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## Comparison of how ambient PM<sub>c</sub> and PM<sub>2.5</sub> influence the inflammatory potential

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### Abstract

Airborne particulate matter (PM) is one of six criteria air pollutants currently regulated by the U.S. Environmental Protection Agency (EPA), with existing ambient standards for PM<sub>2.5</sub> and PM<sub>10</sub>. Currently there are no health-based regulations for the size fraction between 2.5 and 10 μm, commonly known as the coarse fraction (PM<sub>c</sub>). The present study investigates current gaps in knowledge for PM<sub>c</sub> including exposure toxicity and PM ratios (PM<sub>c</sub>:PM<sub>2.5</sub>) in PM<sub>10</sub>. Throughout the world, all three PM size fractions have been shown to be associated with adverse impacts. Recent studies have shown that PM<sub>c</sub> can be more detrimental to susceptible populations when directly compared to PM<sub>2.5</sub>, and that the PM<sub>c</sub> fraction in PM<sub>10</sub> can account for the majority of the inflammatory response from PM<sub>10</sub> exposure.

In our studies we utilized a bone marrow-derived mouse macrophage *in vitro* system to compare the inflammatory potential of PM<sub>c</sub>, PM<sub>2.5</sub>, and mixtures of the two. The result was a linear increase in interleukin(IL) –1β with increasing levels of exposure to winter and summer PM<sub>c</sub>, as compared to PM<sub>2.5</sub>, which exhibited logarithmic growth. Also, exposure to PM<sub>10</sub> as a function of PM<sub>2.5</sub> and PM<sub>c</sub> mass ratios showed that IL-1β and TNF-α levels increased synergistically with a greater burden of PM<sub>c</sub>. Endotoxin content in the PM did not correlate with these results, suggesting that other activators in PM<sub>c</sub> are likely responsible for activating the NF-κB pathway and the inflammasome.

### Keywords

inflammation; pulmonary macrophage; coarse fraction; ambient particulate matter; mixtures; NAAQS; IL-1β; TNF-α; endotoxin; NF-κB

### Introduction

Airborne particulate matter (PM) is a known causative agent in inducing or exacerbating a variety of respiratory and cardiovascular diseases (Pelucchi et al. 2009, Brook et al. 2010, Dvonch et al. 2009, Samet et al. 2009, Weinmayr et al. 2010, Zanobetti et al. 2010, Xu et al. 2011). PM is also one of six criteria air pollutants currently regulated by the United States Environmental Protection Agency (EPA), with existing ambient standards for both PM<sub>2.5</sub> and PM<sub>10</sub> (i.e. particulate matter (PM) with mean aerodynamic diameters <2.5 and <10 micrometers (μm), respectively). Currently there are no health-based regulations for the size fraction between 2.5 and 10 μm, commonly known as the coarse fraction (PM<sub>c</sub>). Compared to ambient PM<sub>2.5</sub> and PM<sub>10</sub> (which are routinely measured across the US), much less is known about PM<sub>c</sub> seasonal concentrations and health effects. In multiple research studies conducted throughout the world, all three PM size fractions have been shown to be

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associated with adverse impacts (Gilmour et al. 2007, Gordian et al. 1996, Host et al. 2008, Mar et al. 2004, Monn and Becker 1999, Ostro et al. 2000, Pelucchi et al. 2009 2007).

PM<sub>c</sub> has been shown to cause a more significant pulmonary inflammatory response than PM<sub>2.5</sub> in animal models (Tong et al. 2010, Wegesser and Last 2009), as well as increased inflammatory potential in multiple respiratory cell lines (Gualtieri et al. 2010). Becker et al. (Becker et al. 2005) reported that compared to PM<sub>2.5</sub>, PM<sub>c</sub> induced higher levels of the inflammation markers IL-6 and Cox-2 mRNA in alveolar macrophages, and higher levels of IL-8 and Cox-2 mRNA in normal human bronchial epithelial cells. These studies suggest that the coarse fraction in PM<sub>10</sub> accounts for 90-95% of the resulting inflammatory response (Becker et al. 2005). These findings may be explained by the biological composition of PM<sub>c</sub>, as the coarse fraction can contain up to 10 times the amount of biological content compared to PM<sub>2.5</sub> (Heinrich et al. 2003). This biological content includes pathogen associated molecular patterns (PAMPS), such as endotoxin, which prime the activation of the inflammasome (Tschopp and Schroder 2010, Yazdi et al. 2010). The inflammasome is a protein complex that once formed can stimulate maturation of pro-IL-1 $\beta$  to its active form, IL-1 $\beta$ . However, a pool of pro-IL-1 $\beta$  will need to be maintained allowing the inflammasome to continