Published in final edited form as:

Toxicol Appl Pharmacol. 2015 April 15; 284(2): 236–245. doi:10.1016/j.taap.2015.02.002.

Regulation of ozone-induced lung inflammation and injury by the β -galactoside-binding lectin galectin-3

Vasanthi R. Sunil^{a,*}, Mary Francis^a, Kinal N. Vayas^a, Jessica A. Cervelli^a, Hyejeong Choi^a, Jeffrey D. Laskin^b, and Debra L. Laskin^a

Mary Francis: maryfranrutgers@gmail.com; Kinal N. Vayas: kinalv5@gmail.com; Jessica A. Cervelli: j.cervelli@pharmacy.rutgers.edu; Hyejeong Choi: choi@eohsi.rutgers.edu; Jeffrey D. Laskin: jlaskin@eohsi.rutgers.edu; Debra L. Laskin: laskin@eohsi.rutgers.edu

^aDepartment of Pharmacology & Toxicology, Rutgers University, Ernest Mario School of Pharmacy, Piscataway, NJ

^bDepartment of Environmental and Occupational Medicine, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ

Abstract

Macrophages play a dual role in ozone toxicity, contributing to both pro- and anti-inflammatory processes. Galectin-3 (Gal-3) is a lectin known to regulate macrophage activity. Herein, we analyzed the role of Gal-3 in the response of lung macrophages to ozone. Bronchoalveolar lavage (BAL) and lung tissue were collected 24–72 h after exposure (3 h) of WT and Gal-3^{-/-} mice to air or 0.8 ppm ozone. In WT mice, ozone inhalation resulted in increased numbers of proinflammatory (Gal-3⁺, iNOS⁺) and anti-inflammatory (MR-1⁺) macrophages in the lungs. While accumulation of iNOS⁺ macrophages was attenuated in Gal-3^{-/-} mice, increased numbers of enlarged MR-1⁺ macrophages were noted. This correlated with increased numbers of macrophages in BAL. Flow cytometric analysis showed that these cells were CD11b⁺ and consisted mainly (>97%) mature (F4/80⁺CD11c⁺) proinflammatory (Ly6G⁻Ly6C^{hi}) and anti-inflammatory (Ly6G⁻Ly6C^{lo}) macrophages. Increases in both macrophage subpopulations were observed following ozone inhalation. Loss of Gal-3 resulted in a decrease in Ly6C^{hi} macrophages, with no effect on Ly6C^{lo} macrophages. CD11b⁺Ly6G⁺Ly6C⁺ granulocytic (G) and monocytic (M) myeloid derived suppressor cells (MDSC) were also identified in the lung after ozone. In Gal-3^{-/-} mice, the response of G-MDSC to ozone was attenuated, while the response of M-MDSC was heightened. Changes in inflammatory cell populations in the lung of ozone treated Gal-3^{-/-} mice were correlated with reduced tissue injury as measured by cytochrome b5 expression. These data demonstrate that Gal-3 plays a role in promoting proinflammatory macrophage accumulation and toxicity in the lung following ozone exposure.

© 2015 Published by Elsevier Inc.

*Corresponding author at: Rutgers University, Ernest Mario School of Pharmacy, 160 Frelinghuysen Road, Piscataway, NJ 08854; Tel: 848-445-6190; sunilva@pharmacy.rutgers.edu.

Conflict of interest

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Ozone; gal-3; macrophage phenotype; flow cytometry; MDSC

Introduction

Ozone is a highly reactive oxidant present in urban air pollution. The toxicological effects of ozone are attributed to its ability to cause oxidation and peroxidation of membrane lipids and proteins, either directly or indirectly, through the generation of reactive intermediates (Pryor and Church, 1991). This leads to damage of the respiratory epithelium, disruption of alveolar epithelial barrier function, edema and inflammation (Hollingsworth *et al.*, 2007; Al-Hegelan *et al.*, 2011). Evidence suggests that inflammatory macrophages accumulating in the lung in response to ozone-induced injury contribute to pulmonary toxicity [reviewed in (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011)]. Thus, following exposure to products released from ozone-injured epithelial cells, lung macrophages are classically activated to release cytotoxic/proinflammatory mediators including reactive oxygen and nitrogen species which promote tissue injury. This is supported by findings that blocking proinflammatory macrophages or cytotoxic mediators they release protects against ozone-induced lung injury (Pendino *et al.*, 1995; Kleeberger *et al.*, 2001; Fakhrzadeh *et al.*, 2002; Toward and Broadley, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b). Macrophages have also been shown to play a protective role in the lung following ozone exposure, clearing oxidized products and cellular debris (Ishii *et al.*, 1998; Dahl *et al.*, 2007), augmenting lung antioxidant activity, and releasing mediators that suppress inflammation and initiate wound repair (Reinhart *et al.*, 1999; Dahl *et al.*, 2007; Backus *et al.*, 2010). These activities are mediated by distinct subpopulations of macrophages that are alternatively activated (Byers and Holtzman, 2011; Laskin *et al.*, 2011). It appears that the outcome of the pathogenic response to ozone depends in part, on the relative numbers of classically (M1) and alternatively (M2) activated macrophage subpopulations in the lung and their level of activation.

Galectin-3 (Gal-3) is a β -galactoside-binding lectin important in diverse biological processes including cell proliferation, adhesion, differentiation and regulation of the immune system (Perillo *et al.*, 1998; Liu, 2005; Liu and Hsu, 2007). Gal-3 is expressed by activated macrophages, and it acts in an autocrine and paracrine manner to promote macrophage release of proinflammatory mediators including tumor necrosis factor, IL-12, CCL3 and CCL4, as well as reactive nitrogen species generated via inducible nitric oxide synthase (iNOS) (Nishi *et al.*, 2007; Papaspyridonos *et al.*, 2008; Jeon *et al.*, 2010; Dragomir *et al.*, 2012a; Dragomir *et al.*, 2012b). In the present studies, mice with a targeted disruption of the *gal-3* gene were used to analyze its role in ozone-induced macrophage activation and tissue injury. We found that loss of Gal-3 resulted in decreased accumulation of proinflammatory/cytotoxic macrophages in the lung following ozone intoxication. This was correlated with increases in anti-inflammatory/wound repair macrophages and M-MDSC, and reduced lung injury. These data provide support for a role of Gal-3 and proinflammatory/cytotoxic macrophages in the pathogenic response of mice to ozone. Identifying specific macrophage

subpopulations involved in ozone toxicity and proteins regulating their activity may lead to the development of new therapeutic approaches for reducing inflammatory lung injury.

Materials and methods

Animals and exposures

Female specific pathogen-free C57Bl6/J wild type (WT) and B6.Cg-Lgals3^{tm1Poi}/J (Gal-3^{-/-}) mice (8–11 weeks; 17–22 g) were obtained from The Jackson Laboratories (Bar Harbor, ME). Animals were housed in filter-top microisolation cages and maintained on food and water *ad libitum*. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Animals were exposed to ultra-pure air (Messer Gas, Allentown, PA) or 0.8 ppm ozone for 3 h in a whole body Plexiglas chamber. Ozone was generated from oxygen gas via an ultraviolet light ozone generator (Gilmont Instruments, Barrington, IL) and mixed with air. Concentrations inside the chamber were monitored using a Photometric ozone analyzer (model 400E, Teledyne Instruments, City of Industry, CA).

Sample collection

Animals were euthanized 24–72 h after exposure by intraperitoneal injection of Nembutal (200 mg/kg). BAL was collected by slowly instilling and withdrawing 1 ml of PBS into the lung three times through a cannula in the trachea. BAL fluid was centrifuged (300 × g, 8 min), supernatants collected, aliquoted, and stored at –80°C until analysis. Cell pellets were resuspended in 1 ml PBS and viable cells counted on a hemocytometer using trypan blue dye exclusion. The lung was then removed and instilled five times with additional PBS (1 ml) while massaging the tissue. Lavage fluid was centrifuged (400 × g, 6 min, 4°C), the cell pellet resuspended in 1 ml PBS and combined with the first BAL lavage cell suspension. Cells were washed twice with PBS and resuspended in 200 µl staining buffer (PBS, 2% FCS and 0.02% sodium azide). Total protein was quantified in cell-free BAL using a BCA Protein Assay kit (Pierce Biotechnologies Inc., Rockford, IL) with bovine serum albumin as the standard. Samples (25 µl) from 8 mice per treatment group were analyzed in triplicate and evaluated at 560 nm on a Vmax MAXlineTM microplate reader (Molecular Devices, Sunnyvale, CA).

Flow Cytometry

Cells were incubated for 10 min at 4°C with anti-mouse CD16/32 (1:200; Biolegend, San Diego, CA) to block nonspecific binding, and then with FITC-conjugated anti-mouse CD11b (1:200; Biolegend), PE-conjugated anti-mouse Ly6C (1:200; Biolegend), PE/Cy7-conjugated anti-mouse F4/80 (1:200; Biolegend), AF 700-conjugated anti-mouse CD11c (1:200, Biolegend), and AF 647-conjugated anti-mouse Ly6G (1:200; Biolegend) antibodies for 30 min at 4°C, followed by eFluor 780-conjugated fixable viability dye (1:1000; eBioscience, San Diego, CA). Cells were analyzed on a Gallios Flow Cytometer (Beckman Coulter, Brea, CA), and data analyzed using Beckman Coulter Kaluza version 1.2 software. The gating strategy and cell populations characterized are shown in Fig. 1.

Immunohistochemistry

The lung was inflated *in situ* via the trachea with PBS containing 3% paraformaldehyde. After 4 h on ice, the tissue was transferred to 50% ethanol. Tissue sections (4 μ m) were prepared, deparaffinized with xylene (4 min, x 2), followed by decreasing concentrations of ethanol (100%–50%) and finally, water. After antigen retrieval using citrate buffer (10.2 mM sodium citrate, 0.05% Tween 20, pH 6.0) and quenching of endogenous peroxidase with 3% H₂O₂ for 15 min, sections were incubated for 2 h at room temperature with 10–100% goat serum to block nonspecific binding. This was followed by overnight incubation at 4°C with rabbit IgG or rabbit polyclonal anti-Gal-3 (1:2000; R&D Systems, Minneapolis, MN), anti-iNOS (1:750; Abcam, Cambridge, MA), anti-mannose receptor (MR)-1 (1:1000; Abcam), or anti-cytochrome b5 (1:250; Abcam) antibodies. Sections were then incubated with biotinylated secondary antibody (Vector Labs, Burlingame, CA) for 30 min at room temperature. Binding was visualized using a Peroxidase Substrate Kit DAB (Vector Labs). Random sections from three mice per treatment group were analyzed by light microscopy using an Olympus BX51 microscope (Olympus America Inc., Center Valley, PA).

Statistical analysis

All experiments were repeated at least 3 times. Data were analyzed using student's t-test and 2-way ANOVA; a p value of <0.05 was considered statistically significant.

Results

In previous studies we demonstrated that both proinflammatory/cytotoxic M1 macrophages and anti-inflammatory/wound repair M2 macrophages accumulate in the lung following ozone exposure (Sunil *et al.*, 2012). To assess the role of Gal-3 in the accumulation of these macrophage subpopulations in the lung, we used Gal-3^{-/-} mice. In air exposed WT mice, low level Gal-3 staining was evident in lung epithelium and Type II cells, but not in macrophages (Fig. 2). Treatment of mice with ozone resulted in a time related increase in numbers of Gal-3⁺ macrophages in the lung, with no effect on epithelial cells. As expected, Gal-3 staining was not detectable in lungs of air or ozone exposed Gal-3^{-/-} mice (Fig. 2). Following ozone exposure, an increase in iNOS⁺ M1 macrophages was observed in the lungs of WT mice at 24 h and 48 h (Fig. 3). At these times, iNOS was also evident in Type II epithelial cells. By 72 h post ozone, iNOS expression was reduced to levels below control. We also observed increased numbers of MR-1⁺ M2 macrophages in the lung at 24 h and 48 h after ozone (Fig. 4). Whereas ozone-induced increases in iNOS⁺ macrophages, as well as Type II cells, were reduced in Gal-3^{-/-} mice relative to WT mice, numbers of MR-1⁺ macrophages were increased. These cells were larger than MR-1⁺ macrophages in lungs of WT mice and most prominent at 24 h and 48 h post-exposure (Fig. 4). Small MR-1⁺ macrophages were also noted in air exposed Gal-3^{-/-} mice, but not in WT mice. Increases in MR-1⁺ macrophages in ozone-exposed Gal-3^{-/-} mice were correlated with increases in BAL cell number (Fig. 5). Differential analysis revealed that these cells were >97% macrophages.

To further characterize lung macrophages responding to ozone and to assess the effects of loss of Gal-3, we used techniques in flow cytometry. In these experiments, cells were first analyzed for expression of CD11b, a beta₂-integrin expressed on infiltrating myeloid cells

(Fullerton *et al.*, 2013). This was followed by analysis of the granulocytic marker, Ly6G (Lee *et al.*, 2013), the macrophage activation marker, Ly6C (Zimmermann *et al.*, 2012; Epelman *et al.*, 2014), and the mature alveolar macrophage markers, F4/80 and CD11c (Grundy and Sentman, 2005; Zaynagetdinov *et al.*, 2013; Ji *et al.*, 2014) (Fig. 1). Consistent with previous studies (Garn *et al.*, 2006; Matthews *et al.*, 2007; Zaslona *et al.*, 2014), resident alveolar macrophages from air exposed mice were identified as CD11b⁺CD11c⁺F4/80⁺Ly6G⁺Ly6C^{lo} (Fig. 1). These cells were largely unaffected by ozone (data not shown). Conversely, ozone caused a significant increase in CD11b⁺ infiltrating myeloid cells in the lung; these cells were comprised of monocytic Ly6G⁺ and granulocytic Ly6G⁺ subpopulations (Table 1 and Fig. 1). In both WT and Gal-3^{-/-} mice, the majority of the infiltrating cells (>97%) were Ly6G⁺ macrophages. Ozone exposure resulted in an increase in both Ly6G⁺ and Ly6G⁺ cells in WT, as well as Gal-3^{-/-} mice. Greater numbers of granulocytic Ly6G⁺ cells were observed in Gal-3^{-/-} mice, when compared to WT mice, 72 h post ozone exposure (Table 1).

Further analysis of the Ly6G⁺ cells revealed that they consisted of two major subpopulations: proinflammatory Ly6C^{hi} and anti-inflammatory Ly6C^{lo} macrophages; most of these cells were mature lung macrophages, expressing F4/80⁺ and CD11c⁺. Treatment of WT mice with ozone resulted in an increase in both Ly6C^{hi} and Ly6C^{lo} macrophages (Fig. 6). While proinflammatory Ly6C^{hi} macrophages increased rapidly (within 24 h) and remained elevated for at least 72 h, increases in anti-inflammatory Ly6C^{lo} macrophages were not evident until 72 h post ozone. Loss of Gal-3 resulted in a significant decrease in numbers of proinflammatory Ly6C^{hi} macrophages in the lung at all post exposure times, with no significant effects on anti-inflammatory Ly6C^{lo} macrophages (Fig. 6).

Flow cytometric analysis of CD11b⁺Ly6G⁺ infiltrating granulocytic cells revealed that they also expressed high levels of Ly6C, a characteristic feature of myeloid derived suppressor cells (MDSC) (Kong *et al.*, 2013). These cells were found to consist of F4/80⁺ granulocytic (G) and F4/80⁺ monocytic (M) subpopulations (Fig. 1). In WT mice, a time related increase in both subpopulations was observed in the lung beginning 24 h after ozone and persisting for at least 72 h (Fig. 7). While loss of Gal-3 resulted in an attenuated response of G-MDSC to ozone at 48 h and 72 h, a heightened response was observed in the M-MDSC at 24 h and 72 h (Fig. 7).

In further studies we determined if changes in lung macrophage subpopulations in Gal-3^{-/-} mice were correlated with alterations in markers of ozone-induced oxidative stress and injury. Treatment of WT mice with ozone resulted in a marked upregulation of cytochrome b5 expression, an indicator of acute lung injury and oxidative stress (Rodriguez-Ariza *et al.*, 2005; Menoret *et al.*, 2012) (Fig. 8). This was most notable in macrophages and epithelial cells 24 h and 48 h post exposure. Loss of Gal-3 significantly attenuated this response. Ozone exposure also resulted in increases in BAL protein content indicating alveolar-epithelial barrier dysfunction (Bhalla, 1999); however, this was largely unaffected by loss of Gal-3 (Fig. 5).

Discussion

Macrophages have been shown to be important in promoting both acute and chronic inflammation, as well as in suppressing inflammatory responses and inducing wound repair (Hollingsworth *et al.*, 2007; Laskin *et al.*, 2011). Evidence suggests that these diverse activities are mediated by distinct subpopulations of macrophages that appear sequentially in tissues in response to injury (Byers and Holtzman, 2011; Laskin *et al.*, 2011). Consistent with this notion are previous reports that, initially, proinflammatory/cytotoxic M1 macrophages, and subsequently anti-inflammatory/wound repair M2 macrophages, accumulate in the lung after ozone, and that these cells contribute to tissue injury and repair, respectively (Pendino *et al.*, 1994; Koike *et al.*, 1998; Hollingsworth *et al.*, 2007; Tighe *et al.*, 2011; Sunil *et al.*, 2012). The present studies identify a novel population of macrophages in the lung after exposure to ozone that is important in promoting proinflammatory signaling and tissue injury. These cells express Gal-3, a carbohydrate-binding lectin, known to regulate macrophage activation (Perillo *et al.*, 1998; Liu, 2005; Liu and Hsu, 2007; Nishi *et al.*, 2007). These findings are important, as they provide new mechanistic insights into the pathogenesis of ozone-induced lung injury.

Gal-3 is thought to regulate macrophage functioning via its interaction with CD98, a membrane glycoprotein involved in promoting inflammation and fibrosis (Dong and Hughes, 1997; Lopez *et al.*, 2006; Maldonado *et al.*, 2011; Mackinnon *et al.*, 2012). Gal-3 has been shown to play a role in regulating inflammation and in the pathophysiology of emphysema and pulmonary fibrosis, as well as in acute hepatotoxicity, chronic kidney disease and cancer, pathologies associated with excessive inflammatory macrophage activity (Nishiyama *et al.*, 2000; Henderson *et al.*, 2008; Dragomir *et al.*, 2012a; Dragomir *et al.*, 2012b; Mukaro *et al.*, 2013; O'Seaghdha *et al.*, 2013; Fortuna-Costa *et al.*, 2014). We found that ozone exposure resulted in a time related increase in Gal-3⁺ macrophages in the lung. Similar increases in Gal-3⁺ macrophages have been observed in the lung after exposure to other pulmonary toxicants including nitrogen mustard, radiation and ultrafine particles (Kasper and Hughes, 1996; Andre *et al.*, 2006; Malaviya *et al.*, 2012; Van der Meeren *et al.*, 2014). Increased Gal-3 expression has also been noted in lungs of humans with inflammatory diseases such as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis (Nishi *et al.*, 2007; Pilette *et al.*, 2007). These findings suggest that upregulation of Gal-3 may represent a common pathway for promoting proinflammatory/cytotoxic activation of macrophages in diverse pulmonary pathologies.

In accord with our earlier studies (Pendino *et al.*, 1995; Fakhrzadeh *et al.*, 2002; Groves *et al.*, 2013), following ozone exposure, we observed increased numbers of both iNOS⁺ and MR-1⁺ macrophages in the lung, markers of proinflammatory and anti-inflammatory macrophages, respectively. For both iNOS⁺ and MR-1⁺ macrophage subpopulations, maximal accumulation was evident 24–48 h post exposure. The fact that ozone-induced increases in MR-1⁺ macrophages were greater in Gal-3^{-/-} mice, when compared to WT mice, suggests a more prominent induction of tissue repair processes in these mice. Gal-3 has previously been shown to upregulate iNOS expression in brain macrophages (Jeon *et al.*, 2010). The present studies suggest a similar regulatory role for Gal-3 in lung macrophage iNOS expression. This is based on our observation that loss of Gal-3 resulted in reduced

numbers of iNOS⁺ macrophages in the lung. Findings that decreases in iNOS⁺ macrophages in Gal-3^{-/-} mice were correlated with increases in MR-1⁺ macrophages support the idea that the pathogenic response to ozone involves a balance in the activity of M1 and M2 macrophages. Increased numbers of MR-1⁺ macrophages in lungs of Gal-3^{-/-} mice following ozone exposure may contribute to reduced lung injury in these mice. This is supported by our findings that in Gal-3^{-/-} mice, MR-1⁺ macrophages are enlarged, relative to cells in WT mice, and foamy in appearance, indicating robust tissue repair activity (Boven *et al.*, 2006). These data suggest that in the absence of Gal-3, there is a shift in the balance of macrophage subpopulations towards an M2 phenotype resulting in more rapid tissue repair.

Cytochrome b5 is a highly conserved protein that participates in the oxidation of endogenous and xenobiotic substances (Vergeres and Waskell, 1995; Schenkman and Jansson, 2003; Menoret *et al.*, 2012). It is rapidly upregulated in the lung after enterotoxin inhalation and is thought to be an early marker of acute lung injury, inflammation and oxidative stress (Menoret *et al.*, 2012). Consistent with ozone-induced oxidative stress and lung injury, we found that cytochrome b5 expression was upregulated in the lung after ozone. The observation that this response was reduced in Gal-3^{-/-} mice, are in accord with a proinflammatory/cytotoxic role of Gal-3 in ozone-induced injury. In contrast, loss of Gal-3 had no significant effect on ozone-induced increases in BAL protein indicating that proinflammatory mediators generated by Gal-3⁺ macrophages do not contribute to alveolar epithelial barrier dysfunction.

In contrast to the lack of effects of Gal-3 on BAL protein, BAL macrophage number was increased in Gal-3^{-/-} mice, relative to WT mice, following ozone exposure. To determine if this was due to a shift in the phenotype of cells accumulating in the lung, we characterized the cells using techniques in flow cytometry. In WT and Gal-3^{-/-} mice, ozone exposure resulted in increased numbers of infiltrating (CD11b⁺) monocytic (Ly6G⁻) and granulocytic (Ly6G⁺) inflammatory cells in the lung. The majority of the cells were Ly6G⁻, consistent with the observation that macrophages are the predominant cell population recovered in BAL (Ji *et al.*, 2014). Whereas there were no major differences between WT and Gal-3^{-/-} mice in the response of Ly6G⁻ monocytic cells to ozone, maximum increases in Ly6G⁺ granulocytic cells were delayed in Gal-3^{-/-} mice relative to WT mice. These differences likely reflect the unique phenotype of the responding cell populations. This is supported by our findings of heterogeneity within the Ly6G⁻ and Ly6G⁺ cell populations. Thus, while Ly6G⁻ cells consisted of mature (F4/80⁺CD11c⁺) proinflammatory Ly6C^{hi} and anti-inflammatory Ly6C^{lo} macrophages, Ly6G⁺ cells consisted of F4/80⁺ monocytic and F4/80⁻ granulocytic Ly6C⁺ MDSC. Each of these cell populations responded distinctly to ozone and was differentially affected by loss of Gal-3. Whereas in WT mice, Ly6C^{hi} macrophages increased rapidly in the lung and persisted for at least 72 h following ozone exposure, the appearance of Ly6C^{lo} macrophages was delayed for 72 h. This is similar to our immunohistochemistry data which showed sequential accumulation of proinflammatory iNOS⁺ and anti-inflammatory MR-1⁺ macrophages in the lung after ozone. Further analysis of lung macrophages revealed that in WT mice, most of the Ly6C^{hi} proinflammatory cells were F4/80⁺CD11c⁺ indicating a mature phenotype. Loss of Gal-3 resulted in a decrease in

proinflammatory Ly6C^{hi} cells in the lung, which correlated with reduced numbers of proinflammatory iNOS⁺ macrophages. These data are consistent with reports that Ly6C^{hi} macrophages express iNOS (Dragomir *et al.*, 2012a), and provide further support for the proinflammatory actions of Gal-3. In contrast to the effects of loss of Gal-3 on Ly6C^{hi} cells, there were no major effects on anti-inflammatory Ly6C^{lo} macrophages, despite immunohistochemical evidence of increased numbers of MR-1⁺ macrophages in the lung after ozone. These data are in accord with the idea that there are multiple phenotypically distinct populations of anti-inflammatory/wound repair macrophages that respond to tissue injury (Alber *et al.*, 2012; Aggarwal *et al.*, 2014; Wang *et al.*, 2014).

MDSC consist of a heterogeneous population of hematopoietic monocytic and granulocytic precursors with immunosuppressive and anti-inflammatory activity (Nagaraj and Gabrilovich, 2012). MDSC have been implicated in wound healing, a process thought to be mediated via the release of TGF- β and IL-10 (Chambers *et al.*, 2013; Sarkar *et al.*, 2013). Under inflammatory conditions, MDSC expand and develop into G-MDSC or M-MDSC, which are thought to contribute to protection against unrestrained inflammation (Chambers *et al.*, 2013; Sarkar *et al.*, 2013). Following ozone inhalation, we identified both G-MDSC and M-MDSC in the lungs of WT mice. MDSC have been shown to play a key role in the resolution of acute inflammation following spinal cord injury and in suppressing autoimmune disease in the central nervous system (Yi *et al.*, 2012; Saiwai *et al.*, 2013), and they may similarly be important in limiting ozone-induced inflammation and injury. Loss of Gal-3 was associated with a decrease in G-MDSC at 48 h and 72 h post ozone, but an increase in M-MDSC, which was most notable at 72 h. These findings suggest that in the absence of Gal-3, M-MDSC play a more prominent role in the resolution of tissue injury.

In summary, the present studies identify multiple monocytic subpopulations accumulating in the lung following ozone inhalation, and demonstrate a role for Gal-3 in promoting proinflammatory macrophage activation which contributes to pulmonary toxicity. These studies also demonstrate that there are increased numbers of inflammatory cells displaying a M-MDSC phenotype in the lungs of ozone-treated Gal-3^{-/-} mice, which we speculate contribute to the resolution of tissue injury. Elucidating specific subpopulations of inflammatory cells responding to ozone and their function may lead to development of novel approaches for mitigating pulmonary toxicity.

Acknowledgments

This work was supported by NIH grants ES004738, AR055073, ES007148 and ES005022.

Abbreviations

Gal-3	galectin-3
BAL	bronchoalveolar lavage
iNOS	inducible nitric oxide synthase
MR-1	mannose receptor-1

MDSC	myeloid derived suppressor cell
WT	wild type
M	monocytic
G	granulocytic

References

- Aggarwal NR, King LS, D'Alessio FR. Diverse macrophage populations mediate acute lung inflammation and resolution. *Am J Physiol Lung Cell Mol Physiol*. 2014; 306:L709–725. [PubMed: 24508730]
- Al-Hegelan M, Tighe RM, Castillo C, Hollingsworth JW. Ambient ozone and pulmonary innate immunity. *Immunol Res*. 2011; 49:173–191. [PubMed: 21132467]
- Alber A, Howie SE, Wallace WA, Hirani N. The role of macrophages in healing the wounded lung. *Int J Exp Pathol*. 2012; 93:243–251. [PubMed: 22774768]
- Andre E, Stoeger T, Takenaka S, Bahnweg M, Ritter B, Karg E, Lentner B, Reinhard C, Schulz H, Wjst M. Inhalation of ultrafine carbon particles triggers biphasic pro-inflammatory response in the mouse lung. *Eur Respir J*. 2006; 28:275–285. [PubMed: 16641123]
- Backus GS, Howden R, Fostel J, Bauer AK, Cho HY, Marzec J, Peden DB, Kleeberger SR. Protective role of interleukin-10 in ozone-induced pulmonary inflammation. *Environ Health Perspect*. 2010; 118:1721–1727. [PubMed: 20826374]
- Bhalla DK. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *J Toxicol Environ Health B Crit Rev*. 1999; 2:31–86. [PubMed: 10081525]
- Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, Aerts JM, Amor S, Nieuwenhuis EE, Laman JD. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. *Brain*. 2006; 129:517–526. [PubMed: 16364958]
- Byers DE, Holtzman MJ. Alternatively activated macrophages and airway disease. *Chest*. 2011; 140:768–774. [PubMed: 21896520]
- Chambers SE, O'Neill CL, O'Doherty TM, Medina RJ, Stitt AW. The role of immune-related myeloid cells in angiogenesis. *Immunobiology*. 2013; 218:1370–1375. [PubMed: 23932437]
- Dahl M, Bauer AK, Arredouani M, Soininen R, Tryggvason K, Kleeberger SR, Kobzik L. Protection against inhaled oxidants through scavenging of oxidized lipids by macrophage receptors MARCO and SR-A/II. *J Clin Invest*. 2007; 117:757–764. [PubMed: 17332894]
- Dong S, Hughes RC. Macrophage surface glycoproteins binding to galectin-3 (Mac-2-antigen). *Glycoconj J*. 1997; 14:267–274. [PubMed: 9111144]
- Dragomir AC, Sun R, Choi H, Laskin JD, Laskin DL. Role of galectin-3 in classical and alternative macrophage activation in the liver following acetaminophen intoxication. *J Immunol*. 2012a; 189:5934–5941. [PubMed: 23175698]
- Dragomir AC, Sun R, Mishin V, Hall LB, Laskin JD, Laskin DL. Role of galectin-3 in acetaminophen-induced hepatotoxicity and inflammatory mediator production. *Toxicol Sci*. 2012b; 127:609–619. [PubMed: 22461450]
- Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. *Immunity*. 2014; 41:21–35. [PubMed: 25035951]
- Fakhrzadeh L, Laskin JD, Gardner CR, Laskin DL. Superoxide dismutase-overexpressing mice are resistant to ozone-induced tissue injury and increases in nitric oxide and tumor necrosis factor- α . *Am J Respir Cell Mol Biol*. 2004a; 30:280–287. [PubMed: 12855403]
- Fakhrzadeh L, Laskin JD, Laskin DL. Deficiency in inducible nitric oxide synthase protects mice from ozone-induced lung inflammation and tissue injury. *Am J Respir Cell Mol Biol*. 2002; 26:413–419. [PubMed: 11919077]

- Fakhrzadeh L, Laskin JD, Laskin DL. Ozone-induced production of nitric oxide and TNF- α and tissue injury are dependent on NF- κ B p50. *Am J Physiol Lung Cell Mol Physiol*. 2004b; 287:L279–285. [PubMed: 15064226]
- Fortuna-Costa A, Gomes AM, Kozłowski EO, Stelling MP, Pavao MS. Extracellular galectin-3 in tumor progression and metastasis. *Front Oncol*. 2014; 4:138. [PubMed: 24982845]
- Fullerton JN, O'Brien AJ, Gilroy DW. Pathways mediating resolution of inflammation: when enough is too much. *J Pathol*. 2013; 231:8–20. [PubMed: 23794437]
- Garn H, Siese A, Stumpf S, Wensing A, Renz H, Gerns D. Phenotypical and functional characterization of alveolar macrophage subpopulations in the lungs of NO₂-exposed rats. *Respir Res*. 2006; 7:4. [PubMed: 16398938]
- Groves AM, Gow AJ, Massa CB, Hall L, Laskin JD, Laskin DL. Age-related increases in ozone-induced injury and altered pulmonary mechanics in mice with progressive lung inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2013; 305:L555–568. [PubMed: 23997172]
- Grundy M, Sentman CL. GFP transgenic mice show dynamics of lung macrophages. *Exp Cell Res*. 2005; 310:409–416. [PubMed: 16171803]
- Henderson NC, Mackinnon AC, Farnworth SL, Kipari T, Haslett C, Iredale JP, Liu FT, Hughes J, Sethi T. Galectin-3 expression and secretion links macrophages to the promotion of renal fibrosis. *Am J Pathol*. 2008; 172:288–298. [PubMed: 18202187]
- Hollingsworth JW, Kleeberger SR, Foster WM. Ozone and pulmonary innate immunity. *Proc Am Thorac Soc*. 2007; 4:240–246. [PubMed: 17607006]
- Ishii Y, Hashimoto K, Nomura A, Sakamoto T, Uchida Y, Ohtsuka M, Hasegawa S, Sagai M. Elimination of neutrophils by apoptosis during the resolution of acute pulmonary inflammation in rats. *Lung*. 1998; 176:89–98. [PubMed: 9500294]
- Jeon SB, Yoon HJ, Chang CY, Koh HS, Jeon SH, Park EJ. Galectin-3 exerts cytokine-like regulatory actions through the JAK-STAT pathway. *J Immunol*. 2010; 185:7037–7046. [PubMed: 20980634]
- Ji WJ, Ma YQ, Zhou X, Zhang YD, Lu RY, Sun HY, Guo ZZ, Zhang Z, Li YM, Wei LQ. Temporal and spatial characterization of mononuclear phagocytes in circulating, lung alveolar and interstitial compartments in a mouse model of bleomycin-induced pulmonary injury. *J Immunol Methods*. 2014; 403:7–16. [PubMed: 24280595]
- Kasper M, Hughes RC. Immunocytochemical evidence for a modulation of galectin 3 (Mac-2), a carbohydrate binding protein, in pulmonary fibrosis. *J Pathol*. 1996; 179:309–316. [PubMed: 8774488]
- Kleeberger SR, Reddy SP, Zhang LY, Cho HY, Jedlicka AE. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol*. 2001; 280:L326–333. [PubMed: 11159012]
- Koike E, Kobayashi T, Nelson DJ, McWilliam AS, Holt PG. Effect of ozone exposure on alveolar macrophage-mediated immunosuppressive activity in rats. *Toxicol Sci*. 1998; 41:217–223. [PubMed: 9520358]
- Kong YY, Fuchsberger M, Xiang SD, Apostolopoulos V, Plebanski M. Myeloid derived suppressor cells and their role in diseases. *Curr Med Chem*. 2013; 20:1437–1444. [PubMed: 23409714]
- Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: agents of defense or destruction? *Annu Rev Pharmacol Toxicol*. 2011; 51:267–288. [PubMed: 20887196]
- Lee PY, Wang JX, Parisini E, Dascher CC, Nigrovic PA. Ly6 family proteins in neutrophil biology. *J Leukoc Biol*. 2013; 94:585–594. [PubMed: 23543767]
- Liu FT. Regulatory roles of galectins in the immune response. *Int Arch Allergy Immunol*. 2005; 136:385–400. [PubMed: 15775687]
- Liu FT, Hsu DK. The role of galectin-3 in promotion of the inflammatory response. *Drug News Perspect*. 2007; 20:455–460. [PubMed: 17992268]
- Lopez E, del Pozo V, Miguel T, Sastre B, Seoane C, Civantos E, Llanes E, Baeza ML, Palomino P, Cardaba B, Gallardo S, Manzarbeitia F, Zubeldia JM, Lahoz C. Inhibition of chronic airway inflammation and remodeling by galectin-3 gene therapy in a murine model. *J Immunol*. 2006; 176:1943–1950. [PubMed: 16424226]

- Mackinnon AC, Gibbons MA, Farnworth SL, Leffler H, Nilsson UJ, Delaine T, Simpson AJ, Forbes SJ, Hirani N, Gauldie J, Sethi T. Regulation of transforming growth factor-beta1-driven lung fibrosis by galectin-3. *Am J Respir Crit Care Med*. 2012; 185:537–546. [PubMed: 22095546]
- Malaviya R, Venosa A, Hall L, Gow AJ, Sinko PJ, Laskin JD, Laskin DL. Attenuation of acute nitrogen mustard-induced lung injury, inflammation and fibrogenesis by a nitric oxide synthase inhibitor. *Toxicol Appl Pharmacol*. 2012; 265:279–291. [PubMed: 22981630]
- Maldonado CA, Sundblad V, Salatino M, Elia J, Garcia LN, Leimgruber C, Croci DO, Rabinovich GA. Cell-type specific regulation of galectin-3 expression by glucocorticoids in lung Clara cells and macrophages. *Histol Histopathol*. 2011; 26:747–759. [PubMed: 21472689]
- Matthews KE, Karabeg A, Roberts JM, Saeland S, Dekan G, Epstein MM, Ronchese F. Long-term deposition of inhaled antigen in lung resident CD11b-CD11c+ cells. *Am J Respir Cell Mol Biol*. 2007; 36:435–441. [PubMed: 17122367]
- Menoret A, Kumar S, Vella AT. Cytochrome b5 and cytokeratin 17 are biomarkers in bronchoalveolar fluid signifying onset of acute lung injury. *PLoS One*. 2012; 7:e40184. [PubMed: 22792238]
- Mukaro VR, Bylund J, Hodge G, Holmes M, Jersmann H, Reynolds PN, Hodge S. Lectins offer new perspectives in the development of macrophage-targeted therapies for COPD/emphysema. *PLoS One*. 2013; 8:e56147. [PubMed: 23441163]
- Nagaraj S, Gabrilovich DI. Regulation of suppressive function of myeloid-derived suppressor cells by CD4+ T cells. *Semin Cancer Biol*. 2012; 22:282–288. [PubMed: 22313876]
- Nishi Y, Sano H, Kawashima T, Okada T, Kuroda T, Kikkawa K, Kawashima S, Tanabe M, Goto T, Matsuzawa Y, Matsumura R, Tomioka H, Liu FT, Shirai K. Role of galectin-3 in human pulmonary fibrosis. *Allergol Int*. 2007; 56:57–65. [PubMed: 17259811]
- Nishiyama J, Kobayashi S, Ishida A, Nakabayashi I, Tajima O, Miura S, Katayama M, Nogami H. Up-regulation of galectin-3 in acute renal failure of the rat. *Am J Pathol*. 2000; 157:815–823. [PubMed: 10980121]
- O'Seaghdha CM, Hwang SJ, Ho JE, Vasan RS, Levy D, Fox CS. Elevated galectin-3 precedes the development of CKD. *J Am Soc Nephrol*. 2013; 24:1470–1477. [PubMed: 23766533]
- Papaspnyridonos M, McNeill E, de Bono JP, Smith A, Burnand KG, Channon KM, Greaves DR. Galectin-3 is an amplifier of inflammation in atherosclerotic plaque progression through macrophage activation and monocyte chemoattraction. *Arterioscler Thromb Vasc Biol*. 2008; 28:433–440. [PubMed: 18096829]
- Pendino KJ, Meidhof TM, Heck DE, Laskin JD, Laskin DL. Inhibition of macrophages with gadolinium chloride abrogates ozone-induced pulmonary injury and inflammatory mediator production. *Am J Respir Cell Mol Biol*. 1995; 13:125–132. [PubMed: 7542894]
- Pendino KJ, Shuler RL, Laskin JD, Laskin DL. Enhanced production of interleukin-1, tumor necrosis factor-alpha, and fibronectin by rat lung phagocytes following inhalation of a pulmonary irritant. *Am J Respir Cell Mol Biol*. 1994; 11:279–286. [PubMed: 8086166]
- Perillo NL, Marcus ME, Baum LG. Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J Mol Med (Berl)*. 1998; 76:402–412. [PubMed: 9625297]
- Pilette C, Colinet B, Kiss R, Andre S, Kaltner H, Gabius HJ, Delos M, Vaerman JP, Decramer M, Sibille Y. Increased galectin-3 expression and intra-epithelial neutrophils in small airways in severe COPD. *Eur Respir J*. 2007; 29:914–922. [PubMed: 17251233]
- Pryor WA, Church DF. Aldehydes, hydrogen peroxide, and organic radicals as mediators of ozone toxicity. *Free Radic Biol Med*. 1991; 11:41–46. [PubMed: 1937128]
- Reinhart PG, Gupta SK, Bhalla DK. Attenuation of ozone-induced lung injury by interleukin-10. *Toxicol Lett*. 1999; 110:35–42. [PubMed: 10593593]
- Rodriguez-Ariza A, Lopez-Sanchez LM, Gonzalez R, Corrales FJ, Lopez P, Bernardos A, Muntane J. Altered protein expression and protein nitration pattern during d-galactosamine-induced cell death in human hepatocytes: a proteomic analysis. *Liver Int*. 2005; 25:1259–1269. [PubMed: 16343079]
- Saiwai H, Kumamaru H, Ohkawa Y, Kubota K, Kobayakawa K, Yamada H, Yokomizo T, Iwamoto Y, Okada S. Ly6C+ Ly6G- Myeloid-derived suppressor cells play a critical role in the resolution of acute inflammation and the subsequent tissue repair process after spinal cord injury. *J Neurochem*. 2013; 125:74–88. [PubMed: 23278273]

- Sarkar J, Chaudhary S, Jassim SH, Ozturk O, Chamon W, Ganesh B, Tibrewal S, Gandhi S, Byun YS, Hallak J, Mahmud DL, Mahmud N, Rondelli D, Jain S. CD11b+GR1+ myeloid cells secrete NGF and promote trigeminal ganglion neurite growth: implications for corneal nerve regeneration. *Invest Ophthalmol Vis Sci.* 2013; 54:5920–5936. [PubMed: 23942970]
- Schenkman JB, Jansson I. The many roles of cytochrome b5. *Pharmacol Ther.* 2003; 97:139–152. [PubMed: 12559387]
- Sunil VR, Patel-Vayas K, Shen J, Laskin JD, Laskin DL. Classical and alternative macrophage activation in the lung following ozone-induced oxidative stress. *Toxicol Appl Pharmacol.* 2012; 263:195–202. [PubMed: 22727909]
- Tighe RM, Li Z, Potts EN, Frush S, Liu N, Gunn MD, Foster WM, Noble PW, Hollingsworth JW. Ozone inhalation promotes CX3CR1-dependent maturation of resident lung macrophages that limit oxidative stress and inflammation. *J Immunol.* 2011; 187:4800–4808. [PubMed: 21930959]
- Toward TJ, Broadley KJ. Airway function, oedema, cell infiltration and nitric oxide generation in conscious ozone-exposed guinea-pigs: effects of dexamethasone and rolipram. *Br J Pharmacol.* 2002; 136:735–745. [PubMed: 12086983]
- Van der Meeren A, Moureau A, Griffiths NM. Macrophages as key elements of Mixed-oxide [U-Pu(O₂)] distribution and pulmonary damage after inhalation? *Int J Radiat Biol.* 2014; 90:1095–1103. [PubMed: 25029673]
- Vergeres G, Waskell L. Cytochrome b5, its functions, structure and membrane topology. *Biochimie.* 1995; 77:604–620. [PubMed: 8589071]
- Wang M, You Q, Lor K, Chen F, Gao B, Ju C. Chronic alcohol ingestion modulates hepatic macrophage populations and functions in mice. *J Leukoc Biol.* 2014; 96:657–665. [PubMed: 25030420]
- Yi H, Guo C, Yu X, Zuo D, Wang XY. Mouse CD11b+Gr-1+ myeloid cells can promote Th17 cell differentiation and experimental autoimmune encephalomyelitis. *J Immunol.* 2012; 189:4295–4304. [PubMed: 23034169]
- Zaslona Z, Przybranowski S, Wilke C, van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, Wilkinson JE, Moore BB, Peters-Golden M. Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma. *J Immunol.* 2014; 193:4245–4253. [PubMed: 25225663]
- Zaynagetdinov R, Sherrill TP, Kendall PL, Segal BH, Weller KP, Tighe RM, Blackwell TS. Identification of myeloid cell subsets in murine lungs using flow cytometry. *Am J Respir Cell Mol Biol.* 2013; 49:180–189. [PubMed: 23492192]
- Zimmermann HW, Trautwein C, Tacke F. Functional role of monocytes and macrophages for the inflammatory response in acute liver injury. *Front Physiol.* 2012; 3:56. [PubMed: 23091461]

Highlights

- Multiple monocytic-macrophage subpopulations accumulate in the lung after ozone inhalation
- Galectin-3 plays a proinflammatory role in ozone-induced lung injury
- In the absence of gal-3, inflammatory cells with a myeloid derived suppressor cell phenotype contribute to tissue repair

Fig. 1.

Flow chart of gating strategy for identifying lung cell populations. Viable cells staining negatively (CD11b⁻) or positively (CD11b⁺) for CD11b were analyzed for expression of Ly6G. Ly6G⁻ and Ly6G⁺ cells were then analyzed for expression of Ly6C, followed by F4/80 and CD11c. Ly6G⁺ cells were analyzed for expression of Ly6C followed by F4/80. G-MDSC, granulocytic myeloid derived suppressor cells; M-MDSC, monocytic myeloid derived suppressor cells.

Fig. 2. Effects of ozone on Gal-3 expression in the lung. Histologic sections, prepared 24–72 h after exposure of WT and Gal-3^{-/-} mice to air or ozone, were stained with antibody to Gal-3. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3–5 mice per treatment group is shown (Original magnification, x600).

Fig. 3. Effects of loss of Gal-3 on ozone-induced increases in iNOS⁺ macrophages in the lung. Histologic sections, prepared 24–72 h after exposure of WT and Gal-3^{-/-} mice to air or ozone, were stained with antibody to iNOS. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3–5 mice per treatment group is shown (Original magnification, x600).

Fig. 4. Effects of loss of Gal-3 on ozone-induced increases in MR-1⁺ macrophages in the lung. Histologic sections, prepared 24–72 h after exposure of WT and Gal-3^{-/-} mice to air or ozone, were stained with antibody to MR-1. Binding was visualized using a peroxidase DAB substrate kit. Arrows indicate alveolar macrophages. One representative section from 3–5 mice per treatment group is shown (Original magnification, x600).

Fig. 5.

Effects of ozone on bronchoalveolar lavage (BAL) cells and protein content in WT and Gal-3^{-/-} mice. BAL was collected 24–72 h after exposure of mice to air or ozone. *Upper panel:* Viable cells were enumerated by trypan blue due exclusion. *Lower panel:* Cell-free supernatants were analyzed in triplicate for protein using a BCA protein assay kit. Bars are the mean $\pm$ SE (n = 8 mice per treatment group). *Significantly different (p < 0.05) from air-exposed animals; #Significantly different (p < 0.05) from WT animals.

Fig. 6.

Effects of ozone on mature proinflammatory and anti-inflammatory macrophage subpopulations in the lung. BAL cells, collected 24–72 h after exposure of WT and Gal-3^{-/-} mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C, F4/80 and CD11c or appropriate isotypic controls, and analyzed by flow cytometry. *Upper panel:* Mature (F4/80⁺CD11c⁺) CD11b⁺Ly6G⁻Ly6C^{hi} proinflammatory macrophages; *Lower panel:* Mature (F4/80⁺CD11c⁺) CD11b⁺Ly6G⁻Ly6C^{lo} anti-inflammatory macrophages. Bars are the mean $\pm$ SE (n = 4–14 mice per treatment group). *Significantly different (p < 0.05) from air-exposed animals; #Significantly different (p < 0.05) from WT animals.

Fig. 7.

Effects of loss of Gal-3 on MDSC. BAL cells, collected 24–72 h after exposure of WT and Gal-3^{-/-} mice to air or ozone, were stained with antibodies to CD11b, Ly6G, Ly6C and F4/80 or appropriate isotypic controls, and analyzed by flow cytometry. *Upper panel:* CD11b⁺Ly6G⁺Ly6C⁺F4/80⁻ granulocytic (G) MDSC; *Lower panel:* CD11b⁺Ly6G⁺Ly6C⁺F4/80⁺ monocytic (M) MDSC. Bars are the means $\pm$ SE (n = 4–14 mice per treatment group). *Significantly different (p < 0.05) from air-exposed animals; #Significantly different (p < 0.05) from WT animals.

Fig. 8. Effects of ozone on cytochrome b5 protein expression in WT and Gal-3^{-/-} mice. Histologic sections, prepared 24–72 h after exposure of mice to air or ozone, were stained with antibody to cytochrome b5. Binding was visualized using a peroxidase DAB substrate kit. One representative section from 3–5 mice per treatment group is shown (Original magnification, x600).

Table 1Effects of ozone on infiltrating CD11b⁺ myeloid cells

Time after ozone (h)	Monocytic (Ly6G ⁻)		Granulocytic (Ly6G ⁺)	
	WT	Gal-3 ^{-/-}	WT	Gal-3 ^{-/-}
Air	11.7 ± 1.9	17.0 ± 5.7	0.2 ± 0.05	0.4 ± 0.1
24 h	45.3 ± 3.8 *	56.7 ± 8.6 *	2.1 ± 0.6	3.2 ± 0.9 *
48 h	70.4 ± 12.6 *	16.0 ± 0.7 [#]	7.5 ± 2.4 *	3.7 ± 1.0 *
72 h	62.0 ± 9.1 *	80.7 ± 8.4 *	5.4 ± 1.1 *	11.9 ± 1.3 *, [#]

Cells, collected 24–72 h after exposure of WT and Gal-3^{-/-} mice to air or ozone, were immunostained with antibodies to CD11b and Ly6G and analyzed by flow cytometry as described in the Materials and Methods. Data are the number of CD11b⁺ cells x 10³ negative (Ly6G⁻) or positive (Ly6G⁺) for Ly6G. Values are mean ± SE (n=4–14 mice/treatment group).

* Significantly different (p<0.05) from Air.

[#] Significantly different (p<0.05) from WT.