

The airway epithelium nucleotide-binding domain and leucine-rich repeat protein 3 inflammasome is activated by urban particulate matter

Jeremy A. Hirota, PhD,^{a,b} Simon A. Hirota, PhD,^c Stephanie M. Warner, BSc,^a Dorota Stefanowicz, BSc,^a Furquan Shaheen, BSc,^a Paul L. Beck, MD, PhD,^c Justin A. MacDonald, PhD,^d Tillie-Louise Hackett, PhD,^{a,b} Don D. Sin, MD,^{a,e} Stephan Van Eeden, MD,^{a,e} and Darryl A. Knight, PhD^{a,b} Vancouver, British Columbia, and Calgary, Alberta, Canada

Background: The airway epithelium is the first line of defense against inhaled insults and therefore must be capable of coordinating appropriate inflammatory and immune responses.

Objective: We sought to test the hypothesis that the nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) inflammasome, an intracellular danger-sensing complex, plays a critical role in airway epithelium-mediated immune responses to urban particulate matter (PM) exposure.

Methods: In this study we (1) identified NLRP3 and caspase-1 expression in human airway epithelium bronchus and primary cells, (2) characterized NLRP3 inflammasome-mediated IL-1 β production from human airway epithelium in response to PM, and (3) performed *in vivo* PM exposure experiments with wild-type and Nlrp3^{-/-} mice.

Results: Our results demonstrate that human airway epithelium contains a functional NLRP3 inflammasome that responds to PM exposure with caspase-1 cleavage and production of IL-1 β . Exposure of Nlrp3^{-/-} and wild-type mice to PM *in vivo* demonstrates NLRP3-dependent production of IL-1 β in the lung, airway neutrophilia, and increases in CD11c⁺^{hi}/MHC class II⁺^{hi} cell numbers in intrathoracic lymph nodes.

Conclusion: Our study is the first to characterize airway epithelial NLRP3 inflammasome-mediated immune responses

to PM exposure, which might have implications in patients with asthma and other lung diseases. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Nucleotide-binding domain and leucine-rich repeat protein 3 inflammasome, particulate matter with mean diameter less than 10 μ m, IL-1 β , mouse, human, oxidative stress, dendritic cell

The nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3) inflammasome is an intracellular danger-sensing protein complex that includes the NLRP3 protein and inactive caspase-1.^{1,2} The NLRP3 inflammasome responds to intracellular danger signals, including reactive oxygen species,³ ATP,⁴ and uric acid crystals,⁵ by inducing caspase-1 autocleavage.² Activation of caspase-1, an inflammatory caspase, leads to cleavage of immature pro-IL-1 β to mature active IL-1 β , which is then secreted.^{2,6} Secreted IL-1 β has been used as a biochemical marker of NLRP3 inflammasome activation in macrophages and could be used as a surrogate in other cell types.⁷⁻¹⁰ Indeed, most reports on NLRP3 biology have focused on inflammatory cells, with few studies characterizing it in structural cells.¹¹⁻¹³ To our knowledge, no reports on direct NLRP3 inflammasome activity in human airway epithelial cells exist.

Urban particulate matter (PM) contributes to cardiorespiratory morbidity, including exacerbations of asthma and chronic obstructive pulmonary disease.¹⁴⁻¹⁷ To date, it is not known whether PM activates the NLRP3 inflammasome.¹⁸⁻²¹ PM is a likely candidate activator of the NLRP3 inflammasome for 3 reasons. First, PM contains a variety of polyaromatic hydrocarbons and transition metals that induce oxidative stress.²²⁻²⁵ Second, PM might contain coal fly ash, diesel emission particles, and road dust, which all share similar physical properties to known activators of the NLRP3 inflammasome.^{26,27} Third, PM has adjuvant properties that facilitate allergic sensitization and induction of adaptive immune responses.^{23,28} The NLRP3 inflammasome is activated by the adjuvant alum and contributes to adaptive immune responses.^{7,9,29} Taken together, PM appears to possess physical and biochemical characteristics that can contribute to NLRP3 inflammasome activation.

The airway epithelium is the first line of defense against PM and other inhaled stimuli and responds by producing innate immune mediators.³⁰ In addition, interspersed within the epithelium reside dendritic cells that are able to sample the external environment and facilitate adaptive immune responses.³¹ We have shown that primary cultures of human airway epithelial cells

From ^athe UBC James Hogg Research Centre and Institute for Heart + Lung Health, St Paul's Hospital, University of British Columbia, Vancouver; ^bthe Department of Anesthesiology, Pharmacology and Therapeutics and ^cthe Department of Medicine, Division of Respiratory Medicine, University of British Columbia, Vancouver; and ^dthe Gastrointestinal Research Group and ^ethe Department of Biochemistry and Molecular Biology, University of Calgary.

J.A.H. is a recipient of CIHR, IMPACT strategic training, and AllerGen NCE postdoctoral training fellowships. D.S. is the Canada Research Chair in Chronic Obstructive Pulmonary Disease and a Michael Smith Foundation for Health Research (MSFHR) Senior Scholar. T.-L.H. is a recipient of a CIHR/Canadian Lung Association/GSK, IMPACT strategic training, and MSFHR postdoctoral fellowship. S.V. is a senior scholar with the MSFHR and CIHR/GSK professor in Chronic Obstructive Pulmonary Disease. D.A.K. is the Canada Research Chair in Airway Disease and an MSFHR career investigator.

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Corresponding author: Jeremy A. Hirota, PhD, Rm 166 UBC James Hogg Research Centre, St Paul's Hospital, Institute of Heart and Lung Health, 1081 Burrard St, Vancouver, BC, Canada, V6Z 1Y6. E-mail: Jeremy.Hirota@hli.ubc.ca.

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Abbreviations used

BAL:	Bronchoalveolar lavage
BEGM:	Bronchial epithelial growth medium
EHC:	Environmental Health Canada
NLRP3:	Nucleotide-binding domain and leucine-rich repeat protein 3
PM:	Particulate matter
PM ₁₀ :	Particulate matter with mean diameter less than 10 μ m
siRNA:	Small interfering RNA
WT:	Wild-type

are capable of PM endocytosis, which is associated with increased IL-1 β production.^{32,33} Consistent with our *in vitro* data, Sousa et al³⁴ have described expression of IL-1 β in airway epithelium from human lung biopsy specimens. Whether the observed airway epithelial production and expression of IL-1 β was mediated by the NLRP3 inflammasome and whether this leads to activation of any resident dendritic cell-mediated adaptive immune responses remain unknown.

In the present study we hypothesized that the NLRP3 inflammasome plays a critical role in airway epithelium-mediated innate immune responses to PM exposure. To address our hypothesis, we first sought to confirm the presence of the NLRP3 inflammasome in human airway epithelium by using whole human bronchus tissue and primary human airway epithelial cells. Next, we tested the hypothesis that PM is an activator of the NLRP3 inflammasome in human airway epithelium. We extended this hypothesis with PM exposure experiments in Nlrp3^{-/-} animals and pharmacologic strategies in wild-type (WT) mice. Lastly, we investigated the possibility that PM exposure could induce increases in activated intrathoracic lymph node dendritic cell numbers in an NLRP3-dependent mechanism. Our results suggest that the NLRP3 inflammasome in airway epithelium is a molecular complex directly involved in the innate immune responses to PM exposure that are associated with changes in dendritic cell phenotype.

METHODS**Human tissue and cell culture**

Human bronchi and primary human airway epithelial cells were collected as previously described^{35,36} and approved by the University of British Columbia's Office of Research Service ethics committee. Characteristics of the patient sample are shown in Table E1 in this article's Online Repository at www.jacionline.org. Each patient was considered an independent n value.

Mice

WT female C57Bl/6 mice 6 to 8 weeks of age were purchased from Harlan (Mississauga, Ontario, Canada) for glyburide intervention studies. Nlrp3^{-/-} mice on C57Bl/6 background were generously provided by Dr Vishva Dixit from Genentech (San Francisco, Calif), with WT littermates used as control animals for knockout studies. All animal experiments were approved by the University of British Columbia's Office of Research Service animal use committee.

Reagents

Bronchial epithelial growth medium (BEGM) was prepared as per the manufacturer's directions (Lonza, Allendale, NJ) and supplemented with penicillin, streptomycin, and Fungizone (Invitrogen, Burlington, Ontario, Canada). Environmental Health Canada (EHC-93) particulate matter with a mean diameter of less than 10 μ m (PM₁₀) was collected as previously described,^{26,32,33,37} with composition details shown in the Methods section in

this article's Online Repository at www.jacionline.org. Glyburide (InvivoGen, San Diego, Calif) was prepared as an inhibitor of the NLRP3 inflammasome.³⁸ The caspase-1 inhibitor Z-YVAD-FMK was purchased from BioVision (Mountain View, Calif). The following antibodies were used: rabbit anti-human NLRP3 (Sigma, Oakville, Calif), rabbit anti-human caspase-1 (Cell Signaling, Danvers, Mass), Alexa Fluor 594-labeled goat anti-rabbit, Alexa Fluor 680-labeled goat anti-rabbit (Invitrogen, Burlington, Calif), IR800-labeled goat anti-mouse (Rockland, Gilbertsville, Pa), mouse anti-human β -tubulin (Millipore, Billerica, Mass), and biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, Calif) antibodies. Alexa Fluor 488-labeled anti-mouse CD11c (clone N418), phycoerythrin-labeled anti-mouse I-Ab (clone AF6-120.1), and Alexa Fluor 488-labeled IgG isotype control (clone HTK888) antibodies were purchased from BioLegend (San Diego, Calif). Phycoerythrin-labeled IgG isotype control (clone X5563) was purchased from Abcam (Cambridge, Mass). Immunoblots were visualized with a Licor imaging system at 700- and 800-nm wavelengths.

***In vitro* validation of oxidative capacity of PM₁₀**

For more information, see the Methods section in this article's Online Repository.

Pharmacologic inhibition studies

Primary airway epithelial cells were seeded and grown to 90% confluency. For inhibitor experiments, glyburide (100 μ mol/L) and Z-YVAD-FMK (2 μ mol/L) were added 30 minutes before PM₁₀ exposure to inhibit the NLRP3 inflammasome³⁸ or caspase-1, respectively. PM₁₀ was suspended and vortexed in BEGM at a concentration of 500 μ g/mL, which did not induce significant changes in cell viability (see Fig E1 in this article's Online Repository at www.jacionline.org). For small interfering RNA (siRNA) experiment methods, see the Methods section in this article's Online Repository. Cell culture supernatants and protein lysates were collected for soluble mediator analysis and protein expression. To control for the consequences of cell growth on soluble mediator analysis, we normalized cytokine levels to protein lysate content.

***In vivo* experiments**

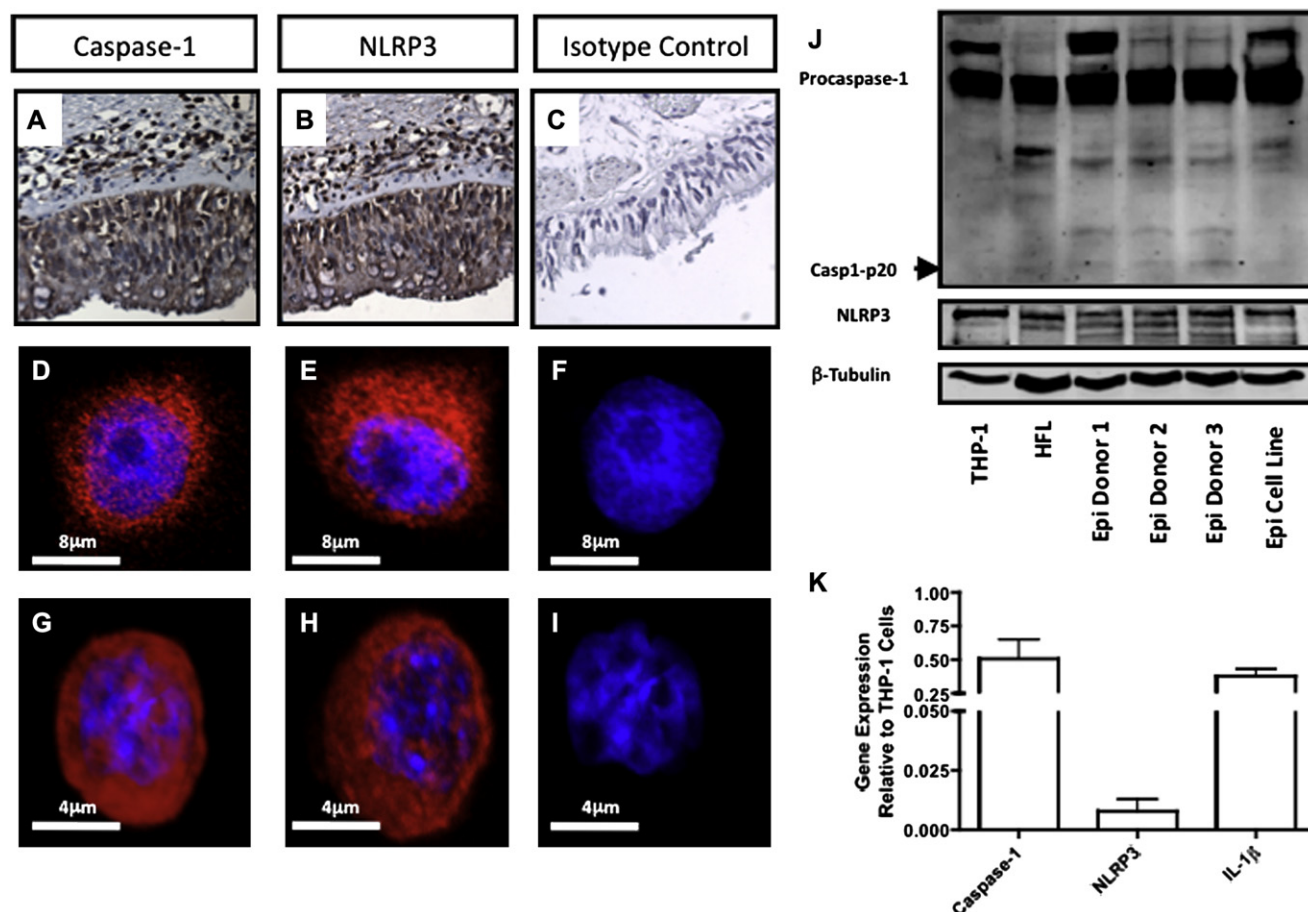
For pharmacologic intervention studies, glyburide at a final concentration of 33.3 mg/mL was dissolved in 2.5% dimethyl sulfoxide/97.5% 2-hydroxypropyl- β -cyclodextrin (10%). Each animal received either vehicle or glyburide intraperitoneal treatment (500 mg/kg) in the morning and evening on the day before PM₁₀ exposure. A third dose of vehicle or glyburide was administered in the morning before PM₁₀ exposure. A single 200 μ g/50 μ L dose of PM₁₀ delivered by means of intratracheal instillation in ketamine/xylazine-anesthetized mice was performed as previously described.²¹ Vehicle (PBS) was administered to control animals. Outcome measurements were performed 4 or 24 hours after exposure. Bronchoalveolar lavage (BAL) and lung specimens were collected as previously described.³⁹

Inflammatory cell and mediator measurements and tissue collection

Levels of IL-1 β in human airway epithelial culture supernatant and murine BAL fluid were measured by using commercially available ELISA kits from R&D Systems (Burlington, Ontario, Canada) designed to detect cleaved mature IL-1 β . Confirmation of ELISA kits was confirmed by means of immunoblotting (see Fig E2 in this article's Online Repository at www.jacionline.org). Total and differential cell counts and tissue collection were performed, as previously described.³⁹

Dendritic cell isolation and flow cytometric methods

Intrathoracic lymph nodes were isolated from mice and prepared for isolation of dendritic cells, as previously described.⁴⁰



Statistical analysis

For all experiments, n = 5 unless otherwise noted in the figure legends. One-way ANOVAs were performed with a *post hoc* Bonferroni correction to correct for multiple comparisons. A corrected *P* value of less than .05 was determined to be a statistically significant difference between compared groups. Data were analyzed with GraphPad Software Version 4.0 (GraphPad Software, Inc, La Jolla, Calif).

RESULTS

The NLRP3 inflammasome is present in human airway epithelium

In situ staining of serial sections of human bronchus demonstrated the expression of caspase-1 and NLRP3 in airway epithelium, with the isotype control showing no positive staining (Fig 1, A-C). Using confocal microscopy, we assessed caspase-1 and NLRP3 expression and localization in primary human airway epithelial cells (Fig 1, D-F). Staining for both caspase-1 and NLRP3 was punctate throughout the cytoplasm. The staining pattern was similar to that observed in THP-1 macrophages, which we used as a positive control (Fig 1, G-I). Expression of caspase-1

and NLRP3 in epithelial cells was confirmed by means of immunoblotting (Fig 1, J). Airway epithelial cells also demonstrate baseline gene expression of caspase-1, NLRP3, and IL-1 β (Fig 1, K).

Functional characterization of the NLRP3 inflammasome in response to PM in airway epithelial cells

We next examined whether the airway epithelial NLRP3 inflammasome was functional. To investigate this process, we used glyburide, a nonselective inhibitor of the NLRP3 inflammasome,³⁸ and Z-YVAD-FMK, a selective caspase-1 inhibitor.⁴¹

Primary human airway epithelial cells exposed to PM₁₀ produced significant IL-1 β (Fig 2, A). Pharmacologic intervention with either glyburide (100 μ mol/L) or Z-YVAD-FMK (2 μ mol/L) significantly attenuated PM₁₀-induced release of IL-1 β . These results were confirmed in THP-1 macrophage cells (see Fig E3 in this article's Online Repository at www.jacionline.org). The endotoxin content in our PM₁₀ mixture is 6.4 EU/mL and does not induce IL-1 β production in airway epithelial cells.³³

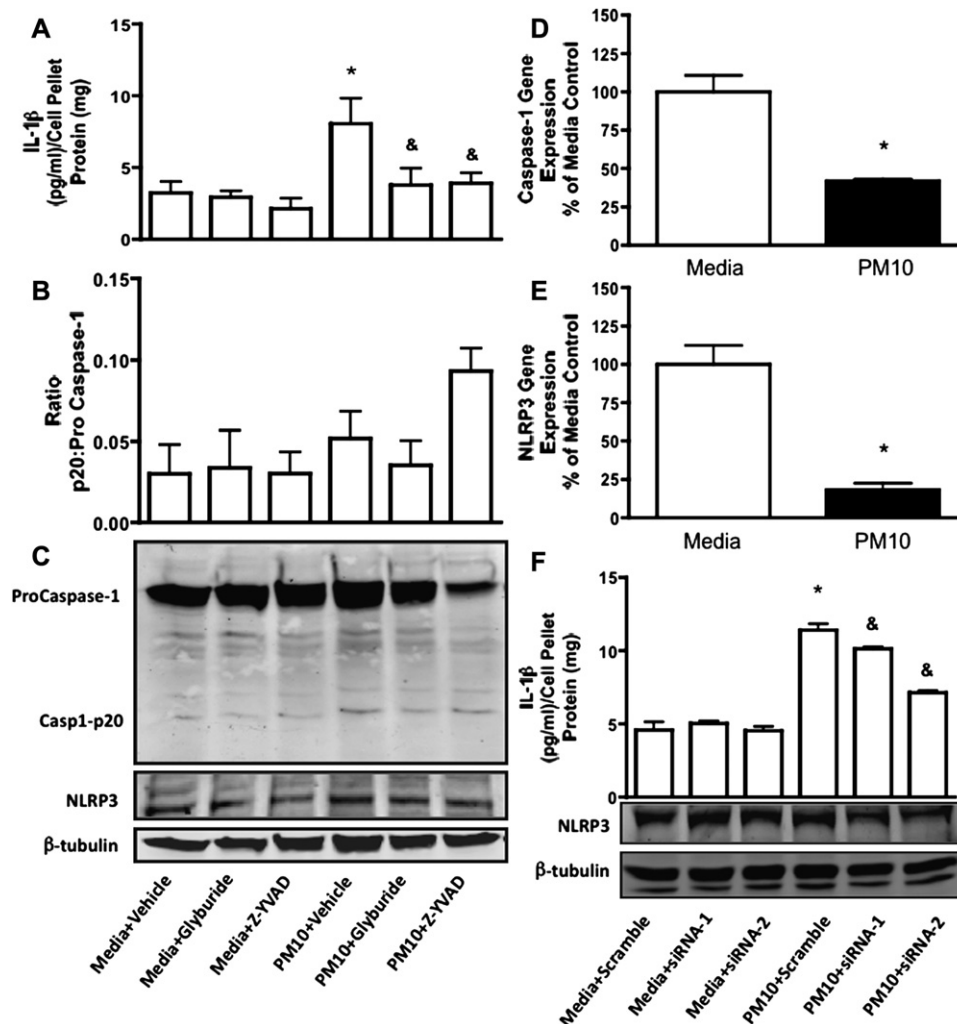


FIG 2. IL-1 β production, caspase-1 activation, and NLRP3 expression in primary airway epithelial cells after incubation with 500 μ g/mL PM₁₀ for 24 hours. **A**, IL-1 β production in epithelial cell culture supernatant normalized to cell pellet protein content (in picograms per milliliter per milligram; glyburide, 100 μ mol/L; Z-YVAD-FMK, 2 μ mol/L). **B**, Ratio of cleaved casp1-p20 subunit to procaspase-1. **C**, Immunoblot of caspase-1 and NLRP3 expression after PM₁₀ exposure. **D** and **E**, Caspase-1 and NLRP3 gene expression after PM₁₀ exposure in epithelial cells. **F**, PM₁₀-induced IL-1 β production after siRNA knockdown of NLRP3 (see the [Methods](#) section in this article's Online Repository). * P < .05 compared with medium plus vehicle. & P < .05 compared with PM₁₀ plus vehicle. All data are expressed as means $\pm$ SEMs (n = 5).

PM₁₀ exposure induced the cleavage of caspase-1 and generated a casp1-p20 subunit (Fig 2, B-C). In contrast, control-treated epithelial cells demonstrated no caspase-1 cleavage. Glyburide (100 μ mol/L) and Z-YVAD-FMK (2 μ mol/L) did not completely inhibit caspase-1 cleavage, which is consistent with the literature.^{38,41} We did not observe significant changes in caspase-1 or NLRP3 protein expression after PM₁₀ exposure at this time point (Fig 2, C), although gene expression was significantly attenuated (Fig 2, D and E). Pharmacologic intervention experiments were confirmed with siRNA NLRP3 gene knockdown experiments (Fig 2, F).

We finally sought to determine whether the EHC-93 PM₁₀ used in our studies could induce changes in redox state because PM₁₀ exposure is associated with oxidative stress.^{19,22,24} By using a cell-permeable redox-sensitive dye that fluoresces on oxidation, we demonstrated a significant increase in staining intensity/unit

area in PM₁₀-treated airway epithelial cells compared with that seen in saline-treated control cells (P < .05, Fig 3).

The NLRP3 inflammasome is required for PM₁₀-induced IL-1 β production *in vivo*

We performed PM₁₀ exposures in 2 independent murine models: (1) Nlrp3^{-/-} and littermate control animals and (2) a glyburide intervention strategy in C57Bl/6 WT mice.

In our Nlrp3^{-/-} study PM₁₀ exposure resulted in significant increases in IL-1 β levels (Fig 4, A) in the BAL fluid of littermate control WT mice. The increases in IL-1 β levels were accompanied by a significant increase in the total number of inflammatory cells, which was driven largely by neutrophils (Fig 4, B and C). We provide confirmatory data that these results are conserved at lower PM₁₀ doses (see Fig E4 in this article's Online Repository

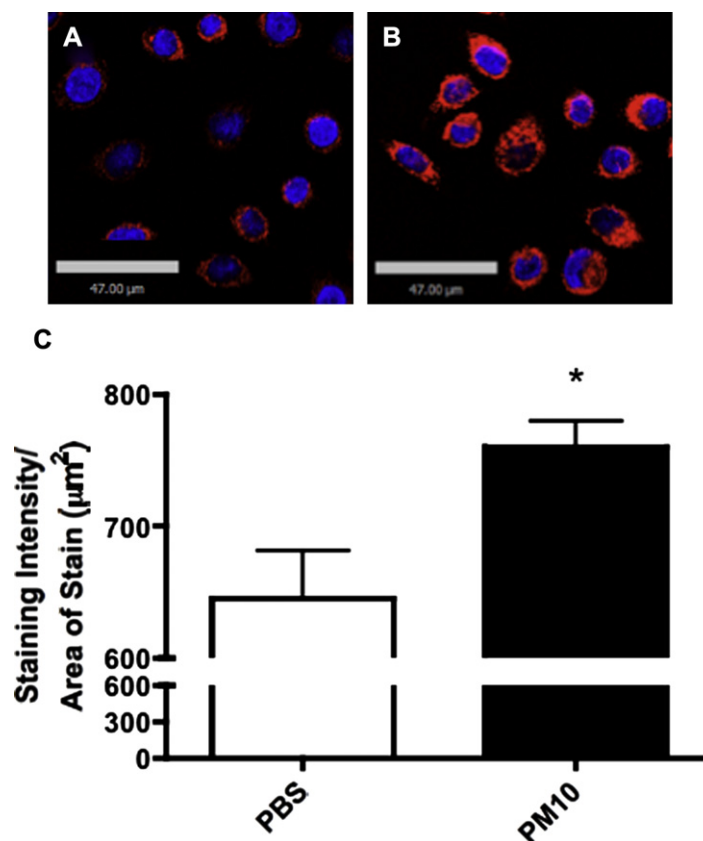


FIG 3. *In vitro* PM₁₀-induced changes in oxidative status of airway epithelial cells. **A** and **B**, Live cell imaging of airway epithelial cells treated with control PBS (Fig 3, **A**) or PM₁₀ (Fig 3, **B**). **C**, Average staining intensity per unit area of stain quantified for 3 random fields of view from 3 independent experiments. **P* < .05 compared with PBS control.

at www.jacionline.org). In stark contrast, Nlrp3^{-/-} mice were protected from PM₁₀-induced increases in IL-1β levels, total inflammatory cell numbers, and neutrophil numbers in BAL fluid (Fig 4). In Nlrp3^{-/-} mice PM₁₀ induced a significant increase in BAL fluid macrophage numbers that was not associated with increased IL-1β levels.

In our glyburide intervention study we pretreated WT C57Bl/6 mice with glyburide or vehicle before PM₁₀ exposure. PM₁₀ exposure to vehicle-treated mice resulted in a trend toward higher IL-1β levels (*n* = 5, *P* = .1; Fig 5, **A**) and a significant increase in inflammatory cell and neutrophil numbers in BAL fluid (Fig 5, **B** and **C**). Glyburide treatment completely inhibited the PM₁₀-induced responses in IL-1β levels, cellular inflammation, and neutrophilia in BAL fluid (Fig 5, **A-C**).

PM₁₀ and IL-1β protein expression are localized to airway epithelium *in vivo*

Despite significant increases in total inflammatory cell numbers detected in BAL fluid from both *in vivo* models, minimal pathologic signs of airway inflammation are observed within lung tissue after PM₁₀ exposure (Fig 6, **A-C**). In the absence of overt cellular infiltrate present in the lung tissue, intracellular PM₁₀ was observed in airway epithelial cells in both WT and Nlrp3^{-/-} PM₁₀-exposed animals (Fig 6, **E-F**). *In vivo* production of IL-1β could be observed in the airway epithelium of WT PM₁₀-exposed mice but not Nlrp3^{-/-} animals (Fig 6, **G-I**).

The NLRP3 inflammasome is required for PM₁₀-induced changes in dendritic cell phenotype

PM₁₀ is known to have adjuvant properties, but it is unknown whether this is mediated through the NLRP3 inflammasome.^{23,28} The NLRP3 inflammasome has been linked to the development of adaptive immune responses by responding to adjuvants.^{7,9,29} To determine whether PM₁₀ exposure resulted in activation of the adaptive immune system in an NLRP3-inflammasome dependent process, we assessed changes in intrathoracic draining lymph node dendritic cell phenotype 4 and 24 hours after exposure to PM₁₀ by means of flow cytometry.⁴⁰

PM₁₀ exposure to WT mice did not result in any significant changes in CD11c⁺^{hi}/MHC class II⁺^{hi} cell numbers 4 hours after exposure (Fig 7, **A**). In contrast, a significant induction of CD11c⁺^{hi}/MHC class II⁺^{hi} cells was observed 24 hours after PM₁₀ exposure (Fig 7, **B-D**) and was NLRP3 inflammasome sensitive. Collectively, these results demonstrate that PM₁₀ can induce changes in a population of CD11c⁺^{hi}/MHC class II⁺^{hi} cells in regional lymph nodes in a process dependent on NLRP3 inflammasome activity.

DISCUSSION

In these experiments we have shown that an NLRP3 inflammasome is present and functional in human airway epithelium. Using primary cultures of human airway epithelial cells, we demonstrated that the NLRP3 inflammasome is activated by PM₁₀.

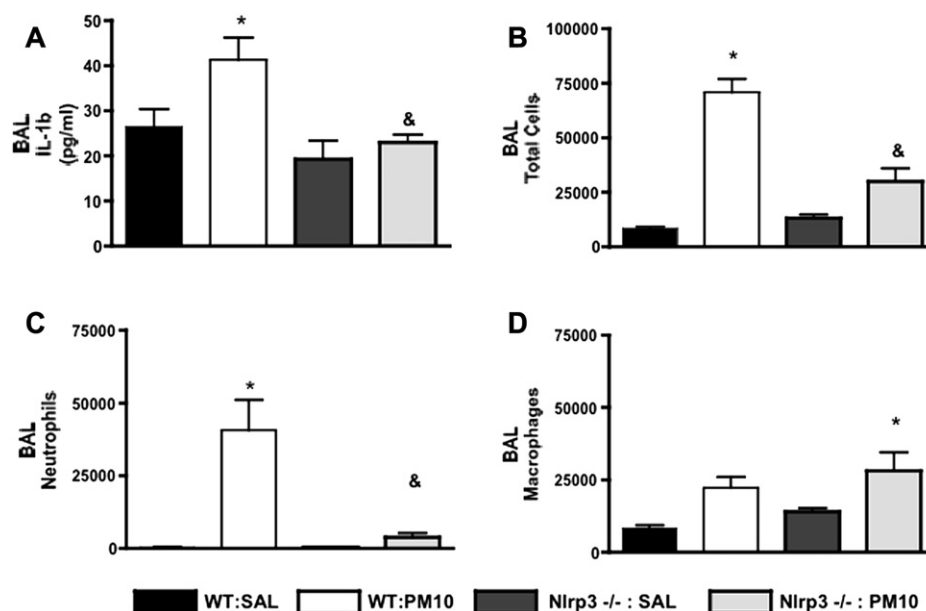


FIG 4. PM₁₀-induced inflammatory responses in lungs of Nlrp3^{-/-} and littermate WT control mice. **A**, BAL fluid IL-1β levels (in picograms per milliliter). **B**, BAL fluid total inflammatory cell numbers. **C**, BAL fluid neutrophil numbers. **D**, BAL macrophage numbers. Data are expressed as means ± SEMs (n = 5). *P < .05 compared with WT saline. &P < .05 compared with WT PM₁₀.

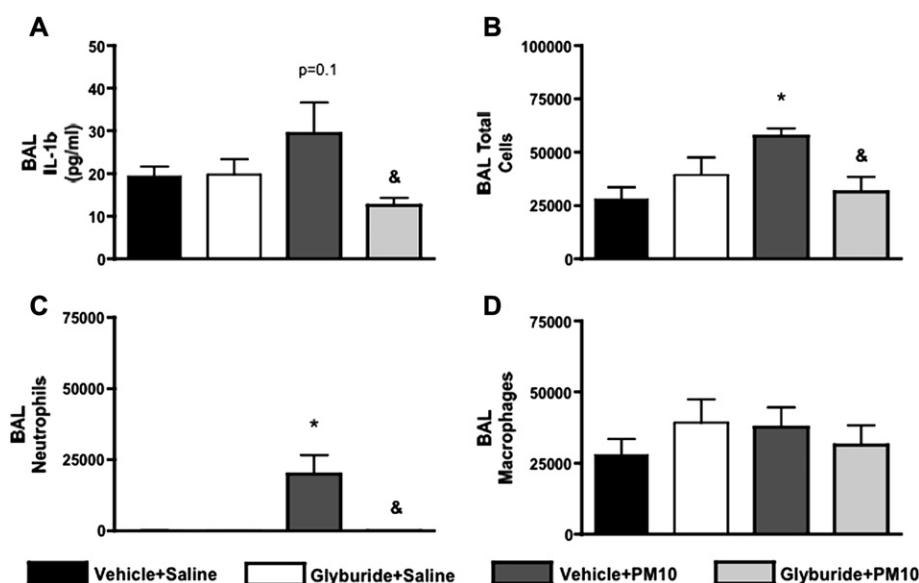


FIG 5. PM₁₀-induced inflammatory responses and effect of glyburide treatment in WT C57Bl/6 mice. **A**, BAL fluid IL-1β (in picograms per milliliter). **B**, BAL fluid total inflammatory cell numbers. **C**, BAL fluid neutrophil numbers. **D**, BAL fluid macrophage numbers. Data are expressed as means ± SEMs (n = 5). *P < .05 compared with vehicle plus saline. &P < .05 compared with vehicle plus PM₁₀.

to produce IL-1β. Using 2 different murine models, we showed that the NLRP3 inflammasome mediates PM₁₀-induced neutrophilic airway inflammation. Lastly, we demonstrate that PM₁₀ induces NLRP3-sensitive downstream changes in dendritic cell phenotype. These data provide *in vitro* and *in vivo* evidence that the airway epithelium contributes to innate immune responses to PM₁₀. Importantly, we showed that inhibition of the NLRP3 inflammasome prevented PM₁₀-induced airway inflammation, suggesting that it might be a useful therapeutic target in mitigating the adverse health consequences of PM exposure.

We initially set out to characterize the expression pattern of the NLRP3 inflammasome in human airway tissue to confirm that this complex existed in airway epithelium. Previous reports by our group have demonstrated IL-1β production from primary human airway epithelial cells, which is consistent with *in situ* positive IL-1β staining in human airway epithelium from bronchial biopsy specimens.³²⁻³⁴ Gene expression profiling of murine whole-lung homogenates has demonstrated the presence of transcripts for NLRP3, although the precise origins of these transcripts were not identified.^{42,43} With respect to the epithelium, caspase-1 expression

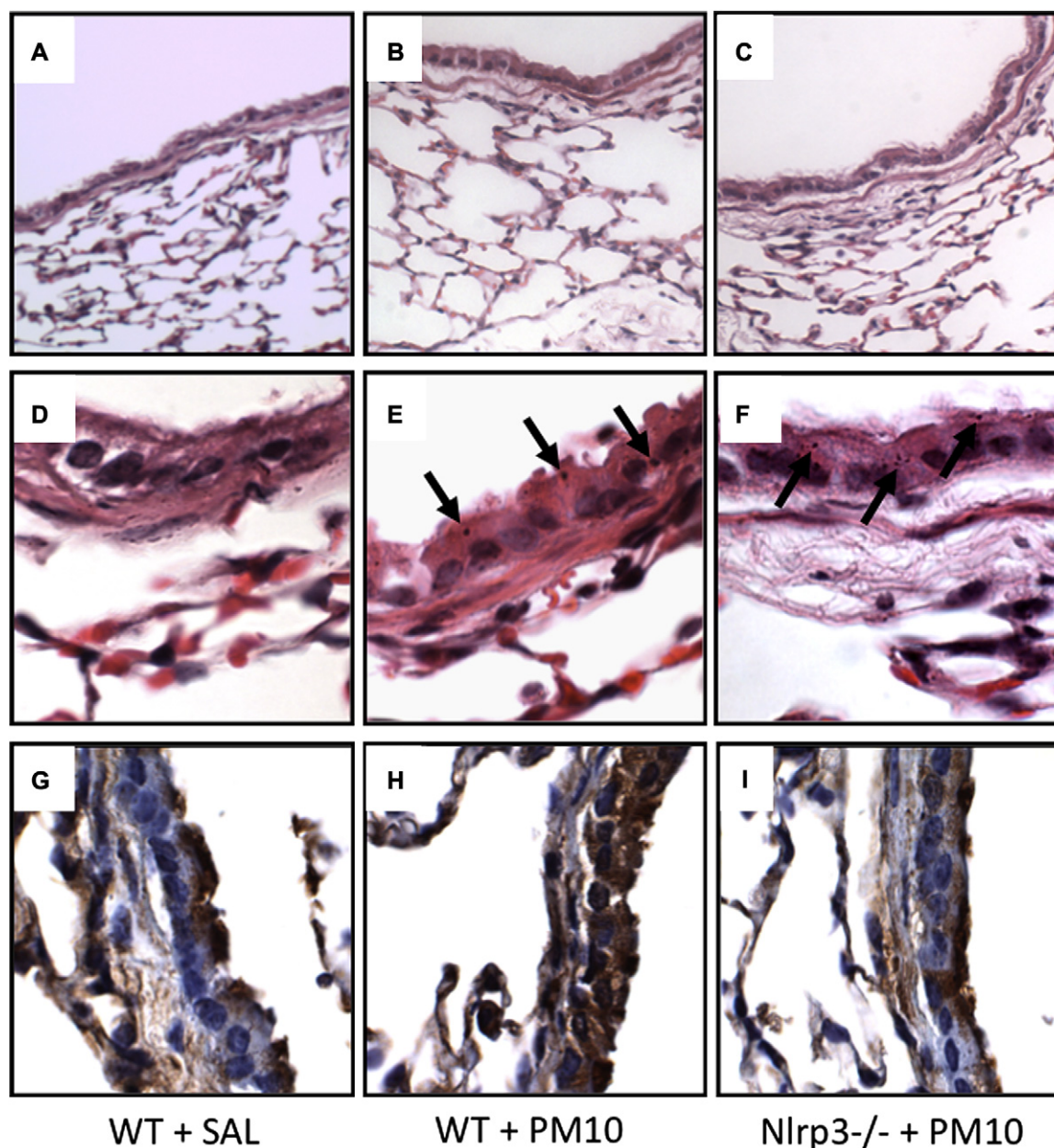


FIG 6. Airway inflammation and PM₁₀ deposition in airway epithelium after exposure. **A-C**, Hematoxylin and eosin staining at $\times 20$ magnification. **D-F**, Hematoxylin and eosin staining at $\times 100$ magnification. *Black arrows point to PM₁₀ in airway epithelial cells.* **G-I**, IL-1 β immunohistochemistry (brown) with hematoxylin counterstaining (blue; $\times 100$ magnification). Representative images from 5 experiments. SAL, Saline.

in airway epithelial cells has been demonstrated in an immortalized type II epithelial cell line (A549).⁴⁴ Because of the absence of direct evidence for NLRP3 inflammasome expression in human airway epithelial cells, we determined gene and protein expression on human bronchial sections and primary human airway epithelial cells. Using these techniques, we provide the first direct evidence of NLRP3 and caspase-1 protein expression in human airway epithelial cells.

The presence of the NLRP3 inflammasome in the human airway epithelium is consistent with the role these cells play in mediating innate and adaptive immune responses to airborne insults, including PM₁₀, allergens, viruses, and bacteria.^{30,45,46}

For the airway epithelial NLRP3 inflammasome to be active in a classical manner, the presence of cleaved caspase-1 and production of mature IL-1 β should be observed.² Indeed, in response to PM₁₀, we observed both total and cleaved caspase-1 and mature IL-1 β levels, confirming that the NLRP3 inflammasome was functional. Our results are consistent with those of Lamkanfi et al,³⁸ who demonstrated significant inhibition of IL-1 β secretion by glyburide at 100 $\mu\text{mol/L}$, which was not accompanied by complete inhibition of caspase-1 cleavage. In contrast to the mechanism of action for glyburide, Z-YVAD-FMK binds irreversibly to active cleaved caspase-1, and therefore it is not surprising that the casp1-p20 subunit is present.⁴¹ The observed

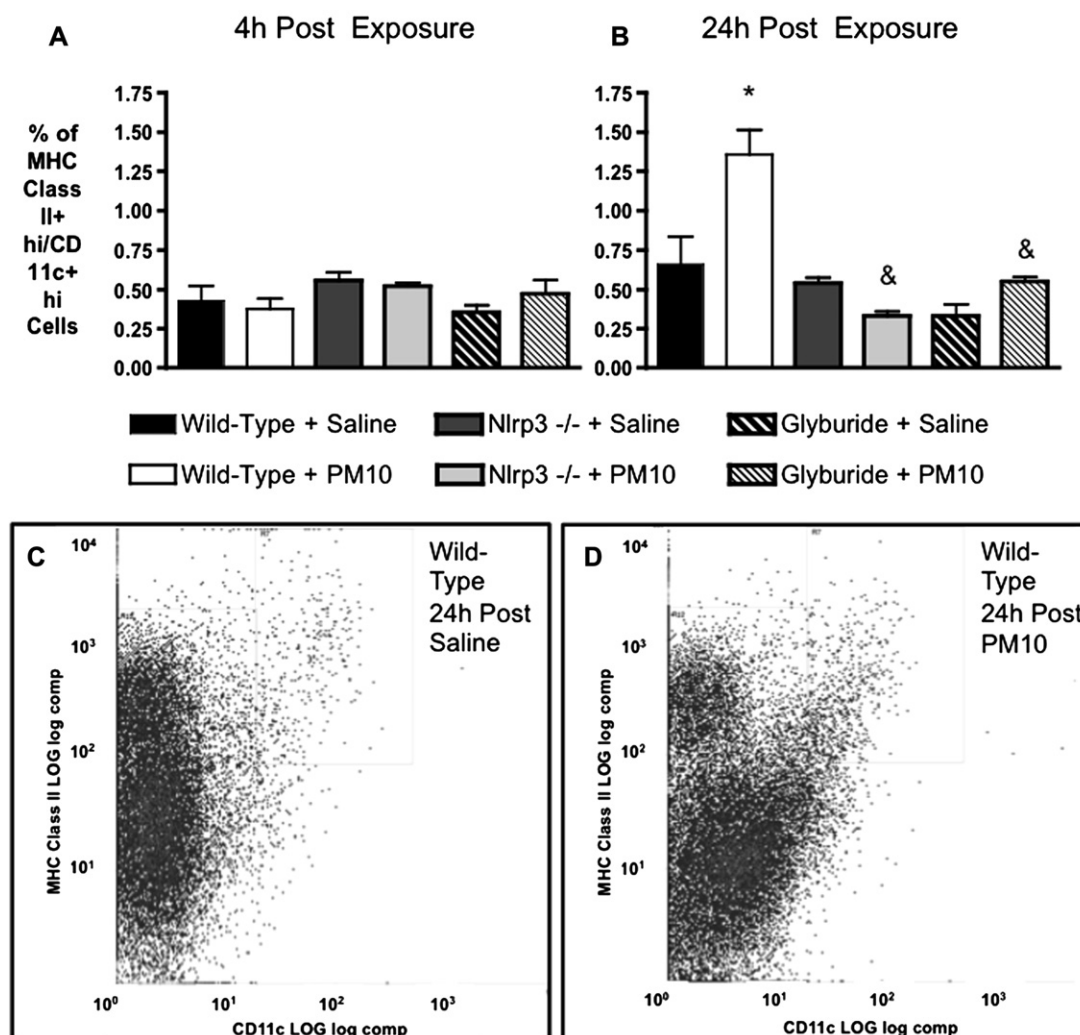


FIG 7. Quantification of PM₁₀-induced changes in dendritic cell surface marker expression in intrathoracic lymph nodes. **A**, Four hours after exposure. **B**, Twenty-four hours after exposure. Data are expressed as means $\pm$ SEMs ($n = 3-5$). * $P < .05$ compared with WT plus saline. & $P < .05$ compared with WT plus PM₁₀. **C** and **D**, Representative 2-parameter histograms demonstrating gating strategies used to identify CD11c^{hi}/MHC class II^{hi} cells 24 hours after exposure: Fig 7, C, WT plus saline; Fig 7, D, WT plus PM₁₀.

