

Neutrophils Mediate Airway Hyperresponsiveness after Chlorine-Induced Airway Injury in the Mouse

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Abstract

Chlorine gas (Cl₂) inhalation causes oxidative stress, airway epithelial damage, airway hyperresponsiveness (AHR), and neutrophilia. We evaluated the effect of neutrophil depletion on Cl₂-induced AHR and its effect on the endogenous antioxidant response, and if eosinophils or macrophages influence Cl₂-induced AHR. We exposed male Balb/C mice to 100 ppm Cl₂ for 5 minutes. We quantified inflammatory cell populations in bronchoalveolar lavage (BAL), the antioxidant response in lung tissue by quantitative PCR, and nuclear factor (erythroid-derived 2)-like 2 (NRF2) nuclear translocation by immunofluorescence. *In vitro*, NRF2 nuclear translocation in response to exogenous hypochlorite was assessed using a luciferase assay. Anti-granulocyte receptor-1 antibody or anti-Ly6G was used to deplete neutrophils. The effects of neutrophil depletion on IL-13 and IL-17 were measured by ELISA. Eosinophils and macrophages were depleted using TRFK5 or clodronate-loaded liposomes, respectively. AHR was evaluated with the constant-phase model in response to inhaled aerosolized methacholine. Our results show that Cl₂ exposure induced neutrophilia and increased expression of NRF2 mRNA, superoxide dismutase-1, and heme-oxygenase 1. Neutrophil depletion abolished Cl₂-induced AHR in large conducting airways and prevented increases in antioxidant gene expression and NRF2 nuclear translocation. Exogenous hypochlorite administration

resulted in increased NRF2 nuclear translocation *in vitro*. After Cl₂ exposure, neutrophils occupied 22 ± 7% of the luminal space in large airways. IL-17 in BAL was increased after Cl₂, although this effect was not prevented by neutrophil depletion. Neither depletion of eosinophils nor macrophages prevented Cl₂-induced AHR. Our data suggest the ability of neutrophils to promote Cl₂-induced AHR is dependent on increases in oxidative stress and occupation of luminal space in large airways.

Keywords: oxidative stress; nuclear factor (erythroid-derived 2)-like 2; anti-granulocyte receptor-1; clodronate-liposomes; eosinophils

Clinical Relevance

Chlorine exposure induces severe neutrophilia and oxidative stress, and is a leading cause of irritant-induced asthma. We present data that suggest how neutrophils mediate lungs' compartment-specific responses to oxidative stress, which may help elucidate the complex role neutrophils play in oxidative stress-driven airway dysfunction.

Irritant-induced asthma (IIA) is a form of occupational asthma that develops without latency in response to single (acute) or multiple (chronic) exposures to an irritant substance (1). Clinical findings are characterized by bronchial hyperresponsiveness, airway

inflammation, edema, and airway damage (2, 3). Causative agents of IIA include diesel exhaust (3), disinfectants (4), preservatives such as formaldehyde (5), and cleaning products like ammonium and amine compounds (6). Exposure to chlorine gas

(Cl₂) and Cl₂-related compounds remains the most reported cause of inhalational accidents resulting in IIA (7). Those at risk include industrial workers (8), swimmers (9), and those exposed through the improper mixing of cleaning agents (6).

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Evidence points to the majority of Cl₂-induced damage originating from its by-products, which include chloramines, hydrochloric acid, chlorine dioxide, and hypochlorous acid (HOCl). Cl₂ is highly soluble in water and is a potent oxidant, having greater toxicity than nitrogen dioxide, oxygen, ozone, and hydrochloric acid (10). Increased oxidant production has been associated with Cl₂ inhalation, including elevated peroxynitrite in the airway tissues and increased carbonylation of proteins (11). However, Cl₂ is scrubbed from the airway fairly rapidly (12), and previous work has shown evidence of ongoing oxidant stress and airway damage, suggesting additional mechanisms for Cl₂-induced airway injury (13, 14). The mechanisms driving this persistent oxidative stress and the associated consequences have not been adequately explored and constitute the rationale for this study. We hypothesize that increased airway hyperresponsiveness (AHR) and oxidative stress are directly related to the presence of neutrophils recruited to the lung after a single Cl₂ exposure.

Neutrophils have been associated with severe asthma and asthma exacerbations (15), and previous work has shown that inhalation of irritant, oxidizing substances such as ozone (16, 17) and phosgene (18) rapidly induce the influx of neutrophils to the airways. After Cl₂ inhalation, studies in rodents (19) and case studies in humans (20) have shown striking neutrophilia, with modest changes in recruitment of other inflammatory cell types, suggesting that neutrophils play an important role in the injury caused by Cl₂ (21). Neutrophils may contribute to oxidant burden through the production of reactive oxygen species because release of myeloperoxidase catalyzes the formation of HOCl from hydrogen peroxide (22).

In the current study, we examined how neutrophils affect Cl₂-induced airway injury, AHR, and the endogenous antioxidant response in a mouse model of IIA. Mice were administered antibodies directed against granulocyte receptor-1 (Gr-1) or Ly6G, both of which have been used to deplete neutrophils *in vivo* (23–29). After Cl₂ exposure, we measured respiratory system mechanics in response to inhaled methacholine (MCh) and assessed airway inflammatory cell profiles, proinflammatory cytokine levels, antioxidant gene expression, and nuclear factor (erythroid-derived 2)-like 2 (NRF-2) epithelial cell nuclear translocation *in*

vivo and *in vitro*. We also assessed the independent contribution of eosinophils and macrophages in the induction of Cl₂-induced AHR and inflammation using an anti-IL-5 antibody (30) or intratracheal and systemic administration of clodronate liposomes (31–33), respectively.

Materials and Methods

Mice

Balb/C male mice (8–10 wk old) from Charles River (Wilmington, MA) were housed in a conventional animal facility at McGill University. All mice were treated in accordance with the guidelines of the Canadian Council for Animal Care, and protocols were approved by the Animal Care Committee of McGill University.

Cl₂ Exposure

Cl₂ exposure was performed as previously described (13). Briefly, mice were exposed to Cl₂ for 5 minutes using a nose-only exposure device at a concentration of 100 ppm. Cl₂ was mixed with room air using a standardized calibrator (Model 230–28A Dynacalibrator; VICI Metronics, Poulsbo, WA).

Measurements of Respiratory System Mechanics and Airway Responsiveness to MCh

Measurement of airway mechanics was performed (13) as previously described 24 hours after Cl₂ exposure using a small animal ventilator (FlexiVent; Scireq, Montreal, Quebec, Canada).

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed at 6 or 24 h after Cl₂ exposure as previously described (14).

Granulocyte and Macrophage Depletion

Mice were injected intraperitoneally with 100 µg/mouse anti-GR1 (Hycult Biotechnology, King of Prussia, PA) in 100 µl sterile PBS 6 hours before Cl₂ exposure. After Cl₂ exposure, mice recovered for 6 or 24 hours. BAL was performed to assess efficacy of anti-GR1. Mouse IgG2a (Hycult Biotechnology) was used as isotype control and injected at the same time points as anti-Gr1. A second neutrophil depletion antibody directed against Ly6G (Bio X Cell, West Lebanon, NH) was also used to confirm key results observed using anti-

Gr1. Anti-Ly6G was injected intraperitoneally at a concentration of 100 µg/mouse 12 hours before Cl₂ exposure. Eosinophils were depleted using TRFK5, and macrophages were depleted using clodronate liposomes. Further details are provided in the online supplement.

RNA Isolation, RT-PCR, and Real-Time Quantitative PCR

RNA isolation, RT-PCR, and real-time PCR were performed at 6 or 24 hours after Cl₂ exposure. Primer sequences are provided in the online supplement.

Tissue Neutrophil Quantification

At 6 or 24 hours after Cl₂ exposure, lung tissue was fixed and embedded in paraffin as previously described (13). Neutrophils were identified by immunohistochemistry with the neutrophil-specific antibody NIMP-R14 (Abcam, Cambridge, MA) and light microscopy using an Olympus BX51 microscope. Images were taken using a QImaging Retina 2000r camera and Image Pro-Plus software. Neutrophils within the luminal space of large airways (> 2.5 mm basement membrane perimeter) were quantified using Image J software.

NRF2 Nuclear Translocation

After NRF2 immunofluorescent staining, tissue sections were visualized using an Olympus BX51 microscope. Images were taken using QImaging Retina 2000r camera. NRF2 nuclear translocation corrected for total NRF2 immunofluorescence was determined using nuclear colocalization commercial software (Image-Pro Plus) and quantified as previously described (34, 35).

NRF2 Luciferase Reporter Assay

BEAS-2B cells were stably transfected with a pGL4.28 plasmid (Promega, Madison, WI) containing an antioxidant response element (G TAC CGC AGT CAC AGT GAC TCA GCA GAA TCG CTA G) upstream of a hygromycin B resistance gene and a firefly luciferase construct. Further details are provided in the online supplement.

Statistical Analysis

Data for respiratory system mechanics were analyzed using a repeated measures ANOVA. Comparisons among several means were performed using ANOVA, and the *post hoc* analysis was performed with a Newman-Keuls test. A *P* value < 0.05 was considered significant.

Results

Changes in BAL of Cl₂-Exposed Mice

Cl₂-induced airway inflammation and epithelial cell shedding were assessed by bronchoalveolar exposure to Cl₂ compared with air-exposed animals (Figure 1A). Macrophages were not significantly altered at either time point (Figure 1B). Neutrophils were significantly increased at 6 and 24 hours compared with control animals but were higher at 6 than at 24 hours after exposure (Figure 1C). Eosinophil numbers were modestly increased at 24 hours but not at 6 hours (Figure 1D). Lymphocytes were increased by 6 hours and remained elevated at 24 hours (Figure 1E). Epithelial cells were highest at 6 hours, but significant numbers of epithelial cells were found in the BAL at 24 hours after Cl₂ exposure (Figure 1F).

Anti-Gr1 Depletes Granulocytes in BAL

Six hours after intraperitoneal administration of 100 µg anti-GR-1 antibody, mice were exposed to Cl₂ and allowed to recover for 6 or 24 hours. After recovery, BAL was performed, and

inflammatory cell profiles were determined. After Cl₂ exposure there is marked neutrophilia, which was absent from the BAL at 6 and 24 hours in anti-GR1-treated mice. At 6 hours, the total number of cells in the BAL fluid was not different between groups that received anti-GR1. By 24 hours, the total cells were fewer in anti-GR1-treated mice compared with untreated animals (Figure 2A). We observed a smaller number of macrophages between air-exposed anti-GR1-treated and nontreated mice at baseline, which then increased slightly at 6 hours in Cl₂-exposed groups and decreased again at 24 hours (Figure 2B). Anti-Gr1 was highly effective at inhibiting BAL neutrophilia at 6 hours after Cl₂ exposure (Figure 3C), but it also caused a decrease in the number of eosinophils and lymphocytes at the same time point (Figures 2D and 2E). There were no changes in the number of shed epithelial cells among any groups (Figure 2F).

Anti-Ly6G Depletes Neutrophils in BAL

Anti-Ly6G was used as a second neutrophil depletion antibody to ensure the AHR

findings using anti-Gr1 were specific to neutrophil depletion. After administration of anti-Ly6G, Cl₂-exposed mice demonstrated BAL inflammatory cell profiles similar to those given anti-Gr1. At 24 hours after Cl₂ exposure, mice treated with anti-Ly6G showed decreased total cell counts (see Figure E1A in the online supplement). There were no differences between Cl₂-exposed and anti-Ly6G + Cl₂-treated mice regarding macrophage numbers (Figure E1B). Like anti-Gr1, anti-Ly6G was highly effective at reducing neutrophil numbers in BAL (Figure E1C). Anti-Ly6G reduced lymphocyte numbers in Cl₂-exposed mice but had no effect on eosinophil or epithelial cells (Figures E1D–E1F).

Anti-GR1 Treatment Reduces Cl₂-Induced AHR

Mice given anti-Gr1 6 hours before Cl₂ exposure had significantly decreased respiratory system resistance and Newtonian resistance (Figures 3A and 3C) compared with Cl₂-exposed mice given isotype control in response to MCh. Anti-Gr1 did not reduce respiratory system elastance, tissue damping, or tissue

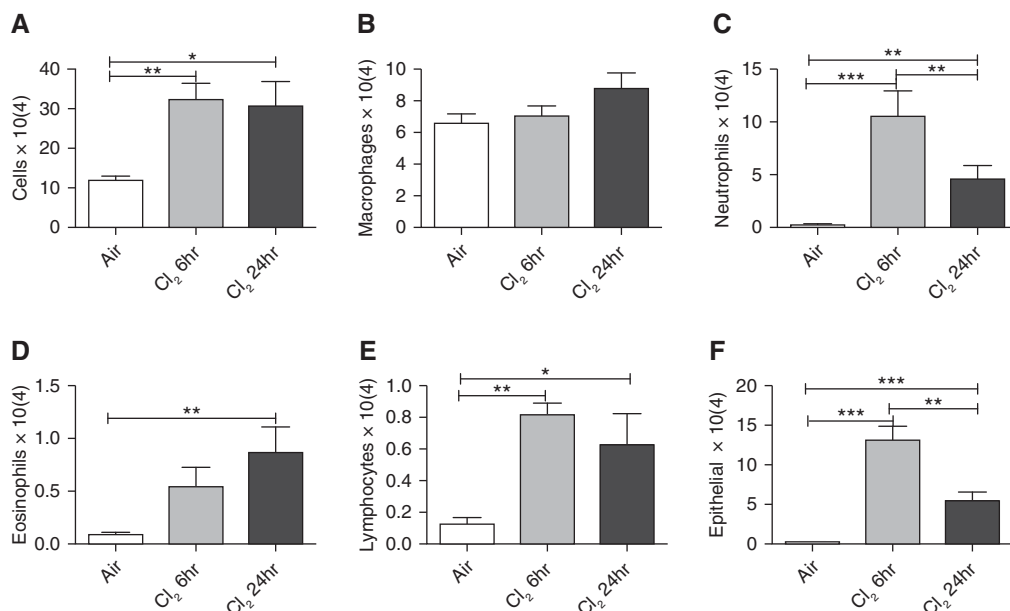


Figure 1. Changes in bronchoalveolar lavage (BAL) of chlorine gas (Cl₂)-exposed mice. At either 6 or 24 hours after Cl₂ exposure, BAL was collected for total and differential cell count analysis. (A) Total cell counts were increased at 6 and 24 hours in Cl₂-exposed mice compared with air-exposed mice. (B) Cl₂ exposure did not induce a significant change in macrophage numbers between the conditions. (C) Neutrophil numbers are increased at 6 and 24 hours after Cl₂ exposure compared with air-exposed mice. Cl₂ exposure resulted in significantly higher neutrophil numbers at 6 hours compared with 24 hours. (D) Cl₂ exposure resulted in a modest increase in eosinophil numbers 24 hours after Cl₂ exposure compared with air-exposed mice. (E) Lymphocyte numbers increased at 6 hours and remained elevated at 24 hours in Cl₂-exposed mice. (F) Cl₂ exposure resulted in increased epithelial cell shedding at 6 and 24 hours. Epithelial cell shedding peaked at 6 hours after Cl₂ exposure. *P < 0.05, **P < 0.01, and ***P < 0.001 (n = 6–10 per group).

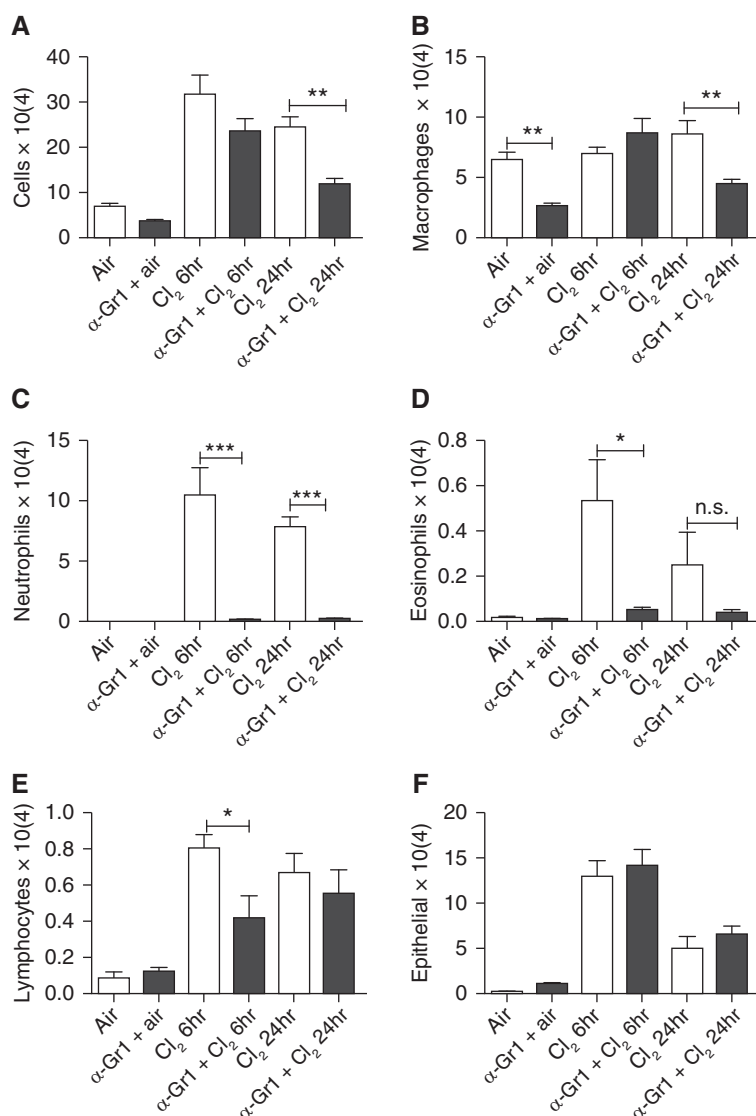


Figure 2. Anti-granulocyte receptor-1 (Gr1) depletes neutrophils from BAL. Six hours before Cl₂ exposure, 100 μg of anti-Gr1 was injected intraperitoneally. At either 6 or 24 hours after Cl₂, BAL was collected and evaluated for inflammatory cells. (A) Treatment with anti-Gr1 reduced total inflammatory cells at 24 hours compared with mice that did not receive anti-Gr1. (B) Anti-Gr1 reduced macrophage numbers in air-exposed mice and in Cl₂-exposed mice at 24 hours. (C) Treatment with anti-Gr1 completely attenuated increased neutrophil numbers seen in the BAL of untreated mice. (D) Anti-Gr1 reduced eosinophil numbers at 6 hours but not in air-exposed mice or at 24 hours after Cl₂ exposure. (E) Lymphocyte numbers were decreased at 6 hours by anti-Gr1 treatment but not in air-exposed mice or at 24 hours after Cl₂. (F) Epithelial cell shedding was not affected by anti-Gr1 treatment. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (*n* = 6–10 per group). n.s., nonsignificant.

elastance (Figures 3B, 3D, and 3E). Anti-Gr1 + air exposure had no effect on AHR for any parameter.

Anti-Ly6G Treatment Reduces Cl₂-Induced AHR

Mice treated with anti-Ly6G 12 hours before Cl₂ exposure demonstrated reduced respiratory system resistance at the three highest doses of

MCh (Figure E2A). Anti-Ly6G reduced respiratory system elastance at 6.25, 12.5, and 25 mg/ml MCh but not at the highest dose of MCh (Figure E2B). Anti-Ly6G reduced Newtonian resistance at the three highest doses of MCh compared with untreated Cl₂-exposed mice (Figure E2C). Anti-Ly6G did not reduce tissue damping or tissue resistance significantly (Figures E2D and E2E).

Neutrophil Quantification in Lung Tissue

In addition to evaluation of the efficacy of anti-Gr1 in reducing BAL neutrophils, lung tissues were stained for a neutrophil-specific marker, NIMP-r14, to ensure depletion was complete from lung tissue. No neutrophils were present in air-treated conditions (Figures 4A and 4B). By 6 hours after Cl₂ exposure, neutrophils could be clearly visualized in the airways and frequently took the form of adherent clumps of neutrophils (Figure 4C). In contrast, very few neutrophils could be identified in anti-Gr1-treated lungs (Figure 4D). By 24 hours, the neutrophil clumps appeared less frequent in Cl₂-treated mice, but abundant neutrophils were still present (Figure 4E). This finding was not seen in anti-Gr1-treated mice, where no neutrophils were seen in either airways or lung parenchymal tissues at 24 hours after Cl₂ exposure (Figure 4F). No neutrophils were seen in the air-exposed isotype control mice (Figure 4G). Quantification of neutrophil occupation of luminal space in large airways showed that neutrophils were significantly present in the lumen at 6 and 24 hours (Figure 4H). All measurements were corrected for airway size.

Effect of Cl₂ Exposure on Proinflammatory Cytokine Levels

Cytokine levels of proinflammatory mediators IL-17A and IL-13 were evaluated by ELISA and quantitative PCR. Chlorine exposure induced a modest increase in IL-17A and IL-13 levels in BAL. Neutrophil depletion using anti-Gr1 or anti-Ly6G did not attenuate the Cl₂-induced increase in IL-17 levels (Figure E3A). Neither anti-Gr1 nor anti-Ly6G attenuated the increase in IL-13 in BAL (Figure E3B). We did not observe significant increases in the transcripts of IL-17A or IL-13 induced by Cl₂ exposure (Figures E3C and E3D).

Anti-GR1 Prevents Increases in Cl₂-Induced Oxidant Gene Up-regulation

At 6 and 24 hours after Cl₂ exposure, antioxidant gene expression in the central lung compartment of mice treated with or without anti-Gr1 was assessed. Cl₂ induced an increase in NRF2 mRNA at 24 hours after exposure (Figure 5A). Treatment with anti-Gr1 prevented increased NRF2 mRNA levels (Figure 5A). SOD-1 was increased at 24 hours after Cl₂ exposure, and this increase was prevented with anti-Gr1 treatment (Figure 5B). GPX-2 decreased in

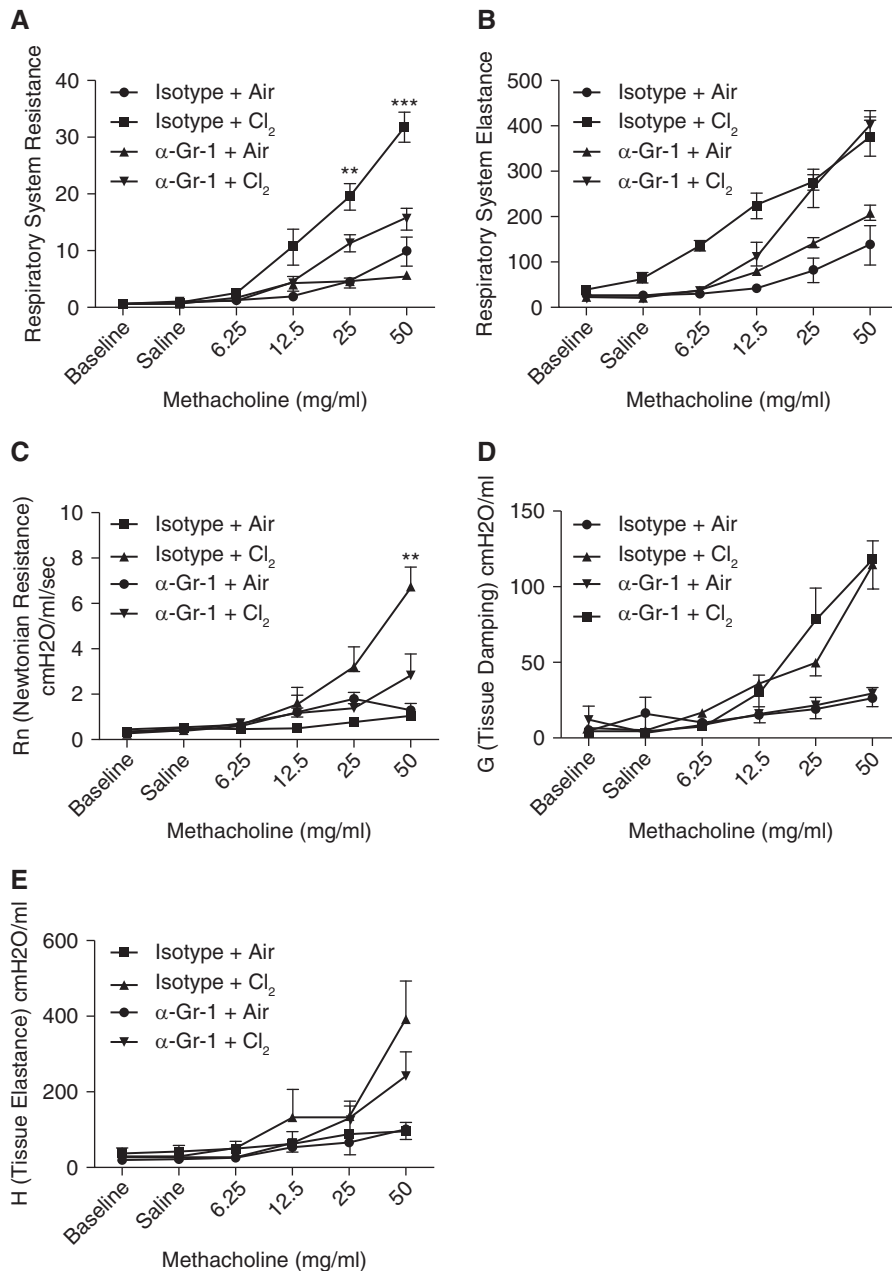


Figure 3. Anti-Gr1 treatment reduces Cl_2 -induced airway hyperresponsiveness. Mice were injected with 100 μg anti-Gr1 6 hours before Cl_2 inhalation. Twenty-four hours later, airway function measurements were taken in response to inhaled methacholine. (A) Neutrophil depletion completely attenuated Cl_2 -induced increases in respiratory system resistance. (B) Anti-Gr1 did not reduce respiratory system elastance. (C) Newtonian resistance was attenuated by anti-Gr1 treatment in Cl_2 -treated mice. (D and E) Anti-Gr1 had no effect on either tissue damping or tissue elastance. Mice given control antibody (IgG2a) did not show any differences in any parameter from mice given PBS only (data not shown). $^{**}P < 0.01$ and $^{***}P < 0.001$ ($n = 6-9$ per group).

mice exposed to Cl_2 , an effect prevented by anti-Gr1 treatment (Figure 5C). Heme oxygenase (HO)-1 levels were increased after Cl_2 exposure at 24 hours, and this effect was prevented by neutrophil depletion using anti-Gr1 (Figure 5D).

Anti-Gr1 Prevents NRF2 Nuclear Translocation

Twenty-four hours after Cl_2 exposure, lungs were fixed and stained for NRF2 to determine if nuclear translocation had taken place in response to Cl_2 . Mice

exposed to air showed clear NRF2 positivity in the cytoplasm and not in the nuclear region (Figure 6A [i, v, and ix]). Visualization of NRF2 for anti-Gr1-treated mice demonstrated similar patterns (Figure 6 [ii, vi, and x]). However, in Cl_2 -exposed mice, NRF2 nuclear translocation was apparent (Figure 6 [iii, vii, and xi]). In mice treated with anti-Gr1, less nuclear translocation took place despite being exposed to Cl_2 (Figure 6 [iv, viii, and xii]). Quantification of NRF2 immunoreactivity confirmed that, after Cl_2 exposure, NRF2 translocated to the nucleus, an effect prevented by anti-Gr1 (Figure 6B).

Hypochlorite Induces NRF2 Nuclear Translocation in BEAS2b Cells

Using an NRF2 luciferase reporter assay, we evaluated if hypochlorite (OCl^-) or H_2O_2 could induce NRF2 nuclear translocation in bronchial epithelial cells (BEAS2b). After incubation of cell lysate supernatant with D-Luciferin and ATP, we found that 0.3 μM OCl^- induces a time-dependent increase in NRF2 nuclear translocation. Nuclear translocation peaked between 3 and 4 hours, showing a 13-fold increase in NRF2 nuclear translocation over non- OCl^- exposed cells, and returned nearly to baseline by 6 hours (Figure 7A). We found a significant increase in NRF2 nuclear translocation at 4 hours (Figure 7B).

Anti-IL-5 Prevents Eosinophilia Selectively after Cl_2 Exposure

To determine if eosinophils, which are important inflammatory cells that may influence AHR, played a role in the changes in airway responsiveness observed in Cl_2 -exposed mice, we depleted eosinophils with anti-IL-5. Anti-Gr1 also caused a reduction in BAL fluid eosinophilia. Therefore, to ensure that the results observed using anti-Gr1 were specific to the depletion of neutrophils, eosinophil-specific depletion was critical. Anti-IL-5 did not affect total inflammatory cells found in BAL after Cl_2 exposure (Figure E4A). It did not influence any of the cell types evaluated, with the exception of eosinophils (Figures E4B-E4F). IgG2a was used as an isotype control and had no effect on eosinophils or any other cell type (data not shown).

Eosinophil Depletion Does Not Affect Cl_2 -Induced AHR

The absence of eosinophils in the airways did not prevent the Cl_2 -induced increase in

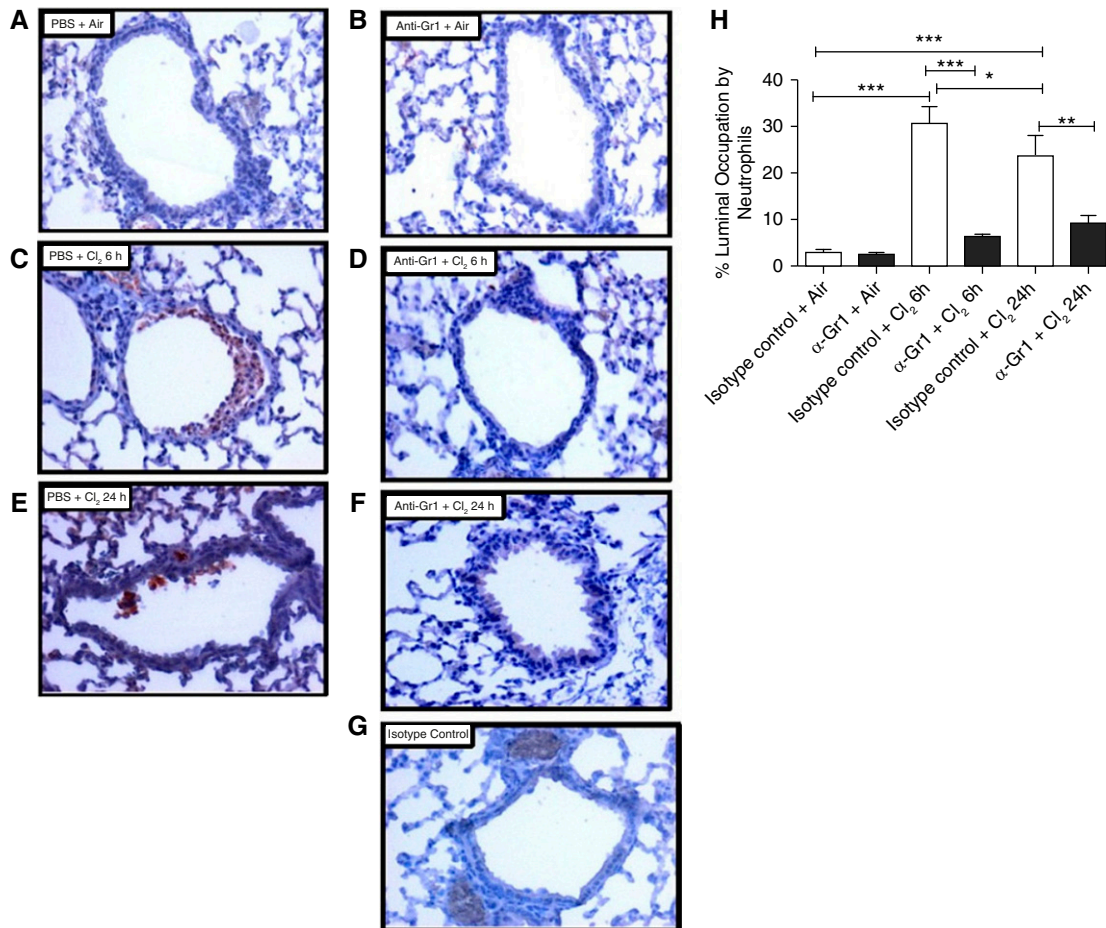


Figure 4. Anti-Gr1 depletes neutrophils from lung tissue. Lung tissue was sectioned at 6 or 24 hours after Cl₂ exposure and with or without pretreatment with 100 μg of anti-Gr1. The presence of neutrophils was identified using the neutrophil-specific antibody Nimp-R14. (A and B) Few neutrophils are seen in the airways or surrounding tissue in air-exposed mice regardless of anti-Gr1 treatment. (C) Six hours after Cl₂ exposure, neutrophils can be easily visualized in the airway lumen. (D) Compared with Cl₂-exposed mice not treated with anti-Gr1, there are very few neutrophils in the airways and surrounding tissue after anti-Gr1 treatment. (E) At 24 hours, neutrophils were still easily identifiable in the airways and surrounding tissue in Cl₂-exposed mice. (F) Anti-Gr1 treatment prevented neutrophil influx to the airways and tissue, and very few neutrophils were visible. (G) Isotype control for Nimp-R14 shows no neutrophil staining. (H) Area of the airway lumen occupied by neutrophils divided by the total lumen area at 6 and 24 hours after Cl₂. Anti-Gr1 prevented this increase. A total of 8 to 10 airways per mouse were measured. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (*n* = 6–10 mice per group).

respiratory system resistance, respiratory system elastance, Newtonian resistance, tissue damping, or tissue elastance (Figures E5A–E5E). Similarly, anti-IL-5 did not affect the airway responsiveness of mice exposed only to air.

Depletion of Macrophages Using Clodronate Liposomes Does Not Prevent Cl₂-Induced AHR

Like neutrophils and eosinophils, macrophages are an important inflammatory cell type that may influence AHR. Therefore, we depleted macrophages using clodronate liposomes 48 hours before Cl₂ exposure. Clodronate administration did not significantly affect total cell counts

(Figure E6A) but reduced significantly the proportion of macrophages in BAL fluid (Figure E6B). In addition to BAL analysis, we confirmed the efficacy of macrophage depletion by FACS analysis of lung tissue (Figure E7). BAL and FACS analysis showed 47 and 42% macrophage depletion using clodronate liposomes, respectively (Figures E6B and E7D), compared with Cl₂-exposed mice treated with encapsosomes. No other cell type was significantly affected by clodronate, and no cell type was depleted by encapsosomes.

Depletion of macrophages did not reduce Cl₂-induced AHR. None of the parameters measured was affected by macrophage depletion in response to

inhaled MCh. Encapsosomes did not have an effect on airway responsiveness.

Discussion

Cl₂ exposure causes oxidative injury that results in a predominantly neutrophilic airway inflammation, AHR to inhaled aerosolized MCh, and oxidative damage. In the current study we assessed the role of the neutrophil in Cl₂-induced AHR and its contribution to oxidative stress. Using neutrophil depletion antibodies anti-Gr1 and anti-Ly6G, we were able to prevent neutrophil migration into the airway and alveolar compartments. Our results suggest

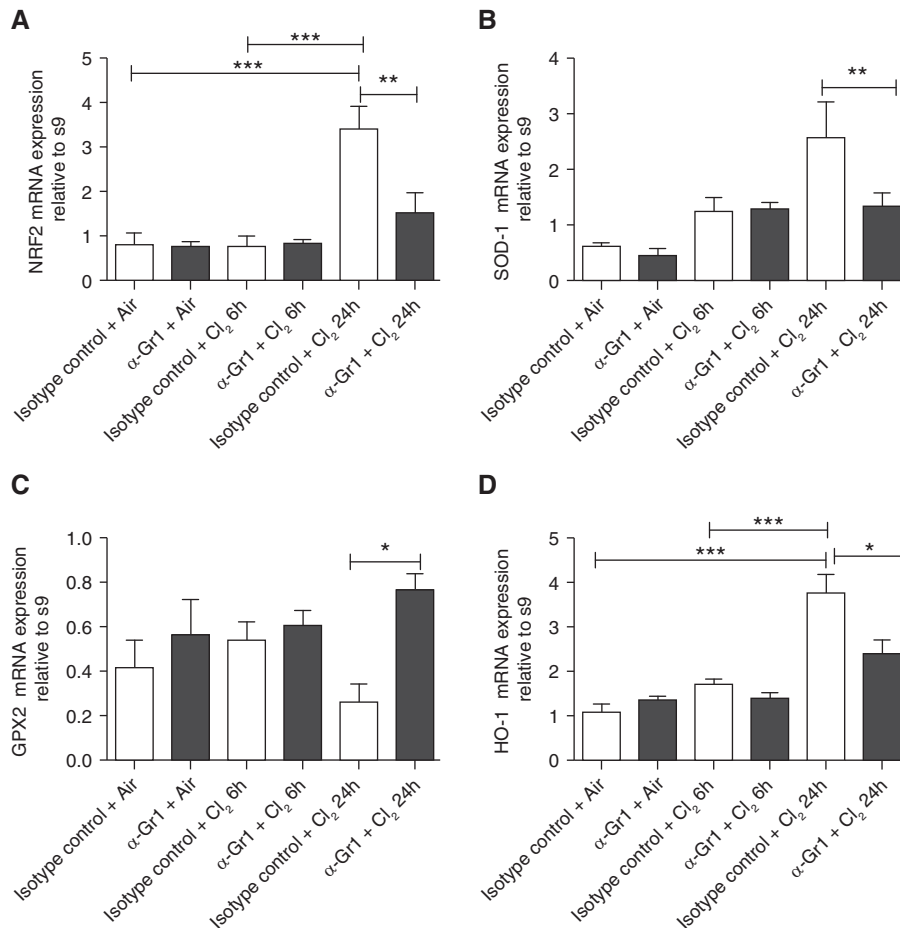


Figure 5. Anti-Gr1 prevents increases in Cl₂-induced oxidant gene up-regulation. Six hours before Cl₂ exposure, 100 μ g of anti-Gr1 was administered intraperitoneally. mRNA was isolated from the central portion of mouse lungs 6 or 24 hours after Cl₂ exposure. (A) Treatment with anti-Gr1 prevented Cl₂-induced increases in nuclear factor (erythroid-derived 2)-like 2 (NRF2), a critical transcription factor involved the endogenous antioxidant response at 24 hours. (B) Anti-Gr1 prevented increases in superoxide dismutase 1 (SOD-1) at 24 hours. (C) Cl₂ exposure led to a decrease in glutathione peroxidase 2 (GPX2) mRNA at 24 hours, an effect prevented by anti-Gr1 treatment. (D) Anti-Gr1 prevented Cl₂-induced increases in heme oxygenase-1 (HO-1) at 24 hours. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ ($n = 6$ –10 per group). s9, ribosomal protein s9.

that the presence of neutrophils accounted for increased airway responsiveness measured by respiratory system mechanical parameters. To assess whether neutrophilic inflammation accounts for increased oxidative stress and induction of endogenous antioxidant mechanisms, we measured antioxidant mRNA levels after Cl₂ exposure and treatment with anti-Gr1. We observed an up-regulation of mRNA associated with antioxidants after Cl₂ exposure, and this effect was prevented by anti-Gr1 treatment. Likewise, translocation of NRF2 to the nucleus, which followed Cl₂ exposure, was prevented by neutrophil depletion. We also assessed the direct effect of OCl⁻, a more stable form of HOCl,

an oxidant by-product of Cl₂ hydrolysis, and a neutrophil by-product on bronchial epithelial cells. We found that OCl⁻ was able to induce nuclear translocation of NRF2 in bronchial epithelial cells, supporting our *in vivo* observations. Assessment of proinflammatory cytokines revealed that Cl₂ exposure induces increases in IL-17A and IL-13; however, neutrophil depletion attenuated neither IL-17A nor IL-13 levels in BAL fluid, suggesting that synthesis of these cytokines may not be neutrophil dependent in this model.

Chlorine is a highly reactive gas and when hydrated forms HOCl, a strong oxidant. There is a substantial body of

literature addressing the role of oxidant injury from chlorine (2, 9, 36–40) and documenting the efficacy of antioxidant treatments (13, 38, 41, 42). Of particular interest is the finding that administration of antioxidants after Cl₂ exposure attenuates airway injury and dysfunction (14). These data suggest that oxidative injury is not limited to the acute effects of Cl₂ but is also related to the ongoing production of oxidant species after exposure, likely resulting from epithelial activation or from the influx of activated leukocytes, as evidenced by the strong NRF2 nuclear translocation observed in Cl₂-exposed mice. NRF2 mRNA was increased after Cl₂ exposure, as was SOD-1 and HO-1 mRNA, which are NRF2-dependent antioxidants. The epithelial antioxidant glutathione peroxidase (GPX)-2 was down-regulated. This is not a unique observation: others have shown down-regulation of the GPX family of antioxidants after severe, acute oxidative stress (43). Because GPX-2 is mostly synthesized in epithelial cells, this down-regulation after Cl₂ exposure may be indicative of excessive cell damage, particularly to epithelial cells, preventing normal function of these cells. Depletion of neutrophils, however, was able to prevent down-regulation of GPX-2, suggesting that neutrophil presence may account for the observed changes.

NRF2 translocation to the nucleus occurred in the mouse airway epithelial cells after Cl₂ exposure, indicative of an altered redox state. This finding was recapitulated *in vitro* using OCl⁻ to stimulate BEAS2B cells directly. Depletion of neutrophils prevented NRF2 translocation, suggesting that their presence increases the level of oxidative stress such that epithelial cells become overwhelmed and engagement of the battery of NRF2-dependent antioxidants is necessitated. This finding further suggests that acute exposure to Cl₂ does not induce the total burden of excess oxidative stress, as previously demonstrated by the efficacy of antioxidant administration after Cl₂ exposure (14), and that oxidative stress induced by neutrophils is sufficient to engage the NRF2 antioxidant system.

AHR after Cl₂ exposure is reflected as exaggerated changes in respiratory system resistance and elastance after inhaled MCh measured at 24 hours after exposure. To assess the relationship between the inflammatory cell influx and AHR, we targeted cells expressing Gr1. Gr1

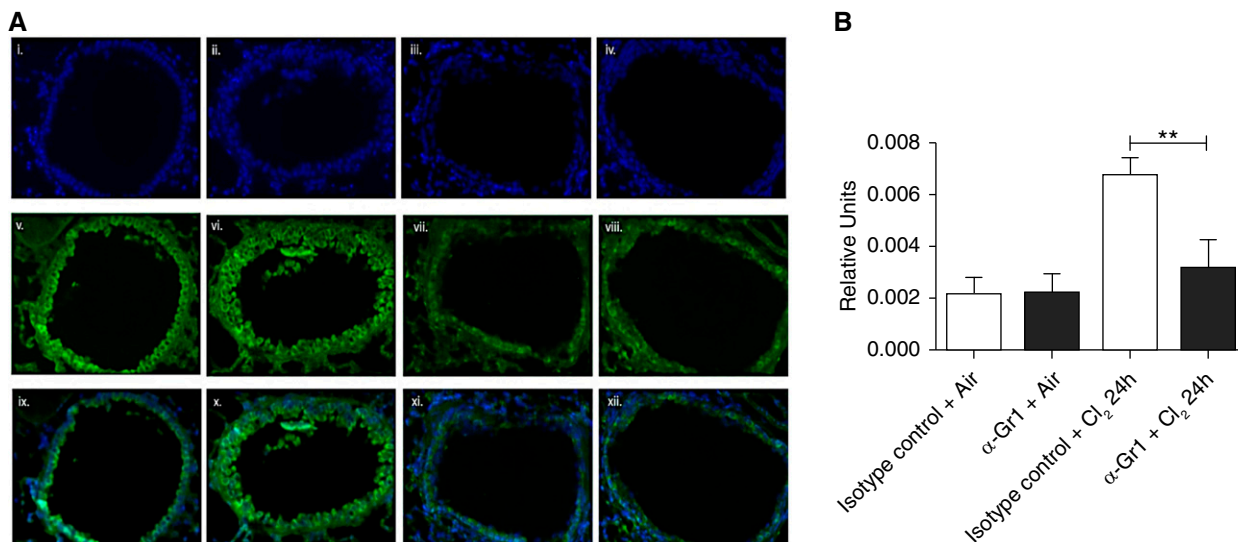


Figure 6. Anti-Gr1 prevents NRF2 nuclear translocation. (A) Immunohistochemistry for NRF2. (i–iv) Nuclear staining with Hoechst stain (blue). (v–viii) NRF2 staining (green). (ix–xii) Merged nuclear and NRF2 staining. (ix) At baseline, distinct green and blue staining is seen, reflecting images of cytoplasmic NRF2 and nuclear staining. (xi) After Cl₂ exposure, there is a reduction of cytoplasmic staining for NRF2 (reduced green). (xii) After anti-Gr1, there is no loss of cytoplasmic staining, consistent with lack of nuclear translocation. (B) Quantification of NRF2 translocation using Image ProPlus software. NRF2 translocation occurred after Cl₂ exposure and this translocation was abrogated by anti-Gr1 treatment. **P < 0.01 (n = 6–10 mice per group; 8–10 airways per mouse).

is expressed in high levels on the surface of neutrophils (24) but also is expressed, albeit at lower levels, on monocytes/macrophages and dendritic cells (44). Eosinophils rose in the BAL fluid after Cl₂, although to levels that were approximately 1/20th of the neutrophils, and were significantly reduced by anti-GR1 treatment. Alveolar macrophages were only reduced under baseline conditions by anti-GR1 antibody and increased to comparable levels after Cl₂ exposure. Lymphocytes were not significantly affected. Anti-IL-5 antibody prevented the increase in eosinophils, and

macrophages were reduced by $\sim 45\%$ after clodronate liposomes, but neither intervention affected AHR significantly. To further confirm that neutrophils, rather than other Gr1-expressing cells, were responsible for the observed changes in AHR, we used an antibody directed against Ly6G and found the effects on AHR to be nearly identical to those of anti-Gr1. It therefore seems probable that the observed effects of anti-GR1 were mediated by a reduction in neutrophils.

The protective effect of neutrophil depletion was most marked against the

changes in resistance and less so against the changes in elastance. A further analysis of respiratory system mechanics using the constant phase model, which captures changes in large airway mechanics (Newtonian resistance), peripheral lung resistance/tissue damping, and tissue elastance (45), indicates that the protective effect of anti-Gr1 is predominantly seen in the large airways because neutrophil depletion was able to reduce respiratory system resistance and Newtonian resistance but not other parameters. These findings suggest potentially different mechanisms of alterations in mechanics in large airways versus peripheral lung compartments. Measurements of large airway parameters clearly showed that neutrophil depletion prevented AHR in Cl₂-exposed mice, maintaining it at levels comparable to their air-exposed counterparts. One potential explanation for these observations may be the mechanical effect of excess cellular influx within the airways. Histological images and analysis demonstrated neutrophil accumulation on the airway walls, and it is possible that these areas that encroach on the airway lumen sufficiently to contribute to the alterations in mechanics because any space occupying elements inside the airway lumen may also augment the increases in resistance evoked by MCh. Preventing the

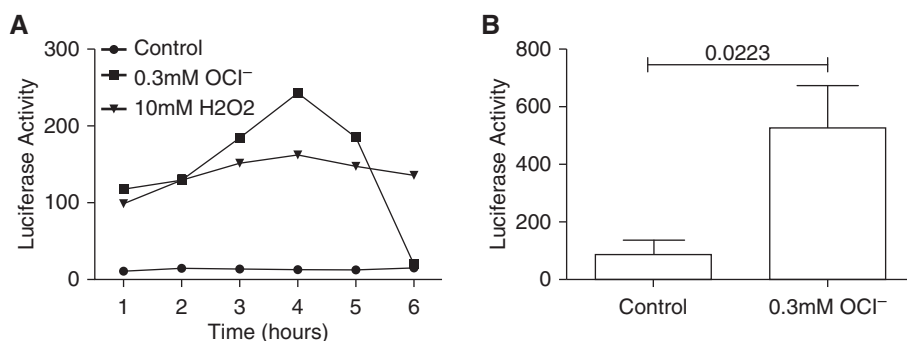


Figure 7. Hypochlorite induces NRF2 nuclear translocation in BEAS2b cells. (A) BEAS2b cells transfected with a luciferin-antioxidant response element reporter showed NRF2 nuclear translocation after administration of 0.3 mM hypochlorite. By 1 hour after stimulation, there was a 10-fold increase in relative fluorescent units over baseline (unstimulated) conditions. NRF2 nuclear translocation peaked at 3 to 4 hours. (B) Peak NRF2 nuclear translocation occurred at 4 hours following stimulation with hypochlorite in BEAS2b cells (n = 3).

influx of the encroaching cells may be sufficient to prevent increased airway resistance in large airways. Furthermore, the absence of neutrophils and the subsequent emigration, which can disrupt epithelial barrier cell function (46), may aid in maintaining normal function of the airways and contribute to preventing AHR. Despite the dramatic effect of neutrophil depletion on large airways, we observed that neutrophil depletion did not prevent AHR in the peripheral lung compartments. Again, these findings suggest different mechanisms mediating the central and peripheral lung compartments.

In conclusion, we have found that Cl₂ exposure alters cytokine levels; alters gene

expression of NRF2, SOD-1, HO-1, and GPX-2; and induces nuclear translocation of NRF2. Anti-Gr1 monoclonal antibody treatment prevented these changes, suggesting that oxidant stress generated by neutrophilia is responsible for a substantial portion of the oxidative burden. Furthermore, Cl₂-augmented airway responses to MCh were prevented by anti-Ly6G and anti-Gr1 treatment, supporting the notion that the causative agent is not wholly responsible for AHR and that these effects are neutrophil specific. The observation of neutrophil occupation in a large portion of the airway luminal space suggests that neutrophil presence may be detrimental

not only by increasing oxidative stress but also through mechanical means. These data provide a model to explore the mechanisms mediating the lungs' compartment-specific responses to oxidative stress and may help elucidate the complex role neutrophils play in oxidative stress-driven airway dysfunction. ■

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

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Title: Neutrophils Mediate Airway Hyperresponsiveness Following Chlorine-Induced Airway Injury in the Mouse

Authors: Toby K McGovern, Madison Goldberger, Benoit Allard, Soroor Farahnak, Yoichiro Hamamoto, Michael O'Sullivan, Nobuaki Hirota, Guy Martel, Simon Rousseau, James G Martin

ONLINE DATA SUPPLEMENT

Material and Methods

Enzyme Linked Immunosorbant Assay of IL-17A and IL-13

Enzyme linked immunosorbant assays (ELISAs) were performed on BAL samples taken 24 hours following Cl₂ exposure. Cytokine levels for IL-17A and IL-13 were determined according to manufacturer's instructions (eBioscience, San Diego, California, USA).

RNA isolation, reverse transcription and Real-Time quantitative PCR

The following primer sequences were used:

NRF2 forward; TCTCCTCGCTGGAAAAAGAA, reverse; AATGTGCTGGCTGTGCTTTA.

SOD-1: forward; CAGGACCTCATTTTAATCCTCAC, reverse; TGCCCAGGTCTCCAACAT.

GPX2: forward; CTGTGTAACCTCTGCTGTTCC, reverse; CCACACTACCTGAGTCTACC.

HO-1: forward; ACATCTATGTGGCCCTGGAG, reverse; TGTTGGGGAAGGTGAAGAAG

IL-17A, forward; TCCAGAAGGCCCTCAGACTA, reverse; ACACCCACCAGCATCTTCTC

IL-13: forward' ATGAGTCTGCAGTATCCCG, reverse; CCGTGGCAGACAGGAGTGTT.

NRF-2 luciferase reporter assay

Cells were seeded into 24 well plates (50,000 cells per well) in DMEM 10% FBS hygromycin B for 24 hours. Cells were serum deprived overnight and stimulated with 0.3 mM NaOCl or 10mM H₂O₂ for various time points. Reporter lysis buffer (Promega, Madison, WI, USA) was used to lyse the cells. Whole cell lysates were collected and spun at 13,000 RPM for 3 minutes. 10 µl of supernatant was transferred to a 96 well plate for reading in the Tecan iControl luciferase system in the presence of 470 µM D-Luciferin and 530µM ATP.

Eosinophil depletion

Eosinophil depletion was achieved by injecting TRFK5 (100µg i.p; BD Biosciences, Mississauga, ON, Canada) in 100µL PBS one hour prior to Cl₂ exposure. Twenty-four hours following Cl₂ exposure, inflammatory cell profiles were evaluated in BAL and airway physiology measurements were assessed.

Macrophage depletion

Macrophage depletion was achieved using clodronate (Encapsula NanoSciences, Nashville, TN, USA) encapsulated in liposomes. Clodronate (250µg) was administered both intra-tracheally in a single, 50µL instillation in PBS 48 hours prior to Cl₂ exposure and intravenously through the tail vein at a concentration of 0.1mg/kg. Empty liposomes (Encapsula NanoSciences) were used as a control.

Quantification of lung macrophages by flow cytometry

Lung macrophages were harvested after lung digestion in RPMI1640 containing collagenase IV (Sigma-Aldrich, USA), 150 CDU/ml. Cells were re-suspended in FACS buffer, 3% FBS and 0.09% sodium azide and stained for surface markers F4/80-APC and CD11c-PE (Biolegend, San Diego, California, USA). Data were acquired by LSRII flow cytometer and were analyzed by Flow Jo software (Flo Jo, Ashland, Oregon, USA).

Supplemental Figure Legends

Figure E1. Anti-Ly6G depletes neutrophils from BAL

100µg of anti-Ly6G was injected i.p. 12 hours prior to Cl₂ exposure. At 24 hours following Cl₂, BAL was collected and evaluated for inflammatory cells. (A) Treatment with anti-Ly6G reduced total inflammatory cells compared to mice that did not receive anti-Ly6G. (B) Anti-Ly6G did not affect macrophage numbers. (C) Treatment with anti-Ly6G completely attenuated increased

neutrophil numbers seen the BAL of untreated mice. (D) Anti-Ly6G did not affect eosinophil numbers. (E) Lymphocyte numbers were decreased at 24 hours by anti-Ly6G compared to Cl₂ exposed mice. (F) Epithelial cell shedding was not affected by anti-Ly6G treatment. *p<0.05, **p<0.01, ***p<0.001; n=6 per group.

Figure E2. Anti-Ly6G treatment reduces chlorine-induced AHR

Mice were injected with 100µg anti-Ly6G 12 hours prior to Cl₂ inhalation. Twenty-four hours later airway function measurements were taken in response to inhaled methacholine. (A) Treatment with anti-Ly6G completely attenuated Cl₂ induced increases in respiratory system resistance at the three highest doses of methacholine. (B) Anti-Ly6G reduced respiratory system elastance at lower doses of methacholine, but not at the highest dose. (C) Newtonian resistance was attenuated by anti-Ly6G treatment at the three highest doses of methacholine compared to Cl₂ treated mice. (D-E) Anti-Ly6G had no effect on either tissue damping or tissue elastance. * = Air vs Cl₂, # = Cl₂ vs Ly6G + Cl₂, \$ = Ly6G + Air vs Ly6G + Cl₂; **p<0.01, ***p<0.001; n=6-9 per group.

Figure E3. Effects of neutrophil depletion on pro-inflammatory cytokines

Twenty-four hours following Cl₂ exposure, BAL or lung tissue was collected and assayed for IL-17A or IL-13 by ELISA and PCR. (A) IL-17A was increased in BAL of Cl₂ exposed mice, but this effect was not prevented by neutrophil depletion. (B) IL-13 was increased following Cl₂ exposure, regardless of neutrophil depletion. (C-D) PCR showed no difference in expression levels of IL-17A or IL-13 at 24 hours. *p<0.05, n=6 mice per group.

Figure E4. Anti-IL-5 prevented eosinophilia selectively following chlorine-exposure

Mice were treated with 100µg anti-IL-5 1 hour prior to Cl₂ inhalation. (A) Anti-IL-5 had no effect on total inflammatory cells found in BAL. (B-C) Anti-IL-5 did not affect macrophage or neutrophil numbers in either air or Cl₂ exposed mice. (D) Anti-IL-5 was effective at reducing eosinophil numbers following Cl₂ exposure. (E-F) Anti-IL-5 did not affect epithelial cell shedding or lymphocyte numbers in BAL. *p<0.05, n=6-7 per group.

Figure E5. Eosinophil depletion does not affect chlorine-induced AHR

Mice were injected i.p. with 100µg of anti-IL5 one hour prior to Cl₂ exposure. Twenty four hours later airway physiology measurements were taken in response to inhaled methacholine. (A-E) Anti-IL-5 had no effect on respiratory system mechanics in any of the parameters examined. n=6-7 per group.

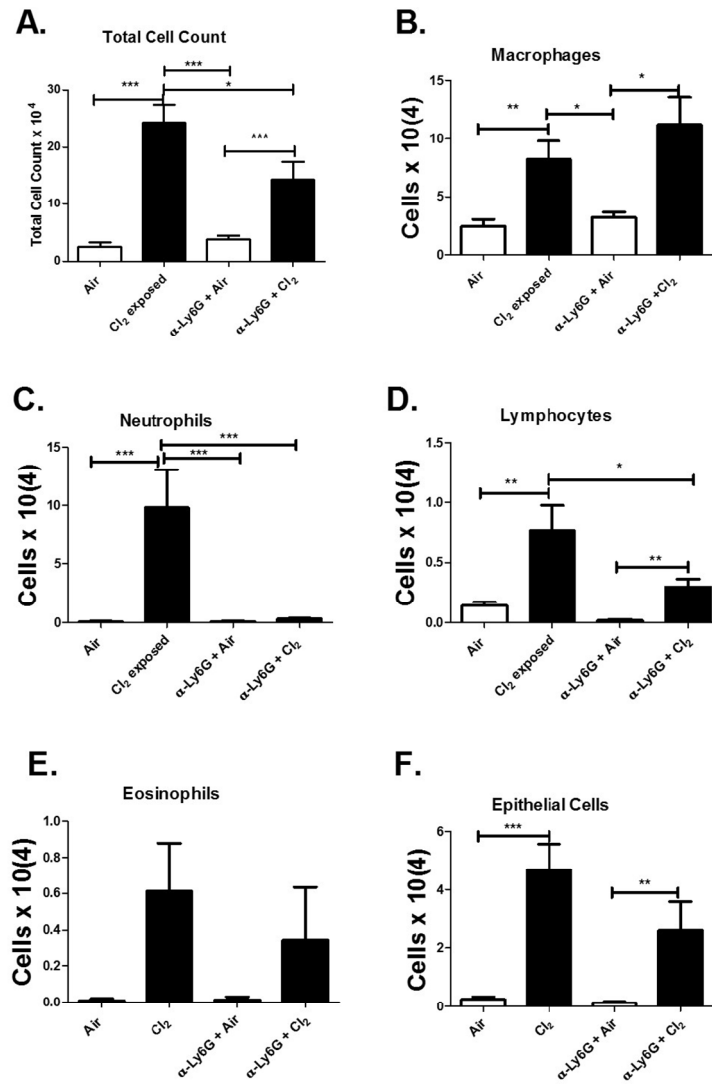
Figure E6. Clodronate liposomes depletion of BAL macrophages.

In order to assess the effect of clodronate on macrophage depletion and airway inflammation following Cl₂, mice were given intratracheal instillations of liposomes either loaded with clodronate or liposomes alone (encapsosomes) 72 hours prior to Cl₂ exposure. Twenty-four hours following Cl₂ exposure, total and differential cell counts were evaluated for efficacy of macrophage depletion. (A) No significant difference in the total cell count in untreated, encapsosome and clodronate treated mice were detected. (B) Clodronate liposomes were effective at depletion macrophages following Cl₂ exposure. (C-F) Clodronate liposomes had no effect on neutrophils, eosinophils lymphocytes or epithelial cells. *p<0.05, n=6 per group.

Figure E7. Depletion of alveolar macrophages in lung tissue

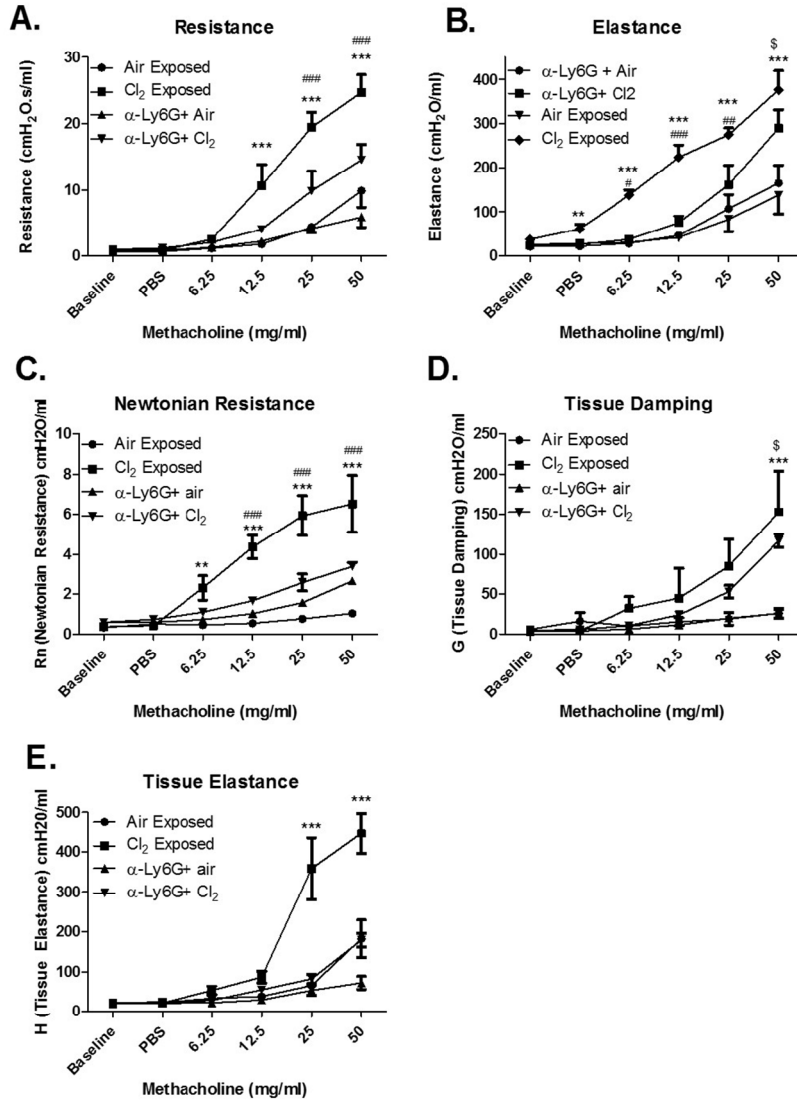
Following clodronate treatment and Cl₂ exposure, lungs were digested and assessed for alveolar macrophage populations in lung tissue. Representative graphs of FACS analysis for lung cells stained for CD11b and F4/80 are shown. (A) Population of CD11c+/F4/80+ in air exposed mice was 5.05%. (B) Air exposed mice following clodronate treatment and CD11c+/F4/80+ at 1.83%. (C) Following Cl₂ exposure CD11c+/F4/80+ at 5.91%. Panel D shows the population of CD11c+/F4/80+ cells at 2.39%. (E) Isotype control. n=4-5.

E1



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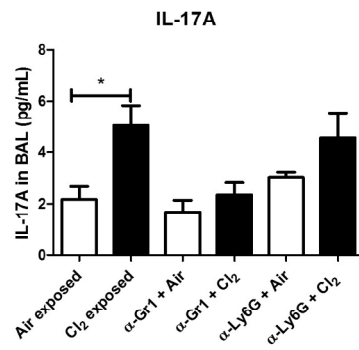
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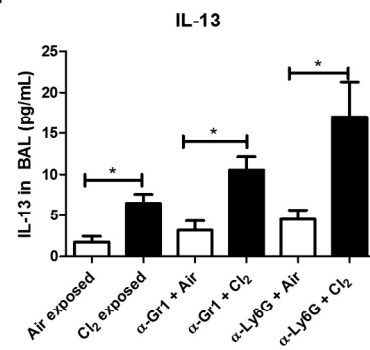
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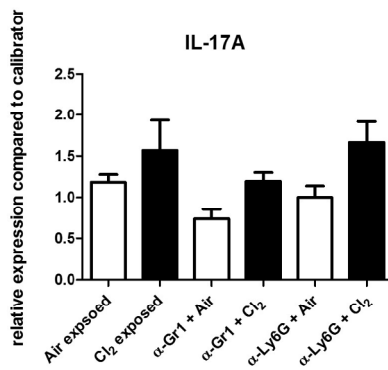
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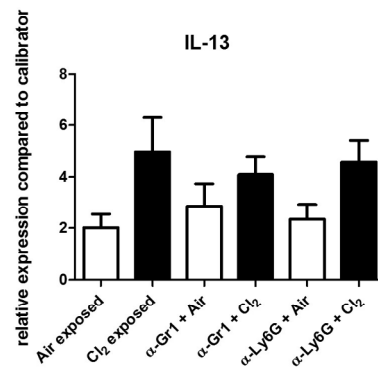
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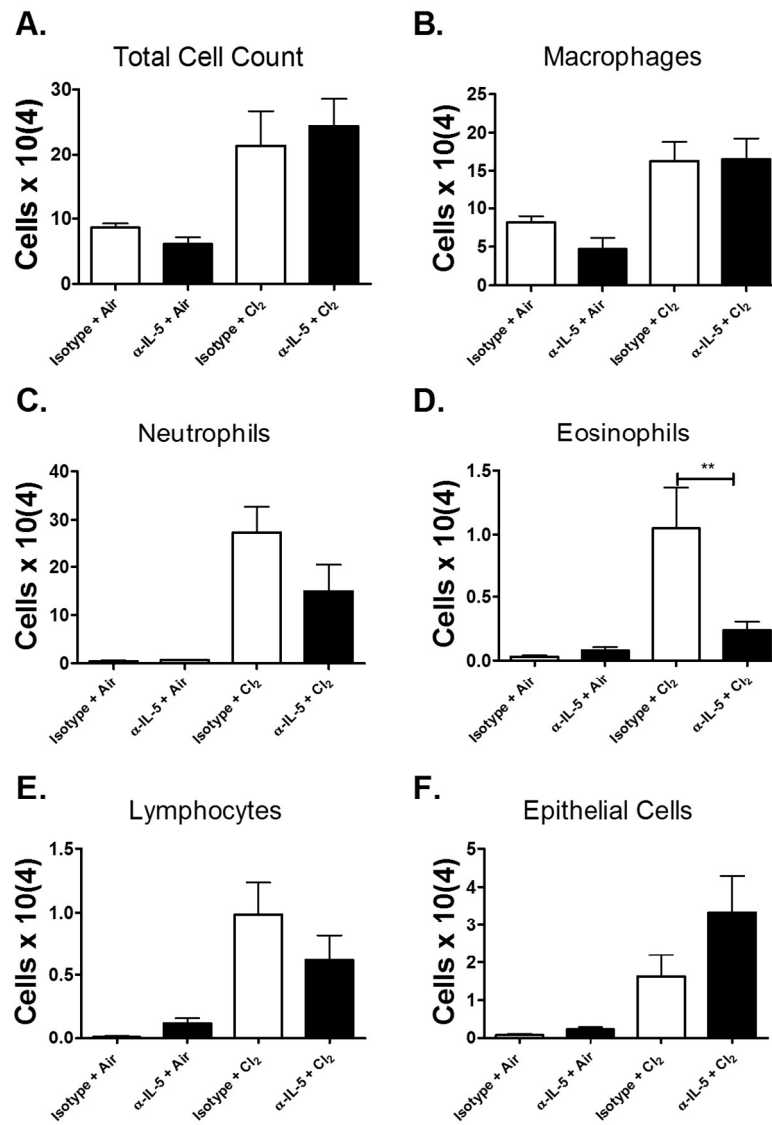


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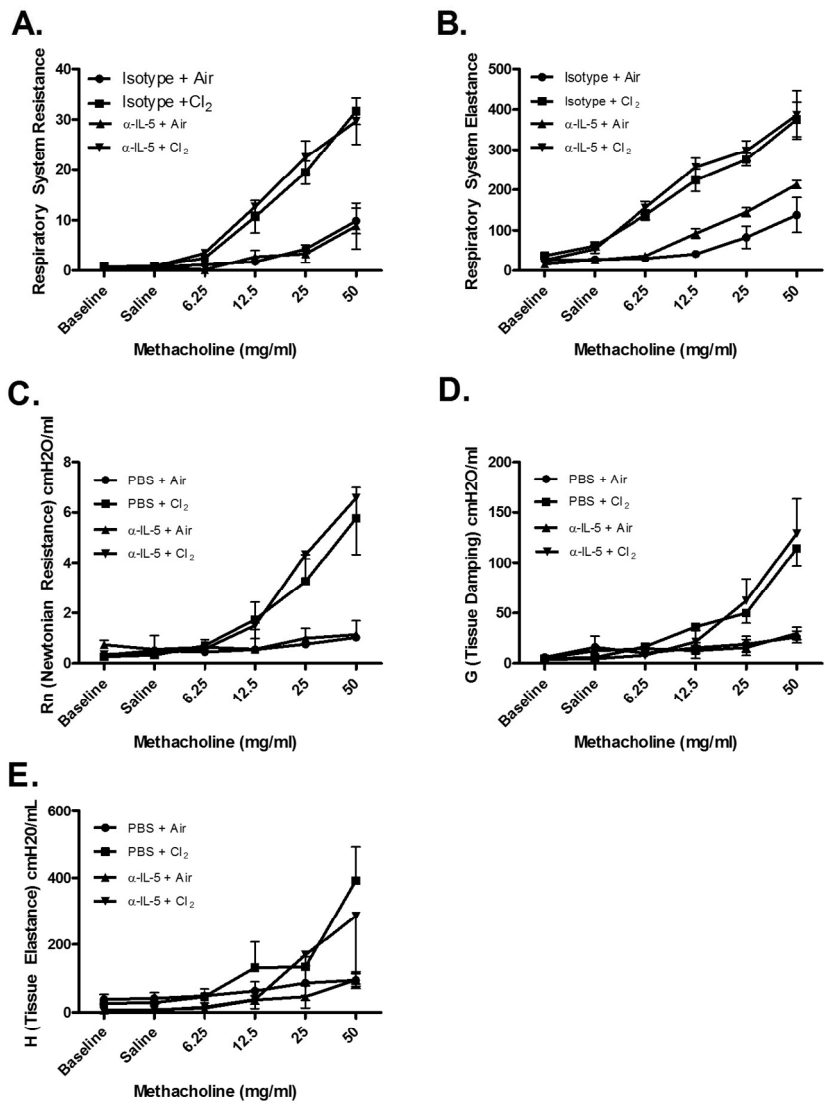
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E4



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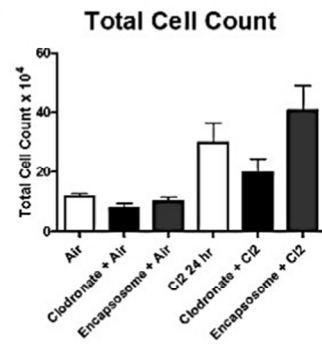
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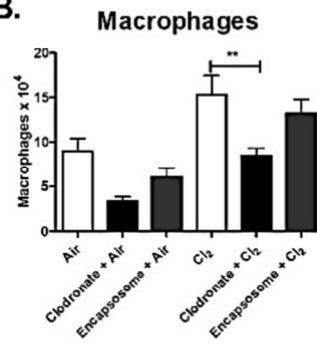
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E6

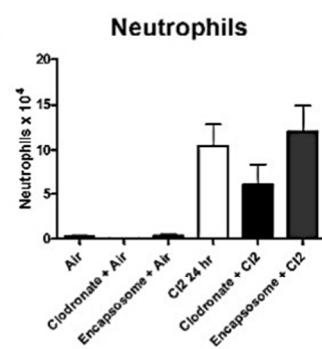
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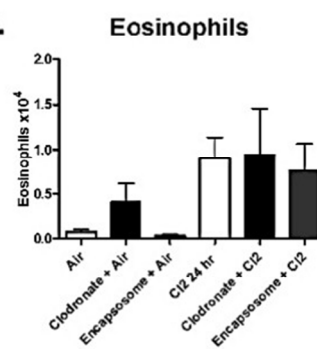
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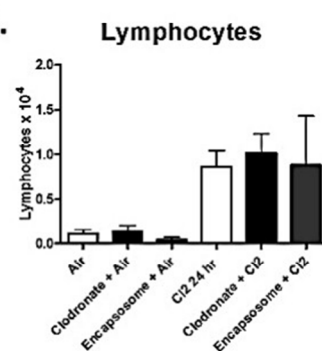
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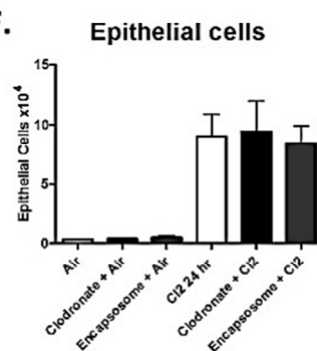
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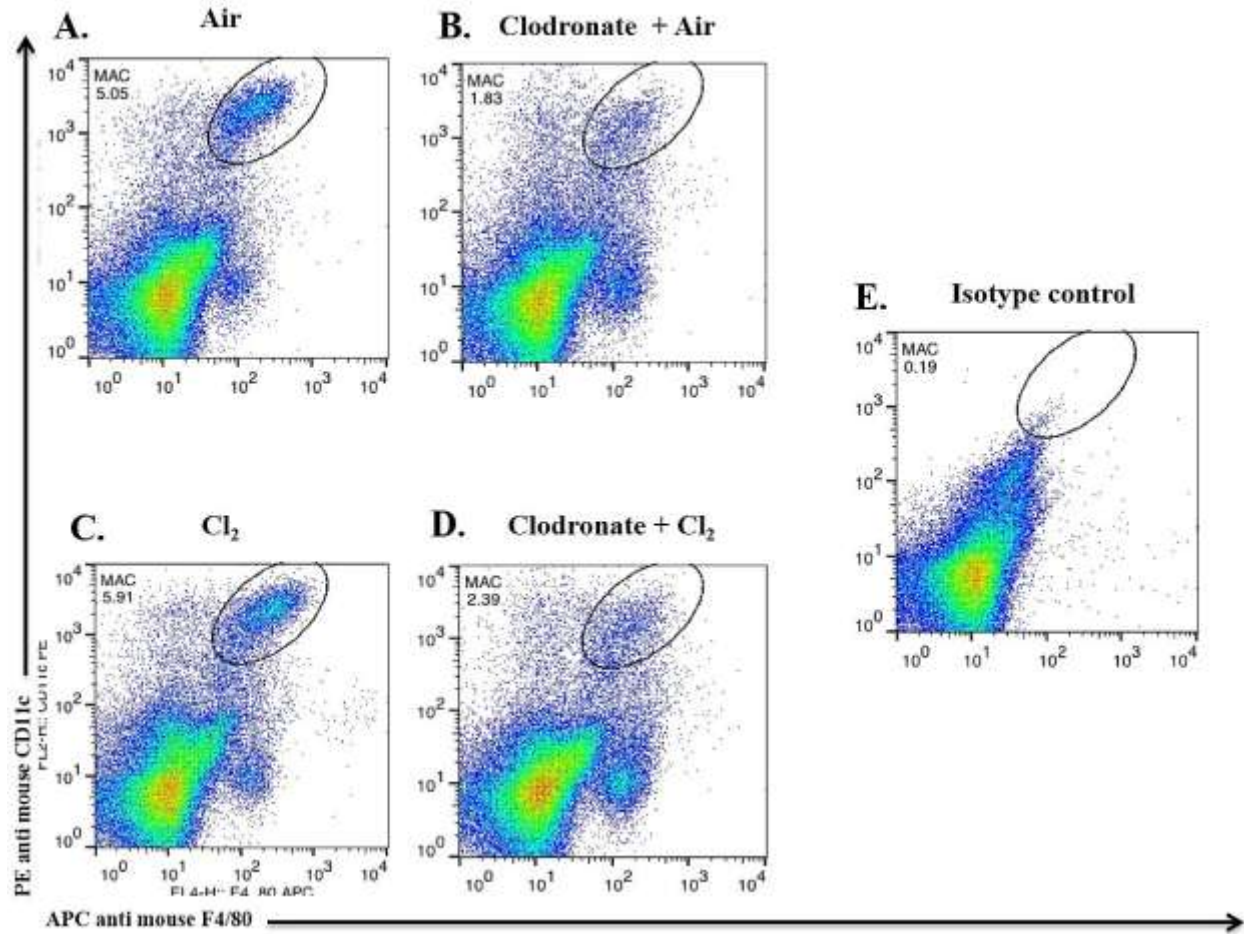


F.



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E7



Lung specific loss of $\alpha 3$ laminin worsens bleomycin-induced pulmonary fibrosis

SUPPLEMENTARY DATA

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