derived either from monocytes or by proliferation of local macrophages. Two unique monocyte populations are present in the circulation (18). Constitutive (Gr-1–) monocytes enter tissues under steady state conditions in the lung and develop into resident tissue interstitial macrophages and alveolar macrophages (AMs) (18–20). This homeostatic mechanism appears to be dependent on the chemokine receptor CX3CR1 (or the fractalkine receptor). In contrast, inflammatory (Gr-1+) monocytes are recruited to the lung during inflammation in a CCR2-dependent manner and develop into either an activated macrophage population known as ExMacs or into monocyte-derived dendritic cells (18, 21). The morphology, phenotype, and effector functions of constitutive monocyte-derived macrophages differ markedly from those of inflammatory monocyte-derived macrophages (16). We and others (16, 17) identified that recruited ExMacs, but not resident lung macrophages, express high levels of MHCII and stimulatory molecules, stimulate T cell activation, and are a major source of inflammatory cytokines and chemokines, such as TNF-α, CXCL2, CXCL10, and NO synthase 2. Despite this characterization of recruited macrophages, little is known about the unique functional roles of resident lung macrophages in the context of noninfectious lung injury.

In the current study, we identify a novel subset of macrophages present in the lung after inhalation of ozone. This subset is separable from resident macrophages and characterized by expression of unique cell surface markers and gene expression. Using cell labeling of resident phagocytic cells, we find that these macrophages are derived from a resident lung intermediate and not a circulating precursor. Finally, we discover that the development of this macrophage subset is diminished in mice deficient in CX3CR1. In the setting of CX3CR1 deficiency and reduced numbers of Gr1+ Macs, mice have enhanced response to ozone. In conclusion, our data support a novel macrophage subpopulation that is derived from resident macrophages and can protect the lungs from oxidant-related lung injury.

Materials and Methods

Mice

C57BL/6 and CX3CR1<sup>GFP/WT</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CX3CR1<sup>GFP/GFP</sup> mice were crossed with C57BL/6 mice to produce CX3CR1<sup>GFP/WT</sup> mice. CCR2<sup>−/−</sup> mice were backcrossed on a C57BL/6 background for >10 generations. Experimental groups consisted of 6- to 10-wk-old female mice for all experiments. Animal experiments were conducted in accordance with National Institutes of Health guidelines and protocols approved by the Animal Care and Use Committee at Duke University.

Exposure protocol

C57BL/6, CCR2<sup>−/−</sup>, CX3CR1<sup>GFP/WT</sup>, and CX3CR1<sup>GFP/GFP</sup> were exposed to either filtered air or ozone. Ozone exposure occurred for 3 h at a dose of 2 ppm. The ozone was generated by directing 100% oxygen through an ultraviolet light generator. This was then mixed with the air supply to the chamber. Temperature and humidity of chamber air were monitored continuously, as was the ozone concentration with an ultraviolet light photometer. The mice receiving filtered air were also placed in chambers for 3 h without ozone instillation. The mice were then removed from the chamber and allowed to recover for 24 h.

Lung digestion and flow cytometry

Mice were euthanized 24 h following ozone or filtered air exposure. The lungs were perfused with HBSS to remove residual RBCs from the pulmonary circulation. The lungs were minced and digested for 40 min at 37 °C in HBSS with 1 mg/ml collagenase A (Roche, Indianapolis, IN) and 0.2 mg/ml DNase 1 (Sigma-Aldrich, St. Louis, MO). The digestion solution was passed through a 70-µm mesh strainer and centrifuged at 535 × g at room temperature over an 18% nycodenz (Accurate Chemical, Westbury, NY) cushion. Low-density cells were collected, underwent RBC lysis, and then washed × 2. The following Abs were used: anti-I-A/I-E FITC, anti-I-AJ/E PE (used in experiments with CX3CR1<sup>GFP/WT</sup> mice), anti-Ly6-G PE, anti- Mac-3 PE, anti–Gr-1 alllophycocyanin, and anti-CD11b alllyacocyanin-Cy7 (all from BD Pharmingen, Franklin Lakes, NJ); anti-CD11c PE Cy5.5 and anti-F480 alllyacocyanin (eBioscience, San Diego, CA); and anti–MHCII FITC, anti–Gr-1 PE, RAd PE (AbD Serotec, Raleigh, NC). Biotin staining was performed for MARCO using a rat IgG1 negative control (catalog no. MCA1211, AbD Serotec), which was conjugated to PE fluorochrome using an Ab conjugation kit (catalog no. LNDK21RPE, AbD Serotec). All staining was performed in PBS with 3% FBS, 10 mM EDTA, 5% normal mouse serum, 5% normal rat serum, and 1% FcBlock (BD Pharmingen). To reduce nonspecific binding, the cells were incubated in staining buffer at 4 °C for 10 min prior to the addition of Abs. Following staining, the cells were washed × 3 and analyzed using a BD Canto II flow cytometer. Data analysis was performed using FlowJo (version 8.8.6, Ashland, OR) software. Cell sorting was accomplished with a BD FACSAria cell sorter.

Resident lung phagocytic labeling

Phagocytic labeling was based on a protocol from Maus et al. (22). In brief, mice were anesthetized using 1–3% isoflurane. PKH26-PCL (Sigma-Aldrich), a red fluorescent dye that labels phagocytic cells, was diluted by a factor of 20, using the diluent B solution (Sigma-Aldrich). Under sterile conditions, 100 µl diluted PKH26-PCL was administered by retro-orbital injection. The mice were allowed to recover for 24 h, then underwent filtered air or ozone exposure and were euthanized and harvested for flow cytometric analysis, as described in the above protocols. In addition to lung harvest, blood was drawn into a 23-gauge needle with a 1-ml syringe containing EDTA. The blood underwent RBC lysis using ACK Lysis Buffer to separate the RBCs from peripheral blood leukocytes. The leukocytes were washed in sterile PBS and then centrifuged at 1600 rpm for 5 min at 4 °C. The whole-blood leukocytes underwent flow cytometric analysis for PKH26-PCL. PKH26-PCL was visualized in the PE channel.

Airway physiology

Direct measurements of respiratory mechanics in response to methacholine were made using the flexiVent system (SCIREQ, Montreal, Canada) and reported as total resistance (R<sub>t</sub>) in H<sub>2</sub>O/ml/s. Anesthesia was achieved with 60 mg/kg pentobarbital sodium injected i.p. Mice were then given a neuromuscular blockade (0.8 mg/kg pancuronium bromide). Mice were ventilated with a computer-controlled ventilator (flexiVent) with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cm H<sub>2</sub>O. Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (0 mg/ml, 10 mg/ml, 25 mg/ml, and 100 mg/ml) was determined by resistance measurements every 30 s for 5 min, ensuring the parameters calculated had peaked. Total lung capacity breaths were administered after each dose of methacholine to maintain patent airways and return the measurements back to baseline. The resistance measurements were then averaged at each dose and graphed (R<sub>t</sub> cm H<sub>2</sub>O/ml/s), along with the initial baseline measurement.

Bronchoalveolar lavage fluid

Immediately after pulmonary function measurements, mice were overdosed with Nembutal (100 mg/kg) to euthanize them. The chest was opened, the trachea was exposed, and bronchoalveolar lavage (BAL) was performed by inserting a catheter into the mouse trachea with PE-90 tubing and instilling saline until the lung reached total lung capacity. This procedure was repeated three times. The total volume returned was the lavage return volume. Cells from the BAL fluid (BALF) were isolated using centrifugation (1500 rpm, 15 min, 15°C), and the supernatant was stored at −80°C for assessment of 8-isoprostanes, protein carbonyls, and cytokines. The cells were used for cell counts and differentials.

Cytokine measurements

Cytokines and chemokines were analyzed using a MILLIPLEX 5-MAP assay kit (Millipore, Billerica, MA) per the manufacturer’s protocol. This is a bead-based suspension array using the Luminox technology (Bio-Rad, Hercules, CA, USA), in which fluorescent-coded beads, known as microspheres, have cytokine capture Abs on the bead surface to bind the protein. CX3CL1 was measured from BALF, using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The plate was read on a plate reader at a wavelength of 450 nm.
8-Isoprostane measurements

8-isoprostanes were measured in both the BALF supernatant and the cell supernatant, using purification columns and an enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI). Samples were briefly diluted 1:2 with column buffer and applied to the purification columns. The sample passed entirely through the column. The column was then washed with column buffer and ultrapure water, and the washes were discarded. Next, 5 ml elution solution was added to the column and allowed to pass through to elute the 8-isoprostane. The solution that passed through the column was then collected in a 5-ml tube, and the elution solution was evaporated to dryness, using a stream of dry nitrogen gas to remove all quantities of organic solvent. The purified samples were then reconstituted with saline and used for the enzyme immunoassay kit (Cayman Chemical), following the manufacturer’s protocol. Samples, standards, buffer, bound 8-isoprostane AChE Tracer, and antiserum were added to the plate and incubated at 40°C for 18 h. The plate was then washed 5 times with wash buffer, and Ellman’s reagent (substrate for AChE tracer) was added. After 90 min, the plate was read on a plate reader at a wavelength of 405 nm. The 8-isoprostane concentrations was calculated by plotting the percent ratio of standard bound/maximum bound for each of the standards, using linear and log axes and performing a 4-parameter logistic fit.

Protein carbonyl

Protein carbonyls were measured in both the BALF supernatant and the cell supernatant using an Oxiselect Protein Carbonyl ELISA kit following the manufacturer’s protocol (Cell Biolabs, San Diego, CA). Briefly, BSA standards or samples were absorbed onto a 96-well plate for 2 h at 37°C. The protein carbonyls present in the sample or standard were derivatized to DNA hydrazone and probed with an anti-DNP Ab, followed by an HRP-conjugated secondary Ab. The plate was then read at 405 nm by a plate reader. The protein carbonyl content in the sample was determined by comparison with a standard curve that was prepared from predetermined reduced and oxidized BSA standards.

Real-time PCR

Total RNA was extracted from flow-sorted macrophages using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. Samples were treated with DNase and removal reagents to eliminate DNA contamination (catalog no. AM1906; Ambion, Austin, TX). RNA samples were then reverse transcribed into cDNA using SuperScript II RT (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. All real-time quantitative PCRs were performed using ABI SDS 7500 and SYBR Green reagent (Applied Biosystems, Foster City, CA). Primers were designed using ABI software and were produced by IDT (Coralville, IA). The sequences were as follows: NAD(P)H:quinone oxioreductase 1 (NQO1) forward, 5'-GTGGTTTGGGGTGCCAGCCAT-3'; NQO1 reverse, 5'-AGGATGCCACCTCTGAGCGCC-3'; CYP1B1 forward, 5'-CCAGGGGTGTCACCTGTC-3'; CYP1B1 reverse, 5'-TGAGAAGACCTGGCCCATGAC-3'; superoxide dismutase 1 (SOD-1) forward, 5'-GCCGGCGGATGAGAAG-3'; SOD-1 reverse, 5'-CCTTTCAGCAGTCATGC-3'; heme oxygenase 1 (HO-1) forward, 5'-CACCGCAACAGGCTCAA-3'; and HO-1 reverse, 5'-TCACGTTGTCATCTCCAGAGTTC-3'. PCR amplification was performed by the following program: 50°C, 2 min; 95°C, 10 min; 95°C 15 s; 60°C 1 min, for 40 cycles. Gene expression values were normalized to the housekeeping gene 18S. The data were presented as a fold change.

Protein expression

Protein carbonyls were measured using an ExMacs (which are a subset of macrophages) and a standard curve that was prepared from predetermined reduced and oxidized BSA standards.

Statistics

Data are expressed as mean ± SE. The statistical difference between groups was assessed by the Student t test. A two-tailed p value of <0.05 was considered statistically significant.

Results

Ozone exposure results in development of a Gr-1–positive subset of macrophages

To further characterize the influx of macrophages after ozone exposure, we performed multicolor flow cytometric analysis on whole-lung macrophages, using a previously described technique (16, 17). Lung macrophages are identified as autofluorescent*, MCHCII*CD11ch by flow cytometry. In previous work, we identified two types of macrophages: resident AMs and ExMacs. ExMacs were separated from AMs by enhanced cell surface expression of both CD11b and CX3CR1. To our surprise, flow cytometric analysis of whole-lung macrophages at 24 h after ozone exposure did not reveal a population of CD11b* macrophages consistent with previously reported ExMacs (Fig. 1A). Rather, we observed a population of autofluorescent* MCHCII*CD11ch macrophages, which were Gr-1lowCD11bhi. These were separate from other AMs, which were Gr-1lowCD11breg. The Gr-1–positive subset was not present in air-exposed mice. AMs and the Gr-1–positive subset both stained positive for the macrophage markers F4/80 and Mac-3 (Supplemental Fig. 1), confirming that they were, in fact, macrophages. Graphic representation of the percentage of Gr-1–positive macrophages as a percentage of total macrophages (Fig. 1B) and of total cells (Fig. 1C) confirmed this identification. This observation suggested that ozone exposure resulted in development of a novel macrophage subset, which was defined by increased Gr-1 expression.

Gr-1–positive macrophages are distinct from resident AMs

We were interested to determine whether unique differences between Gr-1–positive macrophages after ozone and resident macrophages (AMs) could be defined. In addition, we hoped to gain insight into a potential role of this subset in the host response to ozone lung injury. Flow cytometry was performed to identify additional cell surface markers, which could delineate Gr-1–positive macrophages from AMs. Using mice in which an allele for CX3CR1 is replaced, we performed multicolor flow cytometric analysis on a subset of macrophages. Ozone exposure resulted in development of a novel macrophage subset, which was defined by increased Gr-1 expression.

8-Isoprostanes were measured in both the BALF supernatant and the cell supernatant using a standard curve that was prepared from predetermined reduced and oxidized BSA standards. All real-time quantitative PCRs were performed using ABI SDS 7500 and SYBR Green reagent (Applied Biosystems, Foster City, CA). Primers were designed using ABI software and were produced by IDT (Coralville, IA). The sequences were as follows: NAD(P)H:quinone oxioreductase 1 (NQO1) forward, 5'-GTGGTTTGGGGTGCCAGCCAT-3'; NQO1 reverse, 5'-AGGATGCCACCTCTGAGCGCC-3'; CYP1B1 forward, 5'-CCAGGGGTGTCACCTGTC-3'; CYP1B1 reverse, 5'-TGAGAAGACCTGGCCCATGAC-3'; superoxide dismutase 1 (SOD-1) forward, 5'-GCCGGCGGATGAGAAG-3'; SOD-1 reverse, 5'-CCTTTCAGCAGTCATGC-3'; heme oxygenase 1 (HO-1) forward, 5'-CACCGCAACAGGCTCAA-3'; and HO-1 reverse, 5'-TCACGTTGTCATCTCCAGAGTTC-3'. PCR amplification was performed by the following program: 50°C, 2 min; 95°C, 10 min; 95°C 15 s; 60°C 1 min, for 40 cycles. Gene expression values were normalized to the housekeeping gene 18S. The data were presented as a fold change compared with 18S.

Statistics

Data are expressed as mean ± SE. The statistical difference between groups was assessed by the Student t test. A two-tailed p value of <0.05 was considered statistically significant.
with GFP (CX3CR1<sup>GFP/WT</sup>) to track CX3CR1 expression, we identified that Gr-1 macrophages (Gr-1 Macs) had enhanced CX3CR1 expression relative to AMs after ozone exposure. This finding was confirmed both by histogram (Fig. 2A) and by a significant difference in mean fluorescence intensity (MFI) (Fig. 2B). We also demonstrated that Gr-1 Macs had enhanced expression of MARCO by histogram analysis (Fig. 2C). This observation was confirmed by a statistically higher MFI in the Gr-1 Mac population (Fig. 2D). MARCO previously has been suggested to have a role in protection of the host from ozone-induced lung injury (10).

To further understand the difference between Gr-1 Macs and AMs, we performed flow-sorting experiments. Mice were exposed to ozone, and then whole lungs were processed for flow cytometry. Gr-1 Macs were sorted from AMs. The cells then underwent processing for mRNA analysis. Given the findings of MARCO expression, we used real-time PCR to identify antioxidant gene expression. We did not discover significant difference in expression between Gr-1 and AMs for the following genes: thioredoxin reductase, glutathione S-transferase A1, CCAAT/enhancer binding protein-β, CSF-1 receptor, glutathione peroxidase 1, runt-related transcription factor 3, glutathione S-transferase θ 1, glutathione S-transferase M1, and glutathione S-transferase P1 (Supplemental Fig. 3). However, Gr-1 Macs demonstrated significantly enhanced expression of NQO1 (Fig. 3A) and exhibited a trend toward enhanced expression of cytochrome P450, family 1, subfamily B, and polypeptide 1 (CYP1B1) (Fig. 3B). Gr-1 Macs had decreased expression of HO-1 and SOD-1 (Fig. 3C, 3D). Differential cell surface markers and differential mRNA expression support that Gr-1 Macs are, in fact, a unique subset that develops after ozone exposure. In addition, the profile of surface markers and gene expression suggested that this novel subset of macrophages may serve a protective role after inhalation of ozone.

**Gr-1–positive macrophages are derived from a resident lung source**

We and others have previously identified that the recruitment of ExMacs is dependent on recruitment of a CCR2-dependent circulating intermediate. We were therefore interested to determine if the source of Gr-1–positive macrophages after ozone was also due to recruitment into the lung. Recruitment of inflammatory monocytes, which mature into ExMacs and monocyte-derived dendritic cells, is dependent on the chemokine receptor CCR2 and its ligand, CCL2. Using C57BL/6 and CCR2-null mice, we found that no difference could be observed in the number of Gr-1 Macs after ozone exposure in a manner dependent on CCR2 (Supplemental Fig. 3). This observation suggested that our novel macrophage subset was not derived from a circulatory intermediate. On the basis of this result, we considered that Gr-1–positive macrophages could be derived from a resident lung source.

**FIGURE 2.** Cell surface expression of Gr-1<sup>high</sup> macrophages. A and B, CD11b<sup>high</sup> Gr-1<sup>low</sup> and CD11b<sup>low</sup> Gr-1<sup>high</sup> macrophages are analyzed for cell surface expression of CX3CR1, using CX3CR1<sup>GFP/WT</sup> mice after ozone exposure. Gr-1 Macs (red) have enhanced surface expression of CX3CR1 as compared with AM (blue) by histogram analysis (A) and MFI (B). The black open histogram represents unstained FITC channel auto-fluorescence, and the green open histogram represents air-exposed macrophages. C and D, Gr-1 Macs and AMs are analyzed for cell surface expression of MARCO. Gr-1 Macs (red) have enhanced cell surface expression for MARCO by histograms (C) and MFI (D), compared with ozone-exposed AMs (blue), isotype control (black), and air-exposed resident macrophages (green). Histograms are a representative sample of C57BL/6 and CX3CR1<sup>GFP/WT</sup> mice. MFI data are generated from four mice with both filtered air and ozone exposures with two repeats. ***p < 0.0005.