PM₁₀-induced downregulation of both caspase-1 and NLRP3 gene transcripts in epithelial cells contrasts with data for hematopoietic cells stimulated with NLRP3 inflammasome activators,⁴² suggesting that the airway epithelium and inflammatory cells might regulate this gene differently. Our *in vitro* functional data with pharmacologic inhibition and silencing RNA techniques support the presence of a functional NLRP3 inflammasome in airway epithelium and warrants future studies to determine whether additional insults and known NLRP3 activators lead to cleavage of caspase-1, production of IL-1 β , and downstream airway inflammation.

To address the issue of nonselective actions of the pharmacologic inhibitors used in our study, we performed siRNA experiments for NLRP3 mRNA. In these experiments we observed only a mild siRNA-mediated reduction in NLRP3 protein expression, which was consistent with a mild but significant inhibition of PM₁₀-induced downstream IL-1 β production. Incubating airway epithelial cells with greater siRNA concentrations for longer durations might provide more significant NLRP3 inhibition and

conclusive evidence linking the NLRP3 inflammasome with PM₁₀-induced responses, but caution must be taken because off-target siRNA effects have been reported to be vehicle⁴⁷ and dose⁴⁸ dependent. Importantly, the reduction in PM₁₀-induced IL-1 β production after exposure to NLRP3 siRNA was supported by the use of NLRP3 and caspase-1 pharmacologic inhibitors.

Acute immune responses to inhaled PM are associated with exacerbations of existing respiratory and cardiovascular conditions,^{14,16} and our data support that the epithelial NLRP3 inflammasome contributes to these processes. Depending on PM composition, particles have specific inflammatory responses relating to surface area and unique distribution profiles within the airway.^{20,26,49} Our EHC-93 PM₁₀ has been compared with the National Institute for Standards and Technology standard reference material-1649, demonstrating similar transition metal and polycyclic aromatic hydrocarbon content,²⁶ 2 components that induce inflammatory events.^{20,26,49} Independent rodent PM exposure studies using EHC-93 PM₁₀, standard reference material-1649, and other source materials have shown induction of airway inflammatory

responses,^{22,37,50,51} although the precise cellular target and molecular mechanisms have not been elucidated. Our 2 *in vivo* studies demonstrate that PM₁₀ can induce airway epithelial NLRP3 inflammasome-mediated production of IL-1 β and associated airway neutrophilia, which is a novel mechanism for airway inflammation. Our data suggest that the NLRP3 inflammasome or downstream signaling pathways might be suitable targets to prevent PM-induced exacerbations of airway diseases in subjects with risk factors^{52,53} during periods of poor air quality.

In addition to acute innate immune responses mediated by the NLRP3 inflammasome, airway epithelial NLRP3-mediated production of IL-1 β could lead to allergic sensitization.^{23,28} *In vitro* studies exposing airway epithelial cells to either IL-1 β or PM₁₀ have demonstrated epithelial production of GM-CSF and CCL20, 2 factors that influence dendritic cell maturation and migration,^{18,54-56} although it is not clear whether this depends on the airway epithelial NLRP3 inflammasome and downstream IL-1 β signaling. Our *in vivo* data of NLRP3-sensitive increases in MHC class II⁺^{hi}/CD11c⁺^{hi} dendritic cells in regional lymph nodes 24 hours after PM₁₀ exposure supports the role for this molecular complex in mediating PM₁₀-induced activation of these cells, although the airway epithelium cannot be implicated directly with our current results. Clearly, future mechanistic studies with labeled tracer molecules, chimeric mice, and epithelium/dendritic cell coculture models are required to determine the precise contribution of the airway epithelial NLRP3 inflammasome to dendritic cell maturation, migration, and development of allergic adaptive immune responses.

In conclusion, we have used *in vitro*, *in vivo*, and whole human bronchus samples to describe a role for the NLRP3 inflammasome in PM₁₀-induced IL-1 β production from airway epithelial cells. The airway epithelial NLRP3 inflammasome orchestrates innate responses to PM₁₀ and might influence the development of adaptive immune responses. Importantly, our results lay the foundation to examine the role of the NLRP3 inflammasome in airway epithelium in patients with inflammatory airway diseases, such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis, to determine whether this danger sensor contributes to airway disease development and progression.

We thank Dr Vishva Dixit from Genentech for providing us with Nlrp3^{-/-} mice and Dr Renaud Vincent for providing EHC-93 PM₁₀ for our *in vitro* and *in vivo* studies.

Key messages

- Human airway epithelium expresses a functional NLRP3 inflammasome activated by PM.
- *In vivo* PM exposure results in NLRP3 inflammasome-dependent innate immune responses.
- PM exposure induces NLRP3 inflammasome-dependent increases in intrathoracic lymph node MHC class II⁺^{hi}/CD11c⁺^{hi} dendritic cells.

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METHODS

Human tissue and cell culture

EHC-93 PM₁₀ characteristics. The following elemental metals are contained in EHC-93 PM₁₀: aluminum, 10×10^3 $\mu\text{g/g}$ PM; chromium, 42 $\mu\text{g/g}$ PM; copper, 845 $\mu\text{g/g}$ PM; iron, 15×10^3 $\mu\text{g/g}$ PM; lead, 7×10^3 $\mu\text{g/g}$ PM; magnesium, 7×10^3 $\mu\text{g/g}$ PM; nickel, 67 $\mu\text{g/g}$ PM; vanadium, 90 $\mu\text{g/g}$ PM; and zinc, 10×10^3 $\mu\text{g/g}$ PM.^{E1} The elemental and polyaromatic hydrocarbon content of EHC-93 PM₁₀ has been compared with that of National Institute for Standards and Technology standard reference material-1649, showing similarities in both constituents.^{E1}

The endotoxin content in EHC-93 PM₁₀ is 6.4 EU/mL, which does not induce significant production of IL-1 β , IL-8, or GM-CSF from airway epithelial cells when examined directly.^{E2}

In vitro validation of oxidative capacity of PM₁₀.

Primary airway epithelial cells were seeded at a density of 50,000 cells per dish in 50 mm of MatTek (Ashland, Mass) glass-bottom dishes in BEGM and grown to 60% confluency. PM₁₀ was suspended in BEGM at a concentration of 500 $\mu\text{g/mL}$. Cells were exposed to PM₁₀ or BEGM for 24 hours, as previously described.^{E2,E3} After incubation, cells were washed 3 times with BEGM and incubated with a cell-permeable redox-sensitive fluorescent dye (CellROX, Invitrogen), followed by washes with PBS. Live-cell confocal imaging was performed within 30 minutes of washes. Signal intensity per unit area was determined for 3 fields of view from 3 different samples with Volocity Software (PerkinElmer, Waltham, Mass).

Pharmacologic inhibition studies. To confirm our own past results and literature, we performed functional assays and measured cell viability and lactate dehydrogenase activity in a dose-response study with the EHC-93 PM₁₀ used in our study and compared this with National Institute of Standards and Technology Standard Reference Material Particulate Matter (SRM-1649a). For both EHC-93 and SRM-1649a, a dose-dependent response for IL-1 β production was observed, with significant production at the 500 $\mu\text{g/mL}$ concentration (see Fig E1, A). No doses resulted in changes in cell viability or lactate dehydrogenase levels (see Fig E1, B and C).

siRNA NLRP3 gene knockdown studies

Airway epithelial cells were seeded and grown to 60% confluency. Silencing RNA for NLRP3 was purchased from Ambion (Burlington,

Ontario, Canada): (1) siRNA-1 for NLRP3, Ambion Silencer s41554; (2) siRNA-2 for NLRP3, Ambion Silencer s41555; and (3) scrambled siRNA, Ambion 7400808. HiPerfect (Qiagen, Hilden, Germany) was used for transfection of siRNA, according to the manufacturer's directions. Transfection of 25 nmol/L siRNA was performed for 48 hours, followed by washing, and then finished with media/PM₁₀ incubation for 24 hours without addition of fresh siRNA transfection reagents. PM₁₀ was suspended and vortexed in BEGM at a concentration of 500 $\mu\text{g/mL}$. Cell culture supernatants and protein lysates were collected for soluble mediator analysis and protein expression. To control for the consequences of cell growth on soluble mediator analysis, we normalized cytokine levels to protein lysate content.

Inflammatory cell and mediator measurements and tissue collection

Confirmation of *in vitro* ELISA-detected IL-1 β cleavage product was performed by means of immunoblotting (see Fig E2).

RESULTS

In addition to activation of airway epithelial NLRP3 inflammasomes, PM₁₀ induces activation of the NLRP3 inflammasome in THP-1 macrophages.

We provide confirmatory data that increases in lung lavage IL-1 β levels and total inflammatory cell numbers are conserved at lower doses of PM in WT mice (see Fig E4).

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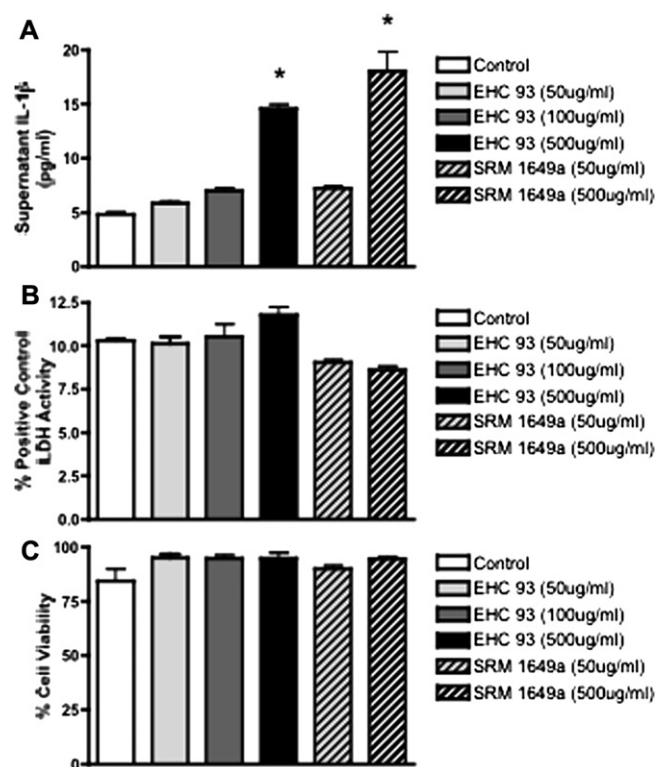


FIG E1. Dose response of EHC-93 and SRM-1649a PM₁₀ on human airway epithelial cells. **A**, IL-1 β production in cell culture supernatant. **B**, Lactate dehydrogenase (LDH) cytotoxicity assay. **C**, Counting of cell viability by using trypan blue exclusion methods. * $P < .05$ compared with control.

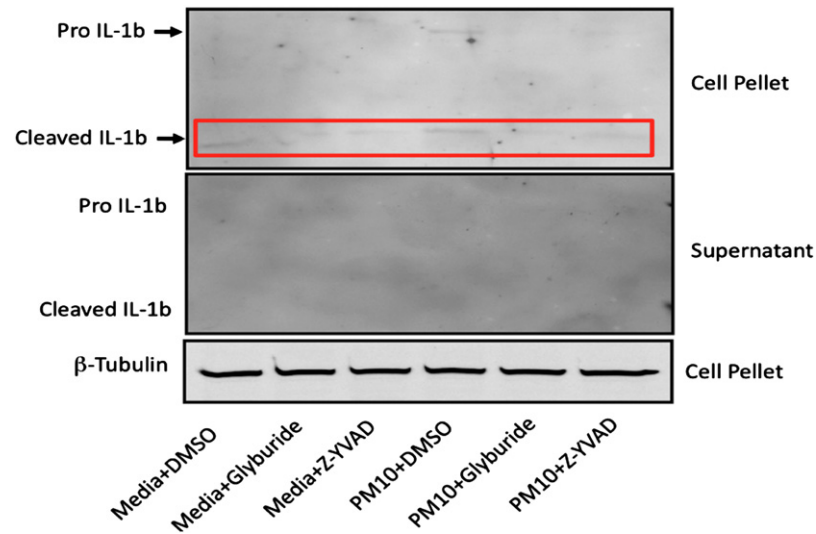


FIG E2. Immunoblot of airway epithelial cell lysates for IL-1 β expression. Equal amounts of protein (30 μ g) were loaded into each well. The red box highlights faintly cleaved IL-1 β . Data are representative of 3 independent blots. Arrowheads denote corresponding IL-1 β forms. *DMSO*, Dimethyl sulfoxide.

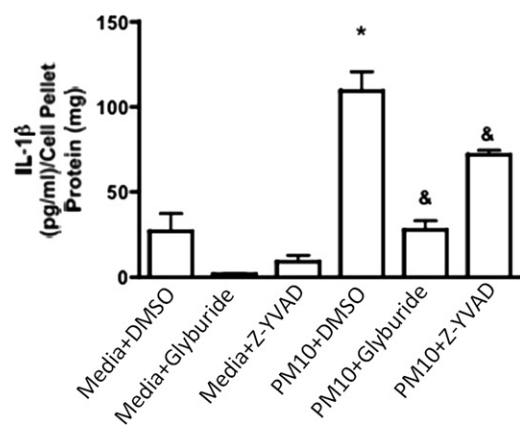


FIG E3. IL-1 β production in THP-1 macrophage cell culture supernatant normalized to cell pellet protein content (in picograms per milliliter per milligram). *DMSO*, Dimethyl sulfoxide. * $P < .05$ compared with media+DMSO. & $P < .05$ compared to PM10+DMSO

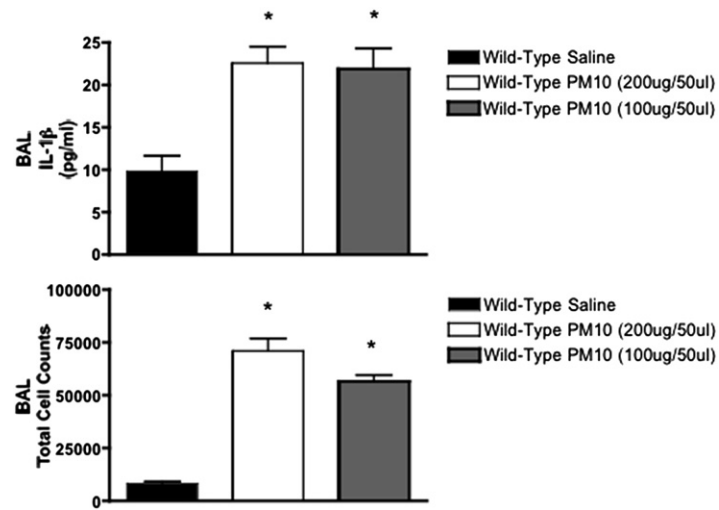


FIG E4. Lung lavage fluid IL-1 β levels and total inflammatory cell numbers in WT C57Bl/6 mice treated with PM₁₀.

TABLE E1. Characteristics of the patient sample

Patient no.	Age (y)	Sex	Smoker (Y/N)	Respiratory disease (Y/N)
1	42	Female	N	N
2	22	Female	N	N
3	24	Female	N	N
4	29	Female	N	N
5	45	Female	N	N

N, No; Y, yes.