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Lung injury, oxidative stress and fibrosis in mice following exposure to nitrogen mustard

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Abstract

Nitrogen mustard (NM) is a cytotoxic vesicant known to cause acute lung injury which progresses to fibrosis. Herein, we developed a murine model of NM-induced pulmonary toxicity with the goal of assessing inflammatory mechanisms of injury. C57Bl6/J mice were euthanized 1–28 d following intratracheal exposure to NM (0.08 mg/kg) or PBS control. NM caused progressive alveolar epithelial thickening, perivascular inflammation, bronchiolar epithelial hyperplasia, interstitial fibroplasia and fibrosis, peaking 14 d post exposure. Enlarged foamy macrophages were also observed in the lung 14 d post NM, along with increased numbers of microparticles in bronchoalveolar lavage fluid (BAL). Following NM exposure, rapid and prolonged increases in BAL cells, protein, total phospholipids and surfactant protein (SP)-D were also detected. Flow cytometric analysis showed that CD11b⁺Ly6G⁺F4/80⁺Ly6C^{hi} proinflammatory macrophages accumulated in the lung after NM, peaking at 3 d. This was associated with macrophage expression of HMGB1 and TNF α in histologic sections. CD11b⁺Ly6G⁺F4/80⁺Ly6C^{lo} anti-inflammatory/pro-fibrotic macrophages also increased in the lung after NM peaking at 14 d, a time coordinate with increases in TGF β expression and fibrosis. NM exposure also resulted in alterations in pulmonary mechanics including increases in tissue elastance and decreases in compliance and static compliance, most prominently at 14 d. These findings demonstrate that NM induces structural and inflammatory changes in the lung that correlate with aberrations in pulmonary function. This mouse model will be useful for mechanistic studies of mustard lung injury and for assessing potential countermeasures.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Keywords

Mustard vesicants; lung injury; macrophages; fibrosis; pulmonary function

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Introduction

Sulfur mustard and the related analog, nitrogen mustard (NM), are cytotoxic vesicants developed as chemical warfare agents. They are known to cause severe and debilitating damage to the respiratory tract, the major cause of morbidity and mortality in exposed victims. Both acute and chronic effects of mustards have been described including inflammation, alveolar-epithelial barrier dysfunction, edema, emphysema and fibrosis (Balali-Mood *et al.*, 2005; Razavi *et al.*, 2013; Weinberger *et al.*, 2011).

Pulmonary injury induced by mustards is associated with an accumulation of inflammatory neutrophils and macrophages in the lung (Malaviya *et al.*, 2010; Malaviya *et al.*, 2012; Sunil *et al.*, 2014). Whereas neutrophils are thought to be involved in the early acute response, macrophages have been implicated in both acute and chronic pathologies associated with mustard exposure (Gustafsson *et al.*, 2014; Malaviya *et al.*, 2016; Sunil *et al.*, 2011). Evidence suggests that the diverse contributions of macrophages to mustard toxicity is due to their ability to respond to localized environmental cues and develop into distinct subpopulations, broadly identified as proinflammatory/cytotoxic M1 macrophages and anti-inflammatory/wound repair M2 macrophages (Arora *et al.*, 2018; Martinez *et al.*, 2014). Prolonged activation and excessive release of inflammatory mediators by M1 and/or M2 macrophages are thought to exacerbate acute toxicity and promote the development of fibrosis (Laskin *et al.*, 2011; Murray *et al.*, 2011).

In the present studies, we describe the pathologic response of mice to pulmonary NM exposure. Our overall goal was to develop a murine model of exposure useful for mechanistic studies and for the assessment of countermeasures that target inflammatory cell subpopulations and mediators they release.

Materials and Methods

Animals and exposures

Male and female specific pathogen-free C57Bl6/J mice (8–12 weeks, The Jackson Laboratories, Bar Harbor, ME) 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 intratracheally to NM (0.08 mg/kg) or PBS control as previously described (Sunil *et al.*, 2018). All instillations were performed by the same individual from Rutgers University Comparative Medicine Resources.

Sample collection

Animals were euthanized 1 d, 3 d, 14 d and 28 d after exposure by intraperitoneal injection of ketamine-xylazine (135 mg/kg; 30 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). Bronchoalveolar lavage (BAL) fluid was collected by slowly instilling and withdrawing 1 ml of ice-cold PBS into the lung three times through a 20-gauge cannula in the trachea. BAL fluid was centrifuged ($300 \times g$, 8 min), supernatants collected, aliquoted, and stored at -80°C until analysis. Cell pellets were resuspended in 250 μl PBS and viable cells enumerated on a hemocytometer using trypan blue dye exclusion. For differential analysis, cytospin preparations of BAL cells were fixed in methanol and stained with Giemsa (Labchem Inc., Pittsburgh, PA). A total of 300 cells were counted by light microscopy.

For isolation of cells for flow cytometry, the lung was perfused *in situ* via the right ventricle with 5 ml of ice-cold PBS. BAL was collected as described above. The lung was then removed and instilled five times with 1 ml HBSS (22 mM HEPES, 4.2 mM NaHCO_3 , pH 7.3; room temperature), while gently massaging the tissue. Lavage fluid was centrifuged ($300 \times g$, 8 min, 4°C), the cell pellet resuspended in 1 ml HBSS and combined with the first BAL lavage cell suspension. Cells were washed three times with HBSS-2% FBS and viable cells counted by trypan blue dye exclusion.

Separate groups of mice were used for tissue collection. The lung was inflated via the trachea with PBS containing 3% paraformaldehyde. After 4 h at 4°C , the tissue was transferred to 50% ethanol. Histological sections (4 μm) were prepared and stained with hematoxylin and eosin (H&E) or Mason's trichrome. Images were acquired at high resolution using an Olympus VS120 Virtual Microscopy System, scanned and viewed using OlyVIA version 2.6 software (Center Valley, PA). All tissues were initially examined at low magnification (2x objective). Subsequently areas of tissue damage were systematically assessed at high magnification (4x objective) and scored blindly by a board-certified veterinary pathologist (Michael Goedken, D.V.M, Ph.D.). The extent of inflammation, including macrophage and neutrophil recruitment, bronchiolar hyperplasia, alterations in alveolar epithelial barriers, fibrin deposition and fibrosis were scored on a scale of 0 to 4, with 0 = no change relative to control; 1 = minimal/very few/small lesions; 2 = few/small lesions; 3 = moderate/moderate size lesions; 4 = large/marked lesions/many changes (Ashcroft *et al.*, 1988). Scores reflect a single, weighted, overall assessment of the entire sample based on severity and distribution.

Measurement of BAL protein, phospholipids and microparticles

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 8–9 mice/treatment group were analyzed in triplicate at 560 nm on a Vmax MAXline™ microplate reader (Molecular Devices, Sunnyvale, CA). For analysis of phospholipids, BAL was centrifuged at $20,000 \times g$ (1 h, 4°C) and pellets containing large aggregate fractions analyzed spectrophotometrically as previously described (Atochina *et al.*, 2004). For microparticle analysis, BAL was centrifuged ($600 \times g$, 8 min) and 30 μl of supernatant transferred to tubes containing 195 μl PBS and 50 μl of counting beads (3 μm ; 10^3 beads/ml; Spherotech, Lake Forest, IL). Samples were incubated for 30 min at 4°C in

the dark and then analyzed on a Beckman-Coulter Gallios flow cytometer (Brea, CA). The microparticle gating strategy was based on forward and side scatter distributions of 0.3, 0.5, and 0.9 μm mega mix beads (Midura *et al.*, 2016). The number of microparticles/ml was quantified in samples containing counting beads using a 90 sec acquisition protocol and calculated as: microparticle events $\times$ number of beads added/bead events $\times$ sample volume, adapted from previous reports (Cointe *et al.*, 2017; McVey *et al.*, 2016).

Western blot analysis of SP-D

Small aggregate BAL fractions (13 μl /well; $\sim$ 2% of total BAL recovered), prepared as previously described (Atochina *et al.*, 2004), were separated on 4–12% Bis-Tris gradient reducing/denaturing gels (Invitrogen, San Diego, CA). Blots were normalized to BAL volume which was not affected by NM exposure. Proteins were transferred to PVDF membranes. Non-specific binding was blocked by incubating the membranes with 10% milk in T-TBS (0.5% Tween 20 in Tris-buffered saline) for 45 min at room temperature. Membranes were then incubated overnight at 4°C with rabbit polyclonal anti-SP-D antibody (DU117, 1:10,000; a gift from Dr. A. Pastva, Duke University). Membranes were washed and incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1:5000, 1% non-fat milk in T-TBS; Bio-Rad, Hercules, CA). Bands were visualized using an ECL Prime detection system (GE Health Care, Piscataway, NJ). Band intensity was assessed using ImageJ software.

Flow cytometry

Cells were resuspended in 100 μl of staining buffer (PBS, 2% FCS and 0.02% sodium azide) and incubated for 10 min at 4°C with anti-mouse CD16/32 (1:100; Biolegend, San Diego, CA) to block nonspecific binding. This was followed by 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, and then with eFluor 780-conjugated fixable viability dye (1:1000; eBioscience, San Diego, CA) for an additional 30 min at 4°C. Cells were washed twice with staining buffer, fixed with 2% para formaldehyde, and analyzed on a Beckman Coulter Gallios flow cytometer. Data were analyzed using Beckman Coulter Kaluza version 1.2 software. Lung cell populations were characterized as previously described (Sunil *et al.*, 2015). Representative examples of our gating strategy and number of events are shown in Supplementary Figure 1. In order to directly compare the two treatment groups, the same gates were used for cell population analysis in PBS and NM treated mice. Of note, subpopulations of lung cells from NM treated mice were more heterogeneous than cells from control mice.

Immunohistochemistry

Tissue sections (4 μm) were deparaffinized with xylene (4 min, $\times$ 2), followed by decreasing concentrations of ethanol (100%–50%) and then 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% H_2O_2 for 10–30 min, sections were incubated for 2 h at room temperature with 10–50% goat serum to block nonspecific binding. This was followed by

overnight incubation at 4°C with rabbit IgG or rabbit polyclonal anti-TNF α (1:200; Abcam, Cambridge, MA), anti-iNOS (1:500; Abcam), anti-Lcn2 (1:100; Abcam), anti-HO-1 (1:50; Enzo Life Sciences, Farmingdale, NY), anti-HMGB1 (1:200; Abcam), anti-mannose receptor (MR)-1 (1:750; Abcam) or anti-TGF β (1:100, 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). Positively staining macrophages were enumerated in all five lung lobes by light microscopy (15 fields/lobe).

Measurement of pulmonary mechanics

Mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). After 5 min, a tracheostomy was performed using an 18-gauge cannula and the animals connected to a SciReq flexiVent (Montreal, Canada) to measure respiratory mechanics, at positive end-expiratory pressure (PEEPs) ranging from 0–9 cm H₂O as previously described (D'Angelo *et al.*, 1992; Smith *et al.*, 2013). Data from the impedance spectra were fit to a constant phase model, allowing for the calculation of frequency-independent resistance, compliance, and the coefficients of tissue damping and elastance in the tissue compartment (Suki *et al.*, 1991). Data were analyzed using flexiVent software version 7.

Statistical analysis

All experiments were repeated at least 3 times. Data were analyzed using 2-way or 3-way ANOVA followed by post hoc analysis using the Mann-Whitney Rank Sum Test; a p value 0.05 was considered significant.

Results

Lung injury, oxidative stress and inflammation following exposure of mice to NM

Treatment of mice with NM resulted in progressive histopathological changes in the lung including hyperplasia of the bronchiolar epithelium, perivascular inflammation, the appearance of enlarged foamy macrophages in alveolar and peribronchiolar regions, and interstitial fibroplasia and fibrosis; these changes were most prominent 14 d post exposure (Figures 1 and 2 and Supplementary Table 1). Increases in BAL protein and cell content were also observed. Whereas BAL protein levels peaked 3 d post-NM, cell numbers peaked at 14 d (Figure 3). Differential staining of BAL cells revealed that the percentage of neutrophils was significantly greater in mice treated with NM relative to control mice, most notably at 3 d post exposure (Supplementary Table 2). Levels of total phospholipids and SP-D were also increased in BAL at 1 d and 3 d after NM (Figures 3 and 4). Whereas phospholipid levels remained elevated for 28 d, levels of SP-D began to decrease after 14 d. We also found that the total number of microparticles in BAL was increased 14 d post-NM (Figure 5).

NM-induced lung injury in mice was associated with oxidative stress as reflected by increased expression of Lcn-2 and HO-1 (Table 1). While persistent increases in Lcn-2 were observed in alveolar macrophages beginning at 3 d post-NM, HO-1 was only upregulated at 1 d and 14 d post-NM.

Effects of NM exposure on lung myeloid cells

In further studies we assessed the phenotype of inflammatory cells responding to NM using techniques in flow cytometry (Sunil *et al.*, 2015). In these experiments, cells were first analyzed for expression of CD11b, a $\beta 2$ -integrin expressed on infiltrating myeloid cells. This was followed by analysis of the granulocytic marker Ly6G, the macrophage activation marker Ly6C, and the mature lung macrophage markers F4/80 and CD11c (Supplementary Figure 1). Treatment of mice with NM resulted in a significant increase in infiltrating CD11b⁺Ly6G⁺ granulocytic cells and CD11b⁺Ly6G⁻ monocytic cells in the lung (Figure 6, top panels). NM-induced increases in granulocytic cells were rapid and transient, while monocytic cells increased gradually reaching a maximum at 14 d. CD11b⁺Ly6G⁻ monocytic cells were found to consist of mature (F4/80⁺CD11c⁺) proinflammatory Ly6C^{hi} and anti-inflammatory Ly6C^{lo} subpopulations. Both subpopulations increased in the lung following NM administration reaching peak levels at 3 d for proinflammatory Ly6C^{hi} macrophages and at 14 d for anti-inflammatory Ly6C^{lo} macrophages (Figure 6, middle panels).

Further analysis of the CD11b⁺Ly6G⁺ granulocytic subpopulation revealed that they also expressed high levels of Ly6C, a characteristic of myeloid-derived suppressor cells (MDSC) (Kong *et al.*, 2013). These cells consisted of F4/80⁺ monocytic (M) and F4/80⁻ granulocytic (G) subpopulations; the majority were G-MDSC. Both MDSC populations were increased at 1 d and 3 d after NM exposure (Figure 6, lower panels). In contrast, resident alveolar macrophages, identified as CD11b⁻CD11c⁺F4/80⁺Ly6G⁻Ly6C^{lo}, decreased after NM exposure, most notably at 1 d and 3 d, returning to control by 28 d (Table 2).

We next determined if inflammatory macrophages responding to NM were functionally activated by analyzing expression of proinflammatory/cytotoxic M1 (TNF α , HMGB1, iNOS), and anti-inflammatory/pro-fibrotic M2 (TGF β , MR-1) activation markers in histological sections (Laskin *et al.*, 2011). TNF α and HMGB1 were upregulated in lung macrophages at all time points post-NM; a small but significant increase in macrophage iNOS staining was also noted after NM, but only at 14 d post-exposure (Figures 7 and 8 and Table 1). Lung macrophages, as well as Type II cells were found to express TGF β at 1 d and 14 d post NM (Table 1 and Supplementary Figure 2). Conversely, NM had no significant effect on expression of MR-1 at any time point analyzed (Table 1).

Effects of NM on lung function

In our next series of studies we analyzed the effects of NM on airway and parenchymal mechanical properties by measuring lung function responses to increasing PEEP (Groves *et al.*, 2012). In control mice, total lung compliance, a measure of lung recruitment, increased over time as a function of PEEP, which is consistent with normal pulmonary function (Figure 9). Increased responsiveness to PEEP was blunted in NM treated mice, becoming statistically significant at 14 d (Figure 9). In control mice, tissue elastance, which was derived from lung impedance, decreased with increasing PEEP dependent recruitment; this was not observed in NM treated mice (Figure 9). These findings, together with our observation that static compliance is reduced 14 d post NM, indicate that the loss of compliance results from parenchymal changes in the lung. A small but significant decrease in PEEP-dependent hysteresis was also observed 1 d post NM, a response that correlated

with increases in BAL SP-D and phospholipids. By 28 d, NM-induced alterations in pulmonary function were largely resolved and lung mechanical properties were similar to control mice (Figure 9).

Discussion

The present studies characterize the progression of NM-induced pulmonary toxicity in mice with the overall goal of developing an experimental model that can be used for mechanistic studies and for the evaluation of potential therapeutics. An advantage of mice is their similarity to humans in terms of anatomy, physiology and genetics. Additionally, murine reagents are more readily available compared to other rodents facilitating an ability to perform mechanistic studies. Early histopathologic changes were noted in the lung within 3 d of NM exposure, which included hyperplasia of the bronchiolar and alveolar epithelium and alveolar wall thickening. These pathologies progressed with time reaching a maximum at 14 d; at this time, there was also evidence of perivascular inflammation, interstitial fibroplasia and fibrosis; enlarged foamy macrophages were also observed. These changes were mostly resolved by 28 d. We previously described similar effects of NM in rats; however, the response in rats was more robust when compared to mice (Venosa *et al.*, 2016). Additionally, in rats, bronchiectasis, squamous cell metaplasia, mesothelial cell proliferation and emphysema-like changes were observed in the lung beginning at 3 d post NM exposure, and prominent collagen deposits and fibrosis was noted at 28 d (Venosa *et al.*, 2016). Thus, while fibrosis progressed in rats, it resolved in mice. Species and/or genetic differences may account for the distinct responses of rats and mice to NM.

NM-induced histopathologic changes in the lungs of mice were correlated with increased numbers of inflammatory cells and protein in BAL, markers of alveolar epithelial barrier dysfunction (Bhalla, 1999; Bhalla *et al.*, 1999). The observation that BAL protein levels peaked 3 d after NM exposure and decreased thereafter, indicates that repair processes begin early in the inflammatory process. This is supported by our findings that early (1 d and 3 d) increases in BAL neutrophils after NM exposure rapidly decline, consistent with the resolution of the acute inflammatory response to tissue injury (Potey *et al.*, 2019; Robb *et al.*, 2016). In contrast, activated (TNF α ⁺, HMGB1⁺) proinflammatory M1 macrophages persisted in the lung for at least 28 d, indicating the presence of chronic inflammation.

Anti-inflammatory M2 macrophages also increased in the lung after NM, peaking at 14 d. M2 macrophages are known to express TGF β , a characteristic of a profibrotic phenotype (Wynn *et al.*, 2010). Our findings that the appearance of TGF β ⁺ M2 macrophages in the lung were coordinate with collagen deposition, are in accord with a role for these cells in the fibrogenic process (Wynn, 2008). Of note are our findings that TGF β ⁺ macrophages were also observed in the lung at 1 d post NM. TNF α has been reported to upregulate TGF β in idiopathic fibrosis and it may play a similar role in NM-induced TGF β expression (Oikonomou *et al.*, 2006). The fact that TGF β was upregulated in the lung 1 d post NM suggests that the fibrogenic process is initiated rapidly. TGF β was also identified in Type II alveolar epithelial cells at 1 d and 14 d after NM administration. Whether these cells also contribute to fibrogenesis after NM exposure remains to be determined. No changes were noted in macrophage expression of the anti-inflammatory M2 macrophage marker, MR-1,

even though increased numbers of Ly6C^{lo} anti-inflammatory macrophages were observed in the lung 14 d following NM treatment of mice. These data suggest that anti-inflammatory macrophages are not functionally activated; this may contribute to the persistence of proinflammatory M1 macrophages in the lung after NM.

Following NM administration, a significant increase in microparticles was noted in BAL collected 14 d post exposure. Microparticles (0.1 –1 µm diameter) are vesicles released by cells upon activation or during apoptosis that function to transport bioactive molecules involved in cell-cell communication and inflammation (Dengler *et al.*, 2013; Neri *et al.*, 2011; Nieri *et al.*, 2016). Microparticles derived from epithelial cells have been reported to stimulate the emigration of macrophages to the lung following injury caused by acid inhalation and hyperoxia (Lee *et al.*, 2017; Lee *et al.*, 2016). Microparticles have also been implicated in fibroblast migration to the lung during the development of COPD and IPF in humans (Bacha *et al.*, 2018; Kubo, 2018). Further studies are required to determine if microparticles play a similar role in macrophage and fibroblast accumulation in the lung following NM-induced injury.

The major phospholipids present in the lung are derived from pulmonary surfactants, key proteins involved in maintaining lung integrity and function (Sorensen, 2018). Our findings of persistent increases in BAL phospholipids following NM exposure are in accord with disruption of surfactant structure, and derangements in pulmonary lipids (Low *et al.*, 1988; Mander *et al.*, 2002; Scaccabarozzi *et al.*, 2015). Alterations in lung lining fluid phospholipids were associated with PEEP-dependent decreases in lung hysteresis 1 d post NM, indicating a reduced capacity for surfactant mediated functional recruitment. We also found SP-D levels were increased in BAL after NM exposure. SP-D is a pulmonary collectin that functions to suppress macrophage inflammatory activity (Sorensen, 2018). Increases in BAL SP-D are considered to be a marker of lung injury and inflammation (Venosa *et al.*, 2016). The fact that SP-D levels decline at 14 d post-NM, are in line with reduced inflammation and initiation of tissue repair processes.

Oxidative stress plays a key role in pulmonary disease pathogenesis induced by mustard vesicants (Malaviya *et al.*, 2016). In response to oxidative stress, cells upregulate Lcn2, a member of the lipocalin superfamily, which regulates inflammatory responses and maintains cellular homeostasis, as well as HO-1, a phase II stress response enzyme with anti-oxidant and anti-inflammatory activity (Bahmani *et al.*, 2010; Borkham-Kamphorst *et al.*, 2013). Consistent with NM-induced oxidative stress in mice, both Lcn2 and HO-1 expression increased in alveolar macrophages (Malaviya *et al.*, 2015; Malaviya *et al.*, 2012; Sunil *et al.*, 2014). While NM-induced HO-1 expression decreased after 14 d, expression of Lcn-2 persisted for at least 28 d. Lcn-2 protects against oxidative stress by upregulating antioxidants (Roudkenar *et al.*, 2008; Roudkenar *et al.*, 2009). It has also been reported to stimulate tissue repair in models of kidney and liver injury and allergic airway disease (Borkham-Kamphorst *et al.*, 2013; Dittrich *et al.*, 2010; Kashani *et al.*, 2015; Schmidt-Ott *et al.*, 2007; Srisawat *et al.*, 2014). We speculate that Lcn-2 plays an analogous role in repair of the lung in mice after NM exposure.

Flow cytometric analysis of lung myeloid cells demonstrated that both proinflammatory CD11b⁺Ly6G⁻Ly6C^{hi} and anti-inflammatory CD11b⁺Ly6G⁻Ly6C^{lo} macrophages accumulate in the lung after exposure of mice to NM. Whereas pro-inflammatory Ly6C^{hi} cells peaked at 3 d post-NM, anti-inflammatory Ly6C^{lo} cells reached a maximum at 14 d post NM exposure, consistent with their respective roles in tissue injury and fibrosis (Wynn *et al.*, 2016). Proinflammatory macrophages accumulating in the lung 3–14 d after NM were found to express TNF α and HMGB-1, indicating that they are functionally active. iNOS expression was also upregulated in lung macrophages, but only at 14 d post NM treatment of mice. These data suggest that there are multiple subpopulations of M1 macrophages that appear in the lung at different times following NM exposure.

MDSC are a heterogeneous population of myeloid cells with diverse functions in the lung (Sendo *et al.*, 2018). Like anti-inflammatory M2 macrophages, they suppress inflammation and induce tissue repair (Kolahian *et al.*, 2016; Sendo *et al.*, 2018). We observed early and rapid increases in numbers of M-MDSC and G-MDSC in the lungs of mice after NM exposure. Further studies are needed to determine if this represents a compensatory attempt to limit proinflammatory macrophage activation in the absence of functionally active anti-inflammatory M2 macrophages.

Resident alveolar macrophages, identified as CD11b⁻CD11c⁺F4/80⁺Ly6G⁻Ly6C^{lo}, were reduced in the lung at early times (1 d - 3 d) after NM exposure. Resident macrophages function to maintain lung homeostasis by recognizing pathogens and noxious stimuli, and initiating an acute inflammatory response; this is thought to be due, in part, to activation of intracellular cell death pathways such as pyroptosis, metosis and necroptosis (Hussell *et al.*, 2014). Resident macrophages originate from embryonic progenitors and are mainly sustained by proliferative self-renewal (Hashimoto *et al.*, 2013). Increases in resident macrophages after 14 d is likely due to proliferation of surviving subpopulations of these cells as the acute inflammatory response resolves.

To assess the effects of NM on pulmonary mechanics, we examined a number of functional parameters at varying levels of PEEP using a small animal ventilator. The most notable effects of NM on lung function occurred 14 d post exposure, when histologic evidence of tissue injury and fibrosis were most prominent. At this time, there was significant tissue consolidation, which we predicted would produce a loss of total lung compliance and indeed, this is what we observed. Of note, NM-induced loss of total lung compliance was most pronounced at high PEEP, indicating a failure of parenchymal recruitment. This is supported by our findings that tissue elastance increased after NM, a response that was also most pronounced at high PEEP. In contrast, static compliance, which is determined primarily by the elastic properties of the tissue, was reduced in NM-treated mice at all levels of PEEP. Importantly, in accord with histological evidence of inflammation and injury resolution at 28 d, changes in lung function were at control levels at this time.

In summary, the present studies show that pulmonary exposure of mice to NM causes an early inflammatory response at 1 d and 3 d followed by fibrosis at 14 d which was mostly resolved by 28 d. These changes were correlated with time-related aberrations in lung function. This mouse model of mustard lung injury will be particularly useful for

mechanistic studies, as murine reagents and transgenic animals are readily available. These studies will be important in the development of therapeutics aimed at treating mustard-induced pulmonary toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Arora S, Dev K, Agarwal B, Das P, Syed MA (2018). Macrophages: Their role, activation and polarization in pulmonary diseases. *Immunobiology*. 223, 383–396. [PubMed: 29146235]
- Ashcroft T, Simpson JM, Timbrell V (1988). Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol*. 41, 467–470. [PubMed: 3366935]
- Atochina EN, Beers MF, Hawgood S, Poulain F, Davis C, Fusaro T, Gow AJ (2004). Surfactant protein-D, a mediator of innate lung immunity, alters the products of nitric oxide metabolism. *Am J Respir Cell Mol Biol*. 30, 271–279. [PubMed: 12871850]
- Bacha NC, Blandinieres A, Rossi E, Gendron N, Nevo N, Lecourt S, Guerin CL, Renard JM, Gaussem P, Angles-Cano E, Boulanger CM, Israel-Biet D, Smadja DM (2018). Endothelial microparticles are associated to pathogenesis of idiopathic pulmonary fibrosis. *Stem Cell Rev*. 14, 223–235.
- Bahmani P, Halabian R, Rouhbakhsh M, Roushandeh AM, Masroori N, Ebrahimi M, Samadikuchaksaraei A, Shokrgozar MA, Roudkenar MH (2010). Neutrophil gelatinase-associated lipocalin induces the expression of heme oxygenase-1 and superoxide dismutase 1, 2. *Cell Stress Chaperones*. 15, 395–403. [PubMed: 19904630]
- Balali-Mood M, Hefazi M (2005). The pharmacology, toxicology, and medical treatment of sulphur mustard poisoning. *Fundam Clin Pharmacol*. 19, 297–315. [PubMed: 15910653]
- Bhalla DK (1999). Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *J Toxicol Environ Health B Crit Rev*. 2, 31–86. [PubMed: 10081525]
- Bhalla DK, Gupta SK, Reinhart PG (1999). Alteration of epithelial integrity, alkaline phosphatase activity, and fibronectin expression in lungs of rats exposed to ozone. *J Toxicol Environ Health A*. 57, 329–343. [PubMed: 10405187]
- Borkham-Kamphorst E, Van De Leur E, Zimmermann HW, Karlmark KR, Tihaa L, Haas U, Tacke F, Berger T, Mak TW, Weiskirchen R (2013). Protective effects of lipocalin-2 (LCN2) in acute liver injury suggest a novel function in liver homeostasis. *Biochim Biophys Acta*. 1832, 660–673. [PubMed: 23376114]
- Cointe S, Judicone C, Robert S, Mooberry MJ, Poncelet P, Wauben M, Nieuwland R, Key NS, Dignat-George F, Lacroix R (2017). Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. *J Thromb Haemost*. 15, 187–193. [PubMed: 27662257]
- D'angelo E, Calderini E, Tavola M, Bono D, Milic-Emili J (1992). Effect of PEEP on respiratory mechanics in anesthetized paralyzed humans. *J Appl Physiol* (1985). 73, 1736–1742. [PubMed: 1474045]

- Dengler V, Downey GP, Tudor RM, Eltzschig HK, Schmidt EP (2013). Neutrophil intercellular communication in acute lung injury. Emerging roles of microparticles and gap junctions. *Am J Respir Cell Mol Biol.* 49, 1–5. [PubMed: 23815257]
- Dittrich AM, Krokowski M, Meyer HA, Quarcio D, Avagyan A, Ahrens B, Kube SM, Witzernath M, Loddenkemper C, Cowland JB, Hamelmann E (2010). Lipocalin2 protects against airway inflammation and hyperresponsiveness in a murine model of allergic airway disease. *Clin Exp Allergy.* 40, 1689–1700. [PubMed: 20412141]
- Groves AM, Gow AJ, Massa CB, Laskin JD, Laskin DL (2012). Prolonged injury and altered lung function after ozone inhalation in mice with chronic lung inflammation. *Am J Respir Cell Mol Biol.* 47, 776–783. [PubMed: 22878412]
- Gustafsson A, Svensson-Elfsmark L, Lorentzen JC, Bucht A (2014). Strain differences influence timing and magnitude of both acute and late inflammatory reactions after intratracheal instillation of an alkylating agent in rats. *J Appl Toxicol.* 34, 272–280. [PubMed: 23625772]
- Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J, Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, Van Rooijen N, Garcia-Sastre A, Stanley ER, Ginhoux F, Frenette PS, Merad M (2013). Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity.* 38, 792–804. [PubMed: 23601688]
- Hussell T, Bell TJ (2014). Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol.* 14, 81–93. [PubMed: 24445666]
- Kashani K, Kellum JA (2015). Novel biomarkers indicating repair or progression after acute kidney injury. *Curr Opin Nephrol Hypertens.* 24, 21–27. [PubMed: 25415614]
- Kolahian S, Oz HH, Zhou B, Griessinger CM, Rieber N, Hartl D (2016). The emerging role of myeloid-derived suppressor cells in lung diseases. *Eur Respir J.* 47, 967–977. [PubMed: 26846830]
- Kong YY, Fuchsberger M, Xiang SD, Apostolopoulos V, Plebanski M (2013). Myeloid derived suppressor cells and their role in diseases. *Curr Med Chem.* 20, 1437–1444. [PubMed: 23409714]
- Kubo H (2018). Extracellular vesicles in lung disease. *Chest.* 153, 210–216. [PubMed: 28684288]
- Laskin DL, Sunil VR, Gardner CR, Laskin JD (2011). Macrophages and tissue injury: agents of defense or destruction? *Annu Rev Pharmacol Toxicol.* 51, 267–288. [PubMed: 20887196]
- Lee H, Zhang D, Wu J, Otterbein LE, Jin Y (2017). Lung epithelial cell-derived microvesicles regulate macrophage migration via microRNA-17/221-induced integrin beta1 recycling. *J Immunol.* 199, 1453–1464. [PubMed: 28674181]
- Lee H, Zhang D, Zhu Z, Dela Cruz CS, Jin Y (2016). Epithelial cell-derived microvesicles activate macrophages and promote inflammation via microvesicle-containing microRNAs. *Sci Rep.* 6, 35250. [PubMed: 27731391]
- Low RB, Adler KB, Woodcock-Mitchell J, Giancola MS, Vacek PM (1988). Bronchoalveolar lavage lipids during development of bleomycin-induced fibrosis in rats. Relationship to altered epithelial cell morphology. *Am Rev Respir Dis.* 138, 709–713. [PubMed: 2462382]
- Malaviya R, Sunil VR, Cervelli J, Anderson DR, Holmes WW, Conti ML, Gordon RE, Laskin JD, Laskin DL (2010). Inflammatory effects of inhaled sulfur mustard in rat lung. *Toxicol Appl Pharmacol.* 248, 89–99. [PubMed: 20659490]
- Malaviya R, Sunil VR, Venosa A, Vayas KN, Heck DE, Laskin JD, Laskin DL (2016). Inflammatory mechanisms of pulmonary injury induced by mustards. *Toxicol Lett.* 244, 2–7. [PubMed: 26478570]
- Malaviya R, Sunil VR, Venosa A, Verissimo VL, Cervelli JA, Vayas KN, Hall L, Laskin JD, Laskin DL (2015). Attenuation of nitrogen mustard-induced pulmonary injury and fibrosis by anti-tumor necrosis factor-alpha antibody. *Toxicol Sci.* 148, 71–88. [PubMed: 26243812]
- Malaviya R, Venosa A, Hall L, Gow AJ, Sinko PJ, Laskin JD, Laskin DL (2012). Attenuation of acute nitrogen mustard-induced lung injury, inflammation and fibrogenesis by a nitric oxide synthase inhibitor. *Toxicol Appl Pharmacol.* 265, 279–291. [PubMed: 22981630]
- Mander A, Langton-Hewer S, Bernhard W, Warner JO, Postle AD (2002). Altered phospholipid composition and aggregate structure of lung surfactant is associated with impaired lung function in

- young children with respiratory infections. *Am J Respir Cell Mol Biol.* 27, 714–721. [PubMed: 12444031]
- Martinez FO, Gordon S (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 6, 13. [PubMed: 24669294]
- Mcvey MJ, Spring CM, Semple JW, Maishan M, Kuebler WM (2016). Microparticles as biomarkers of lung disease: enumeration in biological fluids using lipid bilayer microspheres. *Am J Physiol Lung Cell Mol Physiol.* 310, L802–814. [PubMed: 26944090]
- Midura EF, Prakash PS, Johnson BL 3rd, Rice TC, Kunz N, Caldwell CC (2016). Impact of caspase-8 and PKA in regulating neutrophil-derived microparticle generation. *Biochem Biophys Res Commun.* 469, 917–922. [PubMed: 26707875]
- Murray PJ, Wynn TA (2011). Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol.* 11, 723–737. [PubMed: 21997792]
- Neri T, Armani C, Pegoli A, Cordazzo C, Carmazzi Y, Brunelleschi S, Bardelli C, Breschi MC, Paggiaro P, Celi A (2011). Role of NF-kappaB and PPAR-gamma in lung inflammation induced by monocyte-derived microparticles. *Eur Respir J.* 37, 1494–1502. [PubMed: 21148223]
- Nieri D, Neri T, Petrini S, Vagaggini B, Paggiaro P, Celi A (2016). Cell-derived microparticles and the lung. *Eur Respir Rev.* 25, 266–277. [PubMed: 27581826]
- Oikonomou N, Harokopos V, Zalevsky J, Valavanis C, Kotanidou A, Szymkowski DE, Kollias G, Aidinis V (2006). Soluble TNF mediates the transition from pulmonary inflammation to fibrosis. *PLoS One.* 1, e108. [PubMed: 17205112]
- Potey PM, Rossi AG, Lucas CD, Dorward DA (2019). Neutrophils in the initiation and resolution of acute pulmonary inflammation: understanding biological function and therapeutic potential. *J Pathol.* 247, 672–685. [PubMed: 30570146]
- Razavi SM, Ghanei M, Salamati P, Safiabadi M (2013). Long-term effects of mustard gas on respiratory system of Iranian veterans after Iraq-Iran war: a review. *Chin J Traumatol.* 16, 163–168. [PubMed: 23735551]
- Robb CT, Regan KH, Dorward DA, Rossi AG (2016). Key mechanisms governing resolution of lung inflammation. *Semin Immunopathol.* 38, 425–448. [PubMed: 27116944]
- Roudkenar MH, Halabian R, Ghasemipour Z, Roushandeh AM, Rouhbakhsh M, Nekogoftar M, Kuwahara Y, Fukumoto M, Shokrgozar MA (2008). Neutrophil gelatinase-associated lipocalin acts as a protective factor against H₂O₂ toxicity. *Arch Med Res.* 39, 560–566. [PubMed: 18662586]
- Roudkenar MH, Halabian R, Roushandeh AM, Nourani MR, Masroori N, Ebrahimi M, Nikogoftar M, Rouhbakhsh M, Bahmani P, Najafabadi AJ, Shokrgozar MA (2009). Lipocalin 2 regulation by thermal stresses: protective role of Lcn2/NGAL against cold and heat stresses. *Exp Cell Res.* 315, 3140–3151. [PubMed: 19732769]
- Scaccabarozzi D, Deroost K, Lays N, Omodeo Sale F, Van Den Steen PE, Taramelli D (2015). Altered lipid composition of surfactant and lung tissue in murine experimental malaria-associated acute respiratory distress syndrome. *PLoS One.* 10, e0143195. [PubMed: 26624290]
- Schmidt-Ott KM, Mori K, Li JY, Kalandadze A, Cohen DJ, Devarajan P, Barasch J (2007). Dual action of neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol.* 18, 407–413. [PubMed: 17229907]
- Sendo S, Saegusa J, Morinobu A (2018). Myeloid-derived suppressor cells in non-neoplastic inflamed organs. *Inflamm Regen.* 38, 19. [PubMed: 30237829]
- Smith BJ, Grant KA, Bates JH (2013). Linking the development of ventilator-induced injury to mechanical function in the lung. *Ann Biomed Eng.* 41, 527–536. [PubMed: 23161164]
- Sorensen GL (2018). Surfactant protein D in respiratory and non-respiratory diseases. *Front Med (Lausanne).* 5, 18. [PubMed: 29473039]
- Srisawat N, Murugan R, Kellum JA (2014). Repair or progression after AKI: a role for biomarkers? *Nephron Clin Pract.* 127, 185–189. [PubMed: 25343847]
- Suki B, Bates JH (1991). A nonlinear viscoelastic model of lung tissue mechanics. *J Appl Physiol* (1985). 71, 826–833. [PubMed: 1757318]
- Sunil VR, Francis M, Vayas KN, Cervelli JA, Choi H, Laskin JD, Laskin DL (2015). Regulation of ozone-induced lung inflammation and injury by the beta-galactoside-binding lectin galectin-3. *Toxicol Appl Pharmacol.* 284, 236–245. [PubMed: 25724551]

- Sunil VR, Patel KJ, Shen J, Reimer D, Gow AJ, Laskin JD, Laskin DL (2011). Functional and inflammatory alterations in the lung following exposure of rats to nitrogen mustard. *Toxicol Appl Pharmacol.* 250, 10–18. [PubMed: 20883710]
- Sunil VR, Vayas KN, Cervelli JA, Ebramova EV, Gow AJ, Goedken M, Malaviya R, Laskin JD, Laskin DL (2018). Protective role of surfactant protein-D against lung injury and oxidative stress induced by nitrogen mustard. *Toxicol Sci.* 166, 108–122. [PubMed: 30060251]
- Sunil VR, Vayas KN, Cervelli JA, Malaviya R, Hall L, Massa CB, Gow AJ, Laskin JD, Laskin DL (2014). Pentoxifylline attenuates nitrogen mustard-induced acute lung injury, oxidative stress and inflammation. *Exp Mol Pathol.* 97, 89–98. [PubMed: 24886962]
- Venosa A, Malaviya R, Choi H, Gow AJ, Laskin JD, Laskin DL (2016). Characterization of distinct macrophage subpopulations during nitrogen mustard-induced lung injury and fibrosis. *Am J Respir Cell Mol Biol.* 54, 436–446. [PubMed: 26273949]
- Weinberger B, Laskin JD, Sunil VR, Sinko PJ, Heck DE, Laskin DL (2011). Sulfur mustard-induced pulmonary injury: therapeutic approaches to mitigating toxicity. *Pulm Pharmacol Ther.* 24, 92–99. [PubMed: 20851203]
- Wynn TA (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol.* 214, 199–210. [PubMed: 18161745]
- Wynn TA, Barron L (2010). Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis.* 30, 245–257. [PubMed: 20665377]
- Wynn TA, Vannella KM (2016). Macrophages in tissue repair, regeneration, and fibrosis. *Immunity.* 44, 450–462. [PubMed: 26982353]

Highlights

- NM mustard causes acute lung injury and fibrosis in mice
- Toxicity involves inflammation, oxidative stress and altered lung function
- Proinflammatory and profibrotic macrophages accumulate in the lung after NM
- NM causes activation of macrophage subsets implicated in pulmonary toxicity

Figure 1. Effects of NM on lung histology. Lung sections, prepared 1–28 d after administration of PBS or NM to mice, were stained with H&E. Images were acquired using the VS120 Virtual Microscopy system. One representative section from 3–5 mice/treatment group is shown. Original magnification, 10x; a, alveolar macrophages; b, hyperplasia bronchiolar epithelium; c, hyperplasia alveolar epithelium; d, peri-vascular edema.

Figure 2. Effects of NM on the development of fibrosis. Lung sections, prepared 1–28 d after administration of PBS or NM to mice, were stained with Mason's trichrome. Images were acquired using the VS120 Virtual Microscopy system. One representative section from 3–5 mice/treatment group is shown. Original magnification, 10x; arrows, collagen deposition.

Figure 3.

Effects of NM on BAL protein, cell, and phospholipid content. BAL was collected 1–28 d after administration of PBS or NM to mice. *Upper panel:* Cell-free supernatants were analyzed in triplicate for protein using a BCA protein assay kit. *Middle panel:* Viable cells were enumerated by trypan blue dye exclusion. *Lower panel:* Total phospholipid content was determined as described in Materials and Methods. Bars, mean ± SE (n = 5–14 mice/treatment group). ^aSignificantly different (p<0.05) from PBS treated animals.

Figure 4.

Effects of NM on BAL SP-D levels. BAL was collected 1–28 d after administration of PBS or NM to mice and analyzed by western blotting as described in Materials and Methods. Band density was quantified by ImageJ. *Upper panel:* Each lane represents an individual animal. *Lower panel:* Bars, mean $\pm$ SE (n = 3 mice/treatment group). The experiment was repeated twice using BAL from different set of animals for each treatment group.

^aSignificantly different (p<0.05) from PBS treated animals.

Figure 5.

Effects of NM on BAL microparticles. BAL was collected 1–28 d after administration of PBS or NM to mice. Total microparticles were assessed by flow cytometry as described in Materials and Methods. *Upper panel:* Representative dot plot of BAL from 14 d post NM, showing gating strategy for microparticles. *Lower panel:* Total microparticles. Bars, mean $\pm$ SE (n = 5–14 mice/treatment group). ^aSignificantly different ($p < 0.05$) from PBS treated animals.

Figure 6.

Effects of NM on inflammatory cell accumulation in the lung. Cells, collected by lavage and massage 1–28 d after administration of PBS or NM to mice, were stained with antibodies to CD11b, Ly6G, Ly6C, F4/80 and CD11c or appropriate isotypic controls, and analyzed by flow cytometry. Bars, mean ± SE (n = 4–8 mice/treatment group). ^aSignificantly different (p < 0.05) from PBS treated animals. MP, macrophage; M-MDSC, monocytic-myeloid derived suppressor cell; G-MDSC, granulocytic-myeloid derived suppressor cell; ND, not detected.

Figure 7.

Effects of NM on TNF α expression. Histological sections, prepared 1–28 d after administration of PBS or NM to mice, were stained with antibody to TNF α . Binding was visualized using a DAB peroxidase substrate kit. One representative section from 3–5 mice/treatment group is shown. Original magnification, 60x; arrows indicate macrophage (s) in inset.

Figure 8.

Effects of NM on HMGB1 expression. Histological sections, prepared 1–28 d after administration of PBS or NM to mice, were stained with antibody to HMGB1. Binding was visualized using a DAB peroxidase substrate kit. One representative section from 3–5 mice/treatment group is shown. Original magnification, 60x; arrows indicate macrophage (s) in inset.

Figure 9.

Effects of NM on lung function. Compliance, tissue elastance, static compliance and hysteresis were measured at PEEPs ranging from 0 cm to 9 cm H₂O as described in Materials and Methods. Measurements were made in triplicate, 1–28 d after administration of PBS or NM to mice. Data are the mean ± SE (n = 5–6 mice/treatment group). For each lung parameter, significant differences between the lines were determined by a 3-way

ANOVA comparison of time, treatment and PEEP. ^aOverall line is significantly different ($p < 0.05$) from PBS.

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Table 1.

Semi-quantification of inflammatory marker expression in tissue sections

Macrophages		1 d	3 d	14 d	28 d
TNF α	PBS	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
	NM	20.7 $\pm$ 3.0 ^a	18.5 $\pm$ 2.6 ^a	11.2 $\pm$ 1.8 ^a	20.7 $\pm$ 2.9 ^a
iNOS	PBS	0.4 $\pm$ 0.1	13.8 $\pm$ 1.8	10.0 $\pm$ 1.2	11.9 $\pm$ 1.3
	NM	0.5 $\pm$ 0.1	11.9 $\pm$ 1.8	16.4 $\pm$ 1.7 ^a	13.6 $\pm$ 1.6
Lcn2	PBS	12.5 $\pm$ 1.6	9.2 $\pm$ 1.3	8.2 $\pm$ 1.1	11.3 $\pm$ 1.4
	NM	13.8 $\pm$ 1.6	20.5 $\pm$ 2.6 ^a	21.1 $\pm$ 2.8 ^a	17.4 $\pm$ 2.8 ^a
HO-1	PBS	7.4 $\pm$ 1.0	16.9 $\pm$ 2.8	2.7 $\pm$ 0.5	14.9 $\pm$ n/a
	NM	11.7 $\pm$ 1.8 ^a	17.5 $\pm$ 2.8	16.7 $\pm$ 2.1 ^a	16.1 $\pm$ 1.9
HMGB1	PBS	0.3 $\pm$ 0.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.0
	NM	6.4 $\pm$ 0.8 ^a	9.3 $\pm$ 1.4 ^a	13.1 $\pm$ 1.8 ^a	16.6 $\pm$ 1.6 ^a
MR-1	PBS	9.3 $\pm$ 1.1	13.5 $\pm$ 2.2	6.1 $\pm$ 0.9	8.8 $\pm$ 1.2
	NM	7.2 $\pm$ 0.9	9.7 $\pm$ 1.2	9.1 $\pm$ 1.3	11.8 $\pm$ 1.7
TGF β	PBS	7.3 $\pm$ 1.1	12.3 $\pm$ 2.4	10.7 $\pm$ 1.3	13.4 $\pm$ 2.4
	NM	13.3 $\pm$ 1.7 ^a	18.0 $\pm$ 3.4	22.8 $\pm$ 3.0 ^a	16.1 $\pm$ 3.1
TGF β (TII)	PBS	22.2 $\pm$ 3.4	34.1 $\pm$ 6.0	17.2 $\pm$ 2.4	43.3 $\pm$ 7.6
	NM	43.0 $\pm$ 5.8 ^a	47.2 $\pm$ 8.5	47.9 $\pm$ 7.3 ^a	54.1 $\pm$ 10.1

Lung tissue sections were prepared 1– 28 days after administration of PBS or NM to mice. Numbers of lung macrophages and/or Type II cells staining positively for tumor necrosis factor alpha (TNF α), inducible nitric oxide synthase (iNOS), Lipocalin 2 (Lcn-2), hemeoxygenase (HO)-1, HMGB1, mannose receptor (MR)-1 and transforming growth factor (TGF) β were enumerated in all 5 lung lobes (15 fields/lung lobe). Values are the mean $\pm$ SE of positively stained cells from 3–5 mice/treatment group. Data were analyzed by ANOVA.

^aSignificantly different (p<0.05) from PBS.

n/a, not available

Table 2.

Effects of NM on resident alveolar macrophages (CD11b⁻CD11c⁺F4/80⁺Ly6G⁻Ly6C^{lo}).

Time after exposure (d)	PBS	NM
1	54.0 ± 1.5	6.5 ± 1.2 ^a
3	41.9 ± 0.9	3.1 ± 1.0 ^a
14	43.0 ± 4.5	32.5 ± 2.9 ^a
28	30.0 ± 16.8	36.3 ± 0.9

Cells (10³), collected by BAL and lung massage, 1–28 d after administration of PBS or NM to mice, staining negatively for CD11b (CD11b⁻), were analyzed for expression of CD11c and F4/80, followed by Ly6G and Ly6C. Data are mean ± SE (n = 4–8 mice/treatment group).

^aSignificantly different (p<0.05) from PBS treated animals.