Source of Biomass Cooking Fuel Determines Pulmonary Response to Household Air Pollution

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Abstract

Approximately 3 billion people—half the worldwide population—are exposed to extremely high concentrations of household air pollution due to the burning of biomass fuels on inefficient cookstoves, accounting for 4 million annual deaths globally. Yet, our understanding of the pulmonary responses to household air pollution exposure and the underlying molecular and cellular events is limited. The two most prevalent biomass fuels in India are wood and cow dung, and typical 24-hour mean particulate matter (PM) concentrations in homes that use these fuels are 300 to 5,000 $\mu\text{g}/\text{m}^3$. We dissected the mechanisms of pulmonary responses in mice after acute or subchronic exposure to wood or cow dung PM collected from rural Indian homes during biomass cooking. Acute exposures resulted in robust proinflammatory cytokine production, neutrophilic inflammation, airway resistance, and hyperresponsiveness, all of which were significantly higher in mice exposed to PM from cow dung. On the contrary, subchronic exposures induced eosinophilic inflammation, PM-specific antibody responses, and alveolar destruction that was highest in wood PM-exposed mice. To understand the molecular pathways that

trigger biomass PM-induced inflammation, we exposed Toll-like receptor (TLR)2-, TLR3-, TLR4-, TLR5-, and IL-1R-deficient mice to PM and found that IL-1R, TLR4, and TLR2 are the predominant receptors that elicit inflammatory responses via MyD88 in mice exposed to wood or cow dung PM. In conclusion, this study demonstrates that subchronic exposure to PM collected from households burning biomass fuel elicits a persistent pulmonary inflammation largely through activation of TLR and IL-1R pathways, which could increase the risk for chronic respiratory diseases.

Keywords: smoke; wood; cow dung; lungs; mice

Clinical Relevance

The mechanisms underlying biomass-induced pulmonary responses are poorly understood. This study increases our understanding of acute and subchronic responses to biomass particulate matter exposure and demonstrates that source of biomass fuel affects this pulmonary response.

Approximately half of the world's population uses biomass fuel as their primary household energy source, thus

exposing nearly 3 billion people to the harmful effects of household air pollution (HAP). Indoor combustion of biomass fuels

has been identified as the leading environmental risk factor for cause of death worldwide (1). In 2010, more than

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3.5 million deaths and over 110 million disability-adjusted life years were attributed to HAP (2). The number of people exposed to smoke from biomass fuel far exceeds the one billion people who smoke tobacco, suggesting that exposure to HAP may be the biggest risk factor for respiratory diseases globally (3–5). The burden of disease associated with HAP is attributed to respiratory infections, chronic obstructive pulmonary disease, asthma, lung cancer, cardiovascular disease, low birth weight, cataracts, and tuberculosis (6–8). Although the use of biomass fuels is predominantly a concern in developing nations, it is also present in developed countries, particularly as energy costs rise. For example, a recent study in New Mexico indicated that 26% of participants were exposed to biomass smoke (9).

Wood is the most prevalent source of biomass fuel globally, followed by cow dung, crop residues, and grass (4). In rural India, nearly 90% of the primary energy is derived from biomass (wood, 56%; dung, 21%; crop residues, 16%) (10). Women in rural populations are more adversely affected than men because they spend more time at home and are involved in cooking (11). In addition, young children typically accompany their mother, leading to substantial exposure to their developing lungs. The typical exposure ranges from 3 to 7 hours every day throughout a person's lifetime. The concentration of particulate matter less than 10 μm in size (PM_{10}) due to biomass fuel can reach 30,000 $\mu\text{g}/\text{m}^3$ in rural homes during the peak period of cooking, and the mean concentration is approximately 300 to 5,000 $\mu\text{g}/\text{m}^3$ in a 24-hour period, depending on duration and ventilation (4, 12). Babies born to women exposed to biomass fuel have significantly lower birth weights than babies from women exposed to cleaner fuels (13), and biomass smoke exposure is a major risk factor for lower respiratory tract infections, especially in children. However, there is limited understanding of the mechanistic etiology of biomass smoke-induced toxicity and a lack of knowledge of the relative impacts of the different biomass fuel sources on respiratory health.

Human lungs are exposed to exogenous pollutants and microbes, and many cell types in the lung, including leukocytes and epithelial cells, express pattern recognition receptors such as Toll-like receptors (TLRs) that sense a diverse array of pathogen-associated molecular patterns (PAMPs) and

danger-associated molecular patterns, resulting in activation of transcription factors to initiate cytokine and chemokine responses. PM contains numerous compounds that are capable of activating TLRs, including endotoxin, metals, microbial components, and other organic compounds (14). In addition, PM may increase danger-associated molecular patterns, such as heat shock protein 60 and 70 and high mobility group box 1, to indirectly activate TLRs. Thus, TLR signaling may be an important mediator of the response to biomass PM.

The goal of the current study was to examine the pulmonary outcomes and understand the underlying molecular and cellular events in animal models exposed to PM collected from homes in rural India during cooking with biomass. We identified key differences in the pulmonary responses of mice exposed to wood or cow dung PM and revealed that TLR2/4 and IL-1R signaling pathways play major roles in promoting biomass PM-induced airway inflammation that can potentially cause significant impacts on respiratory health.

Materials and Methods

Collection of Airborne Particulates

We used a rural cohort in Vadu village of the Pune district (Maharashtra, India) that uses biomass fuel as the primary source of cooking fuel. These homes contained closed kitchens that were separated from the rest of the house, thus limiting confounding effects of other household pollutants. The individuals who cooked used wood alone or a mixture of wood and cow dung (referred to herein as “cow dung”). The airborne particulates were collected during cooking for approximately 4 hours per day for 2 weeks via a high-volume pump and a cyclone collector (see Figure E1 in the online supplement). After 2 weeks, this was repeated either in other households or using a different fuel source. This study was approved by the Ethics Committees of KEM Hospital Research Centre Pune, Chest Research Foundation, Pune, India and by the IRB of Johns Hopkins University.

Further details are provided in the online supplement.

Animals

Male C57BL/6 mice were purchased from the National Cancer Institute (Frederick,

MD). $\text{MyD88}^{-/-}$, $\text{TLR2}^{-/-}$, $\text{TLR4}^{-/-}$, $\text{TLR2/4}^{-/-}$, $\text{TLR5}^{-/-}$, $\text{IL-1R}^{-/-}$, $\text{TLR3}^{-/-}$, and wild-type (WT) control mice were originally from the Jackson Laboratory (Bar Harbor, ME). All mice were housed under controlled conditions for temperature and humidity using a 12-hour light/dark cycle. All experimental protocols were performed in accordance with the standards established by the US Animal Welfare Acts as set forth in National Institutes of Health guidelines and in the Policy and Procedures Manual of the Johns Hopkins University Animal Care and Use Committee.

Animal Exposure

Eight-week-old male mice were anesthetized with 40 mg/kg ketamine and 8 mg/kg xylazine, and a 50- μl aliquot of a colloid suspension of biomass PM in PBS was placed on the bridge of the nose for aspiration by the anesthetized animal. Previous studies indicate that this delivery volume and method results in distribution of 55.7% to the lower respiratory tract, and no sample has been detected in the esophagus or stomach (15).

Lung Morphometry and Inflammation

Inflammatory cells were quantified in bronchoalveolar lavage fluid (BALF) as previously described (16). Lung morphometry was quantified as previously described (17).

Cytokine Analysis

In vivo cytokines were quantified by Illuminex from cell-free supernatants from the first 1 ml lavage of PBS. For *ex vivo* analysis of cytokines, 150,000 mouse peritoneal macrophages or human alveolar macrophages were plated in complete media. Biomass PM (50 μg) was suspended in media and added to the cells for 24 hours. Cytokines were quantified in cell-free media.

Pulmonary Mechanics

Airway resistance and methacholine-induced AHR were performed as previously described (18).

Polyaromatic Hydrocarbon Analysis

Polyaromatic hydrocarbons (PAHs) were extracted from samples and quantified by GC/MS/MS, as previously described (19).

Statistical Analyses

The Student's two-tailed *t* test was used to determine statistical significance between

each group. Values are presented as means $\pm$ standard error.

Results

PM Characterization

Biomass PM is a complex mixture of compounds that have been poorly characterized. Therefore, we analyzed wood and cow dung PM for relative differences in size distribution, PAH content, and endotoxin activity, all of which are important mediators of inflammation. The median diameters and geometrical standard deviations for wood and cow dung PM were $2.3 \pm 2.3 \mu\text{m}$ and $3.9 \pm 2.8 \mu\text{m}$, respectively (Figure E2). Thus, most particulates were within the respirable range. PAHs, which are a large group of chemicals that are formed by the incomplete combustion of organic molecules and are often considered to be proinflammatory (20), were shown by gas chromatography–mass spectrometry analysis to be generally more concentrated in wood PM (Table E1) with the exception of naphthalene, which was elevated in cow dung PM. Endotoxin was significantly higher in cow dung PM than in wood PM (55.2 ± 21.6 EU/mg vs. 2.7 ± 0.4 EU/mg, which corresponds to 13.8 ± 5.4 and 0.7 ± 0.1 EU for the 250- μg doses of cow dung and wood and 2.8 ± 1.1 and 0.14 ± 0.02 EU for the 50- μg doses).

Acute Pulmonary Responses in Mice Exposed to Biomass PM

To assess the acute pulmonary responses to biomass, we quantified total inflammatory cells in BALF 24 hours after single intranasal instillations of varying doses of PM generated from burning wood or cow dung. Both cow dung and wood PM elicited dose-dependent inflammatory responses (Figure 1A), although cow dung PM elicited 4- to 6-fold greater cellular inflammatory responses than wood PM ($P < 0.05$ for all doses). We did not detect a significant increase in inflammation with 20 μg wood PM; however, this concentration was sufficient to induce a strong response in mice exposed to cow dung PM. Thus, acutely, cow dung PM is a more potent proinflammatory agent than wood PM.

Exposure of mice to 250 μg PM corresponds to a 24-hour mean concentration of 3,125 $\mu\text{g}/\text{m}^3$, when assuming daily human inhalation volumes

of 16 m^3 (21), and 200-fold higher minute volumes in humans than in mice (21–23). Therefore, this concentration was used to model a relevant high-dose acute exposure. Characterization of the acute inflammatory cell profiles after 250 μg PM revealed that wood and cow dung PM elicited potent neutrophil-mediated responses within the first 24 hours (Figure 1B), which was significantly greater in mice exposed to cow dung PM than to wood PM (13.4-fold at 12 h). We observed small increases in macrophages, lymphocytes, and eosinophils within 24 hours of cow dung PM exposure but not in wood PM-exposed mice. At 72 to 120 hours, both sources of PM elicited subtle increases in macrophages, lymphocytes, and eosinophils, all of which remained significantly higher in mice exposed to cow dung PM than to wood PM.

We collected PM samples from multiple additional households and confirmed that neutrophilic inflammation is consistently and significantly higher in mice exposed to cow dung PM compared with wood PM regardless of kitchen characteristics and fuel storage conditions (Figure 1C). Inflammation was altered only slightly when comparing single exposures with five daily exposures (Figure E3). This acute inflammation was accompanied by significant increases in baseline airway resistance (17.7 and 20.5% for wood and cow dung, respectively) (Figure 1D) and increased responsiveness after treatment with the bronchoconstrictor methacholine (59 and 114% for wood and cow dung, respectively, at 30 mg/ml methacholine) (Figure 1E). Consistent with the elevated neutrophilic inflammation, methacholine-induced resistance was significantly higher in mice exposed to cow dung PM compared with wood PM ($P < 0.05$). Three daily exposures to PM resulted in similar baseline and methacholine-induced resistance (data not shown) as observed after one exposure, again suggesting that multiple high-dose PM exposures do not amplify this response.

Biomass PM Elicits a Diverse Cytokine Response

To identify which inflammatory markers increased after exposure to biomass smoke PM, we examined a panel of 32 cytokines in unconcentrated BALF at 6, 24, and 72 hours after 250- μg biomass PM exposure. Table 1 shows each cytokine at the time

point that gave maximal induction. In general, cytokines peaked after 6 hours, although several cytokines showed maximal induction after 24 hours. Both PM samples elicited similar cytokine profiles: neutrophil chemokines (G-CSF, KC, macrophage inflammatory protein [MIP-2], and MIP-1 α), macrophage chemokines (MIP-1 β , interferon (IFN)- γ -induced protein-10, monocyte chemoattractant protein-1), and cytokines (IL-6, TNF- α , IL-12p70, and MIG); however, the extent of induction was comparatively greater in mice exposed to cow dung PM compared with wood PM (Table 1). We observed slight but significant induction of eosinophilic chemokines (eotaxin, IL-5, and RANTES) (Table 1), and we did not detect any increases in the T_H1 cytokine IFN- γ or in the T_H2 cytokines IL-4 and IL-13. This cytokine profile is consistent with a predominantly neutrophil-mediated inflammation, with little contribution from eosinophils.

Macrophages are primarily responsible for sensing environmental cues. To understand the macrophage inflammatory response to PM, cytokine secretion was quantified from macrophages that were treated *ex vivo* with biomass PM. As with the *in vivo* analysis, we observed stronger induction of cytokines after cow dung PM treatment compared with wood PM treatment, with greater secretion of IL-6, MIP-1 β , KC, G-CSF, and MIP-2 (Table E2). In addition, human alveolar macrophages were harvested from four patients (two smokers, one former smoker, and one nonsmoker), and each patient sample was treated *ex vivo* with PBS, wood PM, or cow dung PM. Similar to the results in mouse macrophages, cytokine secretion was generally higher in human alveolar macrophages treated *ex vivo* with cow dung PM (Table E3).

Subchronic Biomass PM-Induced Inflammatory Responses

Similar to the acute high-dose (250 μg) PM exposures, acute lower (50 μg) PM exposures resulted in neutrophilic inflammation that was significantly higher with cow dung PM (Figure 2A, *top*). However, subchronic 8-week exposure (three times per week) to 50 μg of wood or cow dung PM resulted in significantly elevated eosinophils, macrophages, and lymphocytes when compared with subchronic PBS or acute PM exposure

Figure 1. Cow dung particulate matter (PM) exposure results in a greater acute pulmonary response than wood PM. (A) Total inflammatory cells were determined in bronchoalveolar lavage (BAL) at 24 hours after PM instillation. (B) Differential inflammatory cell profiles were determined at multiple time points after instillation of 250 μg PM. (C) Mice were exposed to different sources of wood and cow dung PM (250 μg), and neutrophilic inflammation was determined in BAL fluid after 24 hours. Closed circles are the same samples used in A and B; open circles are samples collected from additional

Table 1: Cytokine Concentrations in Unconcentrated Bronchoalveolar Lavage Fluid after *In Vivo* Exposure to 250 μg of Particulate Matter

Cytokines*	PBS (pg/ml)	Wood (pg/ml)	Cow Dung (pg/ml)
BAL (6 h)			
KC	59.1 $\pm$ 13.8	372.5 $\pm$ 53.6 [†]	1,163 $\pm$ 132 ^{†‡}
MIP-1 α	13.7 $\pm$ 1.5	46.2 $\pm$ 7.6 [†]	2,61.2 $\pm$ 34.3 ^{†‡}
G-CSF	52.1 $\pm$ 20.0	474.0 $\pm$ 56.9 [†]	5,540 $\pm$ 884 ^{†‡}
MIP-2	36.4 $\pm$ 5.6	236.7 $\pm$ 22.3 [†]	7,51.0 $\pm$ 53.5 ^{†‡}
MIP-1 β	13.4 $\pm$ 1.2	62.2 $\pm$ 10.8 [†]	580.6 $\pm$ 98.7 ^{†‡}
IP-10	16.4 $\pm$ 5.9	55.0 $\pm$ 9.0 [†]	188.8 $\pm$ 31.2 ^{†‡}
RANTES	5.8 $\pm$ 1.0	6.1 $\pm$ 0.5	16.1 $\pm$ 2.2 ^{†‡}
TNF- α	8.2 $\pm$ 0.9	88.7 $\pm$ 20.4 [†]	659 $\pm$ 125.2 ^{†‡}
IL-12P70	2.5 $\pm$ 0.7	8.5 $\pm$ 1.2 [†]	18.4 $\pm$ 1.5 ^{†‡}
IL-6	26.3 $\pm$ 6.8	320.8 $\pm$ 61.7 [†]	1237 $\pm$ 132 ^{†‡}
IL-1 α	56.1 $\pm$ 3.8	55.9 $\pm$ 5.3	95.4 $\pm$ 5.5 ^{†‡}
MCP-1	4.2 $\pm$ 2.1	12.3 $\pm$ 2.2 [†]	18.8 $\pm$ 2.8 ^{†‡}
GM-CSF	23.6 $\pm$ 2.6	34.4 $\pm$ 0.0 [†]	33.2 $\pm$ 1.7 [†]
LIF	3.9 $\pm$ 0.9	23.3 $\pm$ 3.1 [†]	125.3 $\pm$ 11.4 [†]
BAL (24 h)			
M-CSF	2.0 $\pm$ 0.2	2.3 $\pm$ 0.3	13.1 $\pm$ 2.3 ^{†‡}
LIX	0.0 $\pm$ 0.0	19.0 $\pm$ 8.4	72.0 $\pm$ 14.7 ^{†‡}
MIG	5.2 $\pm$ 0.7	60.0 $\pm$ 27.3	491.2 $\pm$ 187.7
VEGF	12.4 $\pm$ 1.9	33.0 $\pm$ 5.7 [†]	166.6 $\pm$ 20.4 ^{†‡}
Eotaxin	1.8 $\pm$ 0.2	2.9 $\pm$ 0.1 [†]	8.0 $\pm$ 0.7 ^{†‡}
IL-5	2.8 $\pm$ 1.0	6.3 $\pm$ 1.3	10.0 $\pm$ 2.0 ^{†‡}
IL-17	0.5 $\pm$ 0.2	0.8 $\pm$ 0.2	2.5 $\pm$ 0.3 ^{†‡}

Definition of abbreviations: BAL, bronchoalveolar lavage; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IP-10, IFN- γ -induced protein 10; KC, killer cells; LIF, leukemia inhibitory factor; LIX, lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; MIG, monokine induced by IFN- γ ; MIP, macrophage inflammatory protein; VEGF, vascular endothelial growth factor.

*Cytokines measured but not included: IFN- γ , IL-10, IL-12p40, IL-15, IL-1 β , IL-4, IL-2, IL-3, IL-7, IL-9, and IL-13.

[†] $P < 0.05$ versus PBS-exposed controls.

[‡] $P < 0.05$ versus wood-exposed group.

(Figure 2A, bottom). The eosinophilic inflammation was significantly higher in mice exposed to wood PM than to cow dung PM. In mice exposed to wood PM, neutrophils were similar after acute and subchronic exposures, whereas in mice exposed to cow dung PM, neutrophils decreased significantly after subchronic exposure. Neutrophils remained significantly higher in mice exposed to cow dung PM than to wood PM. Thus, the inflammatory cell profiles shift dramatically between acute and subchronic exposures, and there are key distinctions between mice exposed subchronically to wood or cow dung PM regarding neutrophilic and eosinophilic responses.

To determine whether these key differences in inflammation between wood

PM-exposed and cow dung PM-exposed mice were reflected in the cytokine profiles, we assessed airway cytokine levels in the BALF 24 hours after the final exposure. Although the acute cytokine profiles revealed substantial overlap in the cytokines elevated after exposure to wood or cow dung PM (Table 1), subchronic cytokine profiles showed distinct differences between wood and cow dung PM (Table 2). Mice subchronically exposed to cow dung PM exhibited increased neutrophil and macrophage chemokines (G-CSF, MIP-1 α , MIP-1 β , IP-10, and monokine induced by IFN- γ), whereas mice subchronically exposed to wood PM exhibited elevated eosinophil cytokines (IL-5 and eotaxin). Thus, the cytokine profiles mirror the inflammation after subchronic exposure.

Subchronic Biomass PM Exposure Induces PM-Specific Antibodies

Because subchronic biomass PM exposures resulted in significant increases in lymphocytes, we sought to determine whether this resulted in the development of biomass PM-specific antibodies. Serum from subchronically exposed mice was incubated with biomass PM, and bound antibodies were stained for IgG. Mice subchronically exposed to wood or cow dung PM exhibited significant increases in PM-specific IgG compared with PBS-exposed control mice (Figure 2B). Serum from mice exposed to wood PM also showed cross-reactivity with cow dung PM, which may be reflective of the fact that the cow dung PM samples are a mixture of wood and cow dung (see MATERIALS AND METHODS). This limitation precludes us from concluding whether one source of biomass is more antigenic than the other, but clear differences exist between biomass PM-exposed mice and control mice. An antigenic response is indicative of the immunogenic nature of PM, and this response may play a role in the pathological progression of chronic diseases associated with such exposure.

Biomass PM-Induced Lung Morphometry

Mice subchronically exposed for 8 weeks to wood or cow dung PM exhibited substantial damage to the airway epithelium and showed evidence of blood vessel thickening (Figure 3A). Although airway epithelial damage was apparent with both PM exposures, only subchronic wood PM exposure resulted in significant increases in airspace enlargement (Figure 3B). Thus, cow dung PM is more inflammatory, but wood PM causes greater destruction of the lungs. Overall, these data indicate that the combination of these types of PM may cause inflammation and lung destruction.

PM-Induced Airway Inflammation Is Dependent on TLR and IL-1R Signaling

In addition to our identification of potent acute inflammation and cytokine responses after exposure to biomass PM, we observed elevated NF- κ B/p65 activity in nuclear

Figure 1. (Continued). households. (D) Baseline resistance was measured 24 hours after biomass exposures. (E) Resistance was measured in response to methacholine challenges 24 hours after 250 μg biomass PM exposure ($n = 5$ –10 mice per group). * $P < 0.05$ versus PBS-exposed controls. [†] $P < 0.05$ versus wood-exposed group.

Figure 2. Subchronic exposure to biomass PM elicits innate and adaptive immune responses. (A) BAL inflammation 24 hours after a single (*top panel*) or 8-week (*bottom panel*) exposure to 50 µg biomass PM. Arrows represent significant changes between acute and subchronic exposures. (B) Relative concentrations of serum IgG that is immunoreactive to cow dung (*top panel*) or wood (*bottom panel*) PM. Serum samples are from mice subchronically exposed to PBS, wood, or cow dung PM ($n = 5-10$ mice per group). * $P < 0.05$ versus PBS-exposed group; † $P < 0.05$ versus wood-exposed group.

fractions of lung homogenates from mice exposed to wood PM (1.54-fold; $P < 0.05$ vs. control) and cow dung PM (2.13-fold; $P < 0.001$ vs. control and $P < 0.05$ vs. wood) (Figure 4A). NF- κ B is activated by a variety of stimuli, including PAMPs, which signal through the TLR/IL-1R adaptor protein MyD88. IRAK1 interacts with MyD88, and activation of MyD88 induces phosphorylation of IRAK1. Using a mouse alveolar macrophage cell line (from Les Kobzik, Harvard University, Cambridge, MA), we observed elevated levels of phosphorylated IRAK1 (pIRAK1) after 30 minutes of treatment with biomass PM (Figure 4B). Furthermore, we transiently transfected a Luciferase–NF- κ B reporter construct into 293-mTLR4 cells, which are stably transfected with murine TLR4, and detected elevated NF- κ B activity in response to biomass PM (Figure 4C). NF- κ B activity was higher in 293-mTLR4 cells than in control HEK293 cells, although cow dung PM significantly increased NF- κ B reporter activity in the control cells. Thus, biomass PM activates TLR4, MyD88, and NF- κ B.

To determine whether biomass PM-induced inflammation is dependent on the TLR/IL-1R pathway, we assessed

inflammation in MyD88^{−/−} mice after acute exposure to biomass PM. MyD88^{−/−} mice exhibited a 95% reduction in neutrophils compared with WT mice after wood PM exposure and a 91% reduction in neutrophils after cow dung PM exposure (Figure 4D). Despite this almost complete lack of neutrophil recruitment in PM-exposed MyD88^{−/−} mice, wood and cow dung PM induced small but significant increases in neutrophils compared with PBS-exposed controls. Therefore, the biomass PM-induced inflammatory response is primarily driven through MyD88 signaling.

We further examined the role of TLR/IL-1R signaling in biomass PM-induced inflammation. Our previous observation that the TLR4 ligand endotoxin was elevated in cow dung PM, coupled with increased NF- κ B activity in HEK-TLR4 cells, led us to hypothesize that the biomass PM-induced inflammation was dependent on TLR4. A concentration of endotoxin similar to that present in 250 µg cow dung PM induced a modest neutrophilic response ($3.65 \pm 0.30 \times 10^5$ neutrophils in WT mice vs. $0.12 \pm 0.03 \times 10^5$ neutrophils in TLR4^{−/−} mice), which accounts for 40% of the response observed with cow

dung PM (data not shown). Similarly, TLR4^{−/−} mice exhibit a 38% reduction in neutrophils compared with WT mice after cow dung PM exposure ($P < 0.05$) (Figure 4D) and a nonsignificant (27%) decrease in neutrophils after wood PM exposure (Figure 4E). Thus, endotoxin contributes significantly to the biomass PM-induced response, but the response was primarily independent of TLR4. We also observed that TLR2/4 double knockout mice elicited significant decreases in neutrophils compared with WT mice or with TLR2^{−/−} or TLR4^{−/−} single knockouts, suggesting that both receptors contribute to wood and cow dung PM-induced inflammation. Biomass PM-induced inflammation was not reduced in TLR5^{−/−} or TLR3^{−/−} mice.

The dependence of this neutrophilic inflammation on MyD88 could not be fully explained by TLR signaling, as indicated by the observation that MyD88^{−/−} mice had significantly fewer neutrophils than TLR2/4^{−/−} mice coupled with no reduction in TLR5^{−/−} or TLR3^{−/−} mice. Thus, other activators of MyD88 are also responsible for the biomass PM-induced neutrophil response. Because MyD88 is

Table 2: Cytokine Concentrations in Undiluted Bronchoalveolar Lavage Fluid after Subchronic Exposure to 50 μ g Biomass Particulate Matter

Cytokines*	PBS (pg/ml)	Wood (pg/ml)	Cow Dung (pg/ml)
G-CSF	7.2 $\pm$ 2.6	18.4 $\pm$ 2.1 [†]	110.3 $\pm$ 8.1 ^{††}
KC	15.0 $\pm$ 3.3	73.8 $\pm$ 13.4 [†]	47.1 $\pm$ 5.1 [†]
IL-6	0.0 $\pm$ 0.0	6.4 $\pm$ 2.0 [†]	2.9 $\pm$ 0.8 [†]
MIP-1 α	14.9 $\pm$ 1.5	22.0 $\pm$ 2.6	68.9 $\pm$ 2.8 ^{††}
MIP-1 β	0.0 $\pm$ 0.0	3.2 $\pm$ 3.2	33.9 $\pm$ 2.0 ^{††}
IP-10	8.7 $\pm$ 2.1	9.6 $\pm$ 1.5	45.3 $\pm$ 7.3 ^{††}
MIG	5.3 $\pm$ 2.9	4.1 $\pm$ 2.0	17.0 $\pm$ 3.0 ^{††}
Eotaxin	2.0 $\pm$ 1.2	7.1 $\pm$ 1.2 [†]	2.2 $\pm$ 1.4 [†]
IL-5	1.3 $\pm$ 1.3	18.5 $\pm$ 5.3 [†]	6.0 $\pm$ 2.8
IL-4	0.0 $\pm$ 0.0	1.7 $\pm$ 1.1	0.0 $\pm$ 0.0
IFN- γ	3.2 $\pm$ 2.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
IL-1 α	1.3 $\pm$ 1.3	4.5 $\pm$ 2.3	1.9 $\pm$ 1.9
IL-2	0.6 $\pm$ 0.5	1.4 $\pm$ 0.9	0.7 $\pm$ 0.6
IL-9	0.0 $\pm$ 0.0	3.8 $\pm$ 3.8	0.0 $\pm$ 0.0
IL-10	1.7 $\pm$ 1.1	0.8 $\pm$ 0.8	0.0 $\pm$ 0.0
IL-12p40	2.2 $\pm$ 2.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
LIF	0.0 $\pm$ 0.0	1.2 $\pm$ 1.2	0.0 $\pm$ 0.0
M-CSF	53.9 $\pm$ 49.9	0.0 $\pm$ 0.0	22.7 $\pm$ 9.0
VEGF	9.2 $\pm$ 1.0	9.2 $\pm$ 1.7	9.6 $\pm$ 0.8
LIX	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	22.1 $\pm$ 22.1
MIP-2	18.7 $\pm$ 18.7	0.0 $\pm$ 0.0	22.7 $\pm$ 22.7

Definition of abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IP-10, IFN- γ -induced protein 10; KC, killer cells; LIF, leukemia inhibitory factor; LIX, lipopolysaccharide-induced CXC chemokine; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; MIG, monokine induced by IFN- γ ; MIP, macrophage inflammatory protein; VEGF, vascular endothelial growth factor.

Cytokines were measured 24 hours after the final exposure.

*Cytokines not detected: GM-CSF, IL-1 β , IL-3, IL-7, IL-12p70, IL-13, IL-15, IL-17, MCP-1, RANTES, and TNF- α .

[†] $P < 0.05$ versus PBS-exposed controls.

^{††} $P < 0.05$ versus wood-exposed group.

also activated by IL-1R, we measured inflammation in IL-1R^{-/-} mice. IL-1R^{-/-} mice exhibited a significant 50% reduction in neutrophils after exposure to cow dung PM and a 27% reduction in

neutrophils after exposure to wood PM (not significant) (Figures 4D and 4E). Thus, biomass PM-induced acute inflammation is driven by MyD88-dependent TLR2/4 and IL-1R signaling.

Discussion

A recent report on the global burden of diseases indicated that 3.5 million deaths are attributed to exposure to HAP (2). Because biomass is used by half the world's population for heating and cooking, there is a need for improved understanding of the cellular and molecular pathways that promote biomass smoke-induced lung disease. In addition, there is a strong rationale for using PM samples generated in real-world settings as opposed to those produced in laboratories. Our analysis revealed that cow dung PM contains greater endotoxin than wood, which is consistent with elevated endotoxin levels measured in homes that cook with dung compared with wood (24). Our data indicate that endotoxin remains active even after combustion. We also observed that both biomass PM sources contain a variety of PAHs, many of which have proinflammatory and cytotoxic properties. PAHs were generally higher in wood PM, with the exception of the simplest PAH, naphthalene. Cow dung has previously been shown to contain a high concentration of naphthalene (25). Despite our characterization of these complex PM samples, it remains difficult to determine which components of biomass smoke contribute to the inflammation and toxicity observed in the current study.

Mice exposed to these biomass PM samples exhibit acute neutrophilic responses, followed by subchronic eosinophilic and lymphocytic responses. A

Figure 3. Biomass PM induces parenchymal destruction. (A) Representative images of lungs 24 hours after subchronic instillation with 50 μ g biomass PM (original magnification, top panel: $\times 40$; bottom panel: $\times 200$). Arrows show damage to airway epithelium, and arrowhead shows thickened blood vessel. Lungs were inflated to 25 cm H₂O with 0.6% agarose and fixed in 10% formalin. Lungs were embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin. (B) Alveolar enlargement was assessed in subchronically exposed mice by measuring mean linear intercept ($n = 9$ –10 mice per group).

Figure 4. Biomass PM-induced inflammation is dependent on Toll-like receptor (TLR) signaling. (A) NF-κB activity in lung homogenates 24 hours after exposure to 250 μg biomass PM. (B) pIRAK1 from B6 macrophage cells treated with 250 μg/ml biomass PM for 30 minutes. (C) 293-mTLR4 and control HEK293 cells were transiently transfected with luciferase–NF-κB reporter and assayed for luciferase after treatment with biomass PM for 24 hours. (D and E) Neutrophils and macrophages in BAL after instillation of 250 μg cow dung PM (D) or wood PM (E). TLR3^{-/-} mice are on a B6.129/F2 background and are presented separately with B6.129/F2 wild-type control mice (*n* = 5–10 mice per group). **P* < 0.05 versus PBS-exposed group; †*P* < 0.05 versus wood-exposed group; ‡*P* < 0.05 versus wild-type mice; §*P* < 0.05 versus TLR2^{-/-} or TLR4^{-/-} groups; ||*P* < 0.05 versus TLR2/4^{-/-} or IL-1R groups.

small number of studies have previously identified elevated neutrophilic inflammation in women (26) and children

(27) who live in homes that use biomass fuels. In mice, PM_{2.5} has been shown to induce an acute neutrophil response,

coupled with a strong increase in IL-6 and TNF-α (28). Furthermore, subchronic cow dung smoke exposure in rats has been shown to induce systemic eosinophilia (25).

Several studies have indicated that exposure to wood smoke (29–31) or cow dung smoke (25, 32) results in inflammation and pulmonary injury, whereas other studies have compared pulmonary responses to diesel exhaust and wood smoke (33), indoor and outdoor air pollution (34), and cow dung and tobacco smoke (35). To our knowledge, our study is the first to compare the differential pulmonary responses of PM that was collected from households burning wood or cow dung, the two most prevalent sources of biomass fuels in India and other developing countries. We showed that cow dung PM elicited a significantly stronger acute response, as indicated by increased inflammation, cytokine secretion, NF-κB activity, and AHR. However, subchronic exposure to wood PM resulted in greater eosinophilic inflammation and increased emphysematous remodeling. In the acute exposures, only cow dung PM elicited a significant eosinophilic response.

Although we do not fully understand why this occurred, previous studies indicate that LPS can stimulate eosinophils at 24 hours (36). The explanation for why subchronic wood PM exposure induces a greater eosinophilic response than cow dung PM is unclear, although repeated exposure to endotoxin may suppress eosinophilic inflammation, as indicated by the observation in humans that endotoxin exposure inversely correlates with incidence of hay fever, atopic asthma, and atopic sensitization (37). In addition, elevated T_H1 responses, which often accompany neutrophilia, may antagonize T_H2 responses, which are associated with eosinophilia (38). It is therefore possible that the significantly higher neutrophilic response observed in cow dung PM-exposed mice may suppress the eosinophilic response in the subchronic model. It is also unclear why subchronic exposure to wood PM, but not cow dung PM, resulted in significant airspace enlargement. It is possible that the elevated PAH concentrations in wood PM or the increased eosinophils in BALF after wood PM exposure could contribute to airspace enlargement. Several PAHs have been shown to damage lung epithelium and promote inflammation.

Mechanistic studies of biomass smoke-induced pulmonary responses are lacking, and data on the relative health impacts associated with biomass smoke exposure from specific fuel sources are limited. In our study, the acute inflammation induced by both biomass PM sources was almost entirely dependent on MyD88. Indeed, MyD88 has previously been shown to be critical for the cytokine responses induced by PM₁₀ and PM_{2.5} (39). Although all TLR and IL-1R family members share a similar intracellular Toll/IL-1 receptor domain, their extracellular domains are more divergent, and each TLR has been shown to recognize distinct PAMPs. Thus, determination of the specific TLRs that mediate the inflammatory response to biomass PM can help to identify those PAMPs that drive the PM-induced inflammatory response. We observed the additive effects of TLR2 and TLR4, which were consistent with previously described nonoverlapping roles of TLR2 and TLR4 in responses to PM_{2.5} and PM₁₀, respectively (39). Considering the elevated endotoxin detected in cow dung PM, a role for TLR4 is not surprising. However, our observation that IL-1R and TLR2 are also partially responsible for the neutrophilic response to biomass PM suggests that other components of PM in addition to endotoxin contribute to this response. This is consistent with a previous report showing that cow dung smoke extract elicited proinflammatory effects that were partially mediated by endotoxin (32).

Many cell types, including leukocytes, epithelial cells, and endothelial cells, express TLRs on their surface. In addition, epithelial cells respond to PM₁₀ and are susceptible to DNA damage and p53-dependent apoptosis after treatment with PM (40). Consistent with these *in vitro* data, we observed epithelial damage in our subchronically exposed mice. Thus, multiple cell types in the lungs may play a role in the TLR-dependent inflammation triggered by biomass PM exposure.

Exposure to a wide range of environmental toxicants, including cigarette smoke (41), traffic-related pollutants (42, 43), and outdoor air pollution (44, 45), is strongly associated with expression of proinflammatory markers. However, biomass-induced inflammatory biomarkers have not been well characterized. Our evidence shows that cow dung and wood PM trigger activation of NF- κ B and a large

array of proinflammatory cytokines. We identified a large number of cytokines that are increased acutely *in vivo* and *ex vivo*, with a surprising high degree of overlap in cytokine profiles between mice exposed to wood PM and cow dung PM. However, subchronic exposures resulted in distinct cytokine profiles, which may be useful as exposure biomarkers. In addition, we observed that subchronic biomass PM exposure elicits an IgG antibody response. Future identification of the specific components of biomass PM that are immunogenic may be very important for the development of biomarkers. These findings warrant further investigations in our cohort to determine if there are antibodies against biomass PM components and if these antibodies play a role in the progression of chronic diseases.

All comparisons between cow dung and wood PM were on the basis of particulate mass. However, normalizing to particulate mass does not account for other differences in burning characteristics that may affect exposure, including combustion efficiency and rate. Previous studies demonstrate that cow dung produces 23% more fine particulates (PM_{2.5}) per kilogram of sample burned (8.5 vs. 6.9 g/kg) and burns at a faster rate than wood (3.5 vs. 2.2 kg/h) (46–48), indicating that cow dung produces nearly twice as much PM as wood. Thus, the differences in acute responses between cow dung and wood exposure presented in this study may be even greater when accounting for these differences in burning and emissions characteristics. An additional limitation of our study is that our cow dung PM samples contained mixtures of wood and cow dung; therefore, the differences between wood and cow dung PM presented in this study are underestimated.

To model biomass exposure, we intranasally delivered biomass particulates that were suspended in PBS. Although inhalation is the natural route of exposure, direct instillation is commonly used for studies of inhalation toxicology. Many factors may make inhalation exposures unfeasible, including the cost of building and maintaining such a system and the requirement for large quantities of PM generated under controlled conditions. The Inhalation Specialty Section of the Society of Toxicology has developed guidelines for the appropriate application of direct instillation (49), which concluded that instillation has

been shown to be a convenient and valid mode of administration of foreign compounds into the airways and is particularly appropriate for comparing relative toxicities of two or more samples. The two modes of exposure yield qualitatively similar results for a variety of biologic endpoints, including pulmonary inflammation (49). Thus, instillation is an appropriate mode of exposure for our present study. Yet, we are mindful of the limitations of this mode of exposure. We did not detect significant increases in oxidative stress in lung homogenates after exposure to biomass PM (data not shown), which may reflect differences between instillation and inhalation exposures. In addition, although we attempted to adjust our doses to account for differences between human and mouse size and breathing patterns, the instillation exposure method delivered a bolus dose that corresponds to a 24-hour inhalation exposure, which may cause an overload of PM.

In conclusion, exposures to PM collected from homes during biomass cooking result in neutrophil responses acutely but shift to eosinophilic inflammation after subchronic exposures. The response to cow dung PM differs from wood PM in the type and extent of inflammation. Both biomass samples are complex mixtures of components, and multiple proinflammatory ligands are present in both samples, yet both samples trigger the TLR/IL-1R pathways to elicit inflammation. A United Nations-sponsored initiative, the Global Alliance for Clean Cookstoves, has pledged to enable 100 million households to adopt clean and efficient cookstoves by 2020. The current study indicates that, in addition to stove efficiency, fuel type is an important contributing factor for improving exposure-related health outcomes. Thus, this study has significant public health implications for designing interventions to improve the health of billions of individuals who are exposed daily to biomass smoke. ■

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