**FIGURE 3.** mRNA expression of Gr-1 Macs and resident Macs after ozone exposure. C57BL/6 mice were exposed to 2 ppm of ozone for 3 h, and then the lungs were harvested at 24 h. After digestion and centrifugation over an 18% nycodenz cushion, the cells are sorted by flow cytometry. Both AMs and Gr-1 Macs are sorted and then processed for mRNA analysis. A and B, Enhanced expression of Gr-1 Macs, compared with AMs, after ozone demonstrates enhanced NQO1 expression (A) and CYP1B1 expression (B). C and D, Reduced expression in Gr-1 Macs over AMs after ozone demonstrates reduced SOD-1 (C) and HO-1 (D). Data are from pooled samples of 15 mice, performed four times. mRNA was analyzed for each sample as fold change versus 18S. *p < 0.05.
intermediate. Therefore, we used a technique described by Maus et al. (22) and used by others (23–25), which uses the fluorescent dye PKH26-PCL (Sigma-Aldrich) to label resident phagocytic cells in the lung. Mice were given PKH26-PCL via retro-orbital injection. At 24 h after injection, the mice were exposed to either ozone or filtered air and processed for flow cytometry. As previously demonstrated by Maus et al., this dye does not label circulating blood cells, as identified by low cell surface expression of the phagocytic label (Fig. 4A, solid red histogram). Resident AMs (defined as MHCII$^\text{int}$ CD11c$^\text{hi}$ Gr-1$^\text{low}$ CD11b$^\text{low}$) successfully stain with the phagocyte label after either filtered air or ozone exposure (Fig. 4A, blue shaded histogram). Interestingly, Gr-1$^+$-positive macrophages (defined as MHCII$^\text{int}$ CD11c$^\text{hi}$ Gr-1$^\text{hi}$ CD11b$^\text{hi}$) also stained for the phagocyte label. To make sure that this staining of the phagocytic label was not due to the autofluorescence of macrophages, we compared both non–phagocyte-labeled (open histogram) and phagocyte-labeled (closed histogram) groups for air-exposed AMs (MHCII$^\text{int}$ CD11c$^\text{hi}$) (Fig. 4B), AMs after ozone (MHCII$^\text{int}$ CD11c$^\text{hi}$ Gr-1$^\text{low}$ CD11b$^\text{low}$) (Fig. 4C), and Gr-1$^+$-positive macrophages (MHCII$^\text{int}$ CD11c$^\text{hi}$ Gr-1$^\text{hi}$ CD11b$^\text{hi}$) (Fig. 4D). These data confirmed that the phagocyte labeling was not due to autofluorescence. These data further support that the Gr-1$^+$-positive macrophages present after ozone exposure are derived from a resident lung source and not recruited or derived from a circulating intermediate.

**CX3CR1 deficiency results in a reduction in Gr-1$^+$ macrophages and in enhanced response to ozone**

The previously described labeling of resident lung cells supports that our macrophage subset was derived from a resident source. Our present understanding of macrophages during lung injury has focused on macrophage differentiation from inflammatory monocytes recruited in a CCR2-dependent fashion. However, maturation of resident macrophages in the context of injury remains poorly understood. CX3CR1 has previously been implicated in the maintenance of resident cell populations. As CX3CR1 expression was enhanced in Gr-1$^+$ macrophages, we were interested to determine the effect of CX3CR1 deficiency on the maturation of Gr-1 Macs. Flow cytometry was performed on CX3CR1$^{GFP/WT}$ [wild-type (WT)] and CX3CR1$^{GFP/GFP}$ (null) after exposure to either air or ozone. Gr-1$^+$ macrophages were then identified. No difference was found in the air-exposed groups (Fig. 5A). However, we observed a significant reduction in the Gr-1$^+$ subpopulation after ozone exposure in the CX3CR1-null mice when compared with the WT mice. These data suggest that CX3CR1 was important for the maturation of Gr-1$^+$ macrophages.

Next, we determined the biological effect of CX3CR1 deficiency and the absence of Gr-1$^+$ macrophages in the context of ozone exposure. The role of CX3CR1 in response to ozone was unknown. CX3CL1 is the only known ligand for CX3CR1. We assessed the expression of CX3CL1 in the BAL of both WT and CX3CR1-null mice. CX3CL1 was increased in the lavage of WT ozone-exposed mice, compared with WT air-exposed mice (Fig. 5B). CX3CR1-null mice, both ozone exposed and air exposed, had elevated levels of CX3CL1 in the BAL. This observation is consistent with prior reports demonstrating the CX3CL1 is increased in CX3CR1-null mice at baseline owing to failed clearance of the ligand by the receptor (26). On the basis of increased expression of CX3CL1 after ozone, we were interested to determine whether CX3CR1-null mice had enhanced airway hyperresponsiveness (AHR) to methacholine challenge. There was no difference in the AHR of air-exposed WT mice and that of CX3CR1-null mice (Fig. 5C). WT mice exposed to ozone were noted to have enhanced AHR, compared with air controls. CX3CR1-null mice had even further enhanced AHR after ozone exposure compared with similarly exposed WT mice. This observation demonstrated that ozone-exposed CX3CR1-null mice develop enhanced AHR to methacholine challenge when compared with ozone-exposed WT mice. On the basis of this observation, we were interested to determine potential mechanisms that could account for this enhanced AHR. Evidence of enhanced total cells and neutrophils in the BAL of CX3CR1-null mice, compared with WT mice, after ozone exposure, was noted, consistent with enhanced inflammation (Fig. 5D). No significant difference in BAL total protein was observed between ozone-exposed WT and CX3CR1-null mice (Fig. 5E). However, evidence of enhanced oxidative stress was present, as measured by both 8-isoprostanes and protein carbonyl levels (Fig. 5F). BAL cytokines, recognized as elevated in the setting of ozone exposure, were further enhanced in ozone-exposed CX3CR1-null mice (Fig. 5G). CX3CR1 deficiency was associated with enhanced

![FIGURE 4. Analysis of macrophages after cell labeling with the dye PKH26-PCL. C57BL/6 mice are given the dye PKH26-PCL or control diluant via retro-orbital injection 24 h prior to exposure to 3 h of filtered air or 2 ppm of ozone. The mice were then harvested and analyzed by flow cytometry. At the same time, whole blood was obtained from the filtered air- and ozone-exposed mice. A, Histogram analysis of PKH26-PCL expression from whole blood (red histogram), AMs (blue histogram), and Gr-1 Macs (green histograms) after filtered air or ozone. B–D, Histogram analysis of phagocytic label with reagent diluant (control) versus PKH26-PCL for AMs after air exposure (B), for AMs after ozone exposure (C), and for Gr-1 Macs after ozone exposure (D). Data are representative of four mice per exposure with one repeat.](http://www.jimmunol.org/beta)
AHR, oxidative stress, and proinflammatory cytokines. Together these findings suggest that CX3CR1-dependent Gr-1+ macrophages are protective to the lung after ozone exposure.

**Discussion**

Our results demonstrate that inhalation of ozone results in the development of a novel macrophage subset characterized by enhanced expression of the cell surface marker Gr-1. These macrophages are not recruited from a circulating intermediate in a manner dependent on CCR2, but rather appear to be derived from a resident lung source. We demonstrate that this novel macrophage subset has enhanced cell surface expression of CX3CR1 and MARCO and a unique mRNA expression profile when compared with other resident macrophages. The development of this novel subset of macrophages is dependent on the chemokine receptor CX3CR1. Furthermore, CX3CR1-null mice have enhanced biological response to inhaled ozone. Our observations, in total, support a novel macrophage subset, which appears to protect the lung from the biological response to ambient ozone.

Ambient ozone remains a significant health burden, which can exacerbate chronic respiratory conditions such as asthma. Macrophages appear to be critical in the response to inhaled ozone. Pendino et al. (7) demonstrated that when the macrophage inhibitor gadolinium chloride was provided to rats prior to ozone exposure, these rats were protected from lung injury, as measured by decreased lavage fluid protein levels, inflammatory cells, cytokines, and NO-inducible NO synthase. We and others (27, 28) have identified macrophage-derived mediators present in the lung after ozone exposure. However, this observation remains somewhat inconsistent and may be dependent on the intensity and duration of exposure, as other groups have demonstrated that ozone exposure results in a decrease in macrophage-derived cytokines (29–31). Macrophages can also serve a protective role in the setting of ozone-induced lung injury. Work by Dahl et al. (10) showed that macrophage expression of both MARCO and scavenger receptor AI and AII was critical to protection against ozone injury in the lung via scavenging of oxidized lipid moieties. These ostensibly conflicting observations suggest that individual macrophage subpopulations may be present after ozone exposure and have divergent roles in the development of the host response.

Heightened interest in macrophage heterogeneity has evolved as a result of our understanding that macrophages have a variety of functions dependent on the local cytokine microenvironment to which they are exposed (12, 32). We previously showed that after both infectious and noninfectious lung injury ExMacs are recruited from a blood-borne intermediate (16, 17). We anticipated recruitment of ExMacs into the lung after ozone exposure. To our surprise, no significant accumulation of CD11b+ ExMacs was noted at 24 h after inhalation of ozone. In addition, no CCR2-dependent recruitment of macrophages was observed. This finding suggests that ExMacs are not recruited to the lung in the setting of ozone exposure. This lack of recruitment could be an issue of the timing after injury or may reflect the difference in the severity of the injury models between ozone and bleomycin or influenza.

In this study, we identified a macrophage subset that was not dependent on CCR2 and was delineated from other macrophages.
by expression of Gr-1, not CD11b. The Gr-1 epitope is expressed on the two molecules Ly-6G and Ly-6C. The expression of Ly-6G is restricted to granulocytes; therefore, Gr-1<sup>+</sup>/Ly-6G<sup>+</sup> cells are defined as neutrophils (33). Excluding neutrophils, Gr-1<sup>+</sup> expression within the lung has been identified with monocytes, with monocyte-derived dendritic cells, and, under certain injury conditions, with ExMacs (16, 17). It is generally accepted that macrophages and dendritic cells derived from monocytes in the setting of inflammation have increased expression of Gr-1, which reflects this recent differentiation from monocytes. In this setting, all of these cells are also characterized by being CD11b<sup>+</sup> and dependent on CCR2 for their migration. Several other descriptions of pulmonary macrophages with low levels of Gr-1 expression have been made. Gr-1<sup>dim</sup> macrophages, which also express CD11c, and low levels of both CD11b and F4/80 were identified after pulmonary infection with *Streptococcus pneumoniae* and were responsible for early production of TNF-α (34). Recent work described Gr-1<sup>dim</sup>/Ly-6G<sup>dull</sup> cells, which were also low in expressing for F4/80 (35). The presence of these cells was associated with the progression of *Mycobacterium tuberculosis* pulmonary infection. Although several descriptions of Gr-1 Macs and their function have been made, it remains unclear if a common cell exists among the various descriptions. Gr-1 Macs are associated with a proinflammatory, not protective, function in these experimental models. None of the prior studies that we are aware of have identified a Gr-1<sup>+</sup>-positive macrophage population from resident cells. This observation suggests that the Gr-1 Macs we speak of are different from those previously described. It remains unknown whether Gr-1 Macs have functional implications or represent a useful marker for identifying subpopulations of myeloid cells.

The appearance of this novel subpopulation after ozone suggested that it may have unique functions when compared with previously described macrophages. We demonstrated that Gr-1 Macs had enhanced cell surface expression of both CX3CR1 and MARCO. The high level of expression of MARCO suggested that these macrophages may be protective in the response to ozone, based on prior observations that MARCO served to scavenge oxidized lipid species produced after ozone exposure to the epithelial lining fluid (10). Drawing on this finding, we hypothesized that the expression profiles of Gr-1 Macs and the Gr-1<sup>+</sup>-negative lung macrophages would each be unique. After sorting, real-time PCR analysis revealed that these Gr-1 macrophages had enhanced expression of NQO1 and reduced expression of both SOD-1 and HO-1. NQO1 is a detoxifying 2-electron reductase that can act as an antioxidant through regeneration of antioxidant forms of ubiquinone and vitamin E quinine. Toxicology studies identify a cytoprotective role for NQO1. For example, NQO1 detoxifies quinones derived from the oxidation of phenolic metabolites of benzene (36), and the absence of NQO1 results in increased toxicity to menadione (37). These results established a role for NQO1 in protection against quinone toxicity. Myeloid expression of NQO1 is both inducible by the antioxidant response element NF-erythroid factor 2 and associated with resistance to oxidant-induced cell injury (38, 39). However, NQO1 has previously been reported to function as either an antioxidant or a pro-oxidant, depending on the quinine substrate and cell type. The high level of expression of both MARCO and NQO1 is suggestive of a proinflammatory, not protective, function in these macrophages. It is also clear that some macrophages function to limit pulmonary inflammation by clearance of inflammatory mediators such as oxidized lipid species and apoptotic cells (10, 44, 45). To better understand the complex functional role of lung macrophages after environmental challenges, delineation of macrophage subpopulations in the lung will be necessary.

We made the interesting discovery that Gr-1 Macs had enhanced CX3CR1 expression. CX3CR1 expression on blood monocytes defines them as constitutive monocytes important for the development and replenishment of resident niches of monocytes and macrophages (18). CX3CR1 is expressed on monocytes, macrophages, mucosal DCs, NK cells, CD8 T cells, and CD4 T cells (18, 46, 47). CX3CR1 has only one known ligand, CX3CL1 (also known as fractalkine). Despite the characterization that blood monocytes express CX3CR1 and data from other organ systems that suggest a critical role for the chemokine axis in inflammation (48–50), until recently there has been a dearth of information on the effect(s) this receptor/ligand axis exercises on the recruitment of cells into the lung during lung injury. CX3CL1 is constitutively expressed on lung macrophages (51), bronchial epithelium (52), and pulmonary endothelium (53). Multiple stimuli, including IFN-γ (52), chronic cigarette smoke (54), and allergen challenge (55), will cause increased pulmonary CX3CL1 expression. We identified that levels of CX3CL1 were increased in the lavage fluid of ozone-challenged C57BL/6 mice when compared with filtered air controls. The precise effect of CX3CL1 after inhalation of ozone remains unknown. Our findings suggest that CX3CL1 may contribute to the maturation of Gr-1 Macs. Prior work demonstrated that increased CX3CL1 appeared to be associated with the recruitment of CX3CR1<sup>+</sup> mononuclear phagocytes during chronic smoke exposure (54). A follow-up study revealed that CX3CL1, in fact, failed to recruit significant numbers of CX3CR1<sup>+</sup> cells into the airspaces, but rather was required for amplifying a subset of CD11b-expressing macrophages, which were spatially confined to the interstitium and were a source of IL-6 and TNF-α (51). This description of cells most consistently fits with prior descriptions of ExMacs (16, 17). We demonstrated the presence of a CX3CR1<sup>+</sup> macrophage population from the whole lung that did not have enhanced CD11b expression but instead had enhanced Gr-1. Gr-1<sup>+</sup> macrophages also seem to be dependent on CX3CR1 for their development after ozone. Gr-1<sup>+</sup>macrophages appear to be a unique macrophage subset when compared with circulatory-derived phagocytes present in the lung following exposure to cigarette smoke (54).

We found that CX3CR1 deficiency was associated with enhanced ozone-induced lung inflammation. This observation is in apparent conflict with prior reports demonstrating protection from the development of emphysema in a model of chronic smoke exposure (51) and protection from allergic airways disease (56).
We suspect that the discrepancy is related to the different models. Allergen challenge is likely to affect the adaptive immune system, whereas the acute response to ozone is predominantly dependent on the innate immune system (6). Chronic smoke exposure appears to cause chronic inflammation, which may affect different cell types and mechanisms. Cigarette smoke seems to stimulate or induce a CX3CR1-dependent recruitment of macrophages from circulating intermediates, and a similar process does not appear to evolve in our acute ozone exposure model. Our results provide strong support that, following exposure to environmental toxicants such as ozone, CX3CR1 is protective and, in addition, contributes to maturation of resident macrophages.

In summary, we identify a novel macrophage subset in the lungs of mice after inhalation of ozone. This unique macrophage subset is characterized by enhanced Gr-1 expression and enhanced cell surface expression of both MARCO and CX3CR1. These macrophages have a unique mRNA expression profile when compared with resident residual macrophages. The mRNA profile is characterized by enhanced NQO1 expression, which is suggestive of a detoxification phenotype. This macrophage subpopulation appears to be derived from resident lung phagocyte cells, not from a circulating intermediate. Finally, we establish that the presence of this macrophage subpopulation is dependent on CX3CR1. Furthermore, following ozone, CX3CR1 adds a protective element to lung defenses, because in the absence of CX3CR1 both biological (neutrophilic inflammation, oxidant stress, and proinflammatory cytokine production) and physiologic (AHR) phenotypes associated with ozone are enhanced. Together these observations support the existence of a novel subpopulation of protective de-toxifying lung macrophages that we now term ToxMacs. We speculate that ToxMacs limit the biological and physiologic responses to ozone owing to their ability to scavenge oxidized species and/or manage oxidant balance. Improved understanding of macrophage subpopulations may provide insights into potential treatments to protect against oxidant-induced lung injury or disease.

Disclosures
The authors have no financial conflicts of interest.

References
Corrections


Following an inquiry at Duke University, we have been informed that FlexiVent data provided to us by the animal pulmonary physiology laboratory may have been unreliable. The data in Figs. 1–4 are not affected. We have replicated the studies in Fig. 5A–F, and the new data support the originally published findings. We were unable to repeat the experiments in Fig. 5G; therefore, to maintain the accuracy of the scientific record, a corrected Fig. 5 and figure legend, representing the new data, are shown below.

![Corrected Figure 5](image-url)
Supplemental Figure 1

A

F4/80

AM - FA  AM - Ozone  Gr-1 Mac - Ozone

B

Mac-3

AM - FA  AM - Ozone  Gr-1 Mac - Ozone
Supplemental Figure 2
Supplemental Figure Legends

**Figure S1:** AMs and Gr-1 positive macrophages stain positive for F4/80 and Mac-3. Flow cytometry was performed on C57BL/6 mice 24 hours after 3 hours of filtered air or 2ppm of ozone. Macrophages were identified as MHCII<sup>int</sup> and CD11c<sup>+</sup>. Subgate analysis of macrophages was performed using CD11b vs. Gr-1 expression. This identified AM and Gr-1 positive macrophages. F4/80 (A) and Mac-3 (B) were identified to confirm that these stain for additional macrophage markers in the AM –FA, AM – Ozone and Gr-1 Mac – Ozone groups. The staining is compared to unstained controls. The experiment is representative from an experiment with 5 FA mice and 5 ozone exposed mice.

**Figure S2:** mRNA profile of flow cytometry sorted Gr-1 positive and resident macrophages after ozone exposure. C57BL/6 mice were exposed to 2ppm of ozone for three hours and then the lungs were harvested at 24 hours. After digestion and centrifugation over an 18% Nicodenz cushion, the cells are sorted by flow cytometry. Both resident alveolar macs (AM) and Gr-1 Macs are sorted and then processed for mRNA analysis. Analysis was performed for the following genes: TrxR – thioredoxin reductase, GSTA1 – glutathione s-transferase A1, C/EBP-beta – CCAAT/enhancer binding protein-beta, CSF1R – colony stimulating factor 1 receptor, GPX1 – glutathione peroxidase 1, RUNX3 – runt-related transcription factor 3, GSTT1 – glutathione s-transferase theta 1, GSTM1 – glutathione s-transferase M1, GSTP1 – glutathione s-transferase P1. Data is from pooled samples of 15 mice which were performed 4 times. mRNA was analyzed for each sample as fold change vs. 18s.

**Figure S3:** Gr-1+ Macrophages develop independent of CCR2. Flow cytometry analysis was performed at 24 hours after 3 hours of 2ppm ozone exposure. Macrophages were identified as
MHCII$^{\text{int}}$ and CD11c$^+$. Subgate analysis of macrophages was performed using CD11b vs. Gr-1 expression. A graphic representation of Gr-1 positive macrophages as a percentage of total cells in WT (open box) and CCR2 null mice (closed box) demonstrates no difference in the development of Gr-1 positive macrophages. Data were derived from 3-4 mice with both exposures and are representative of two separate experiments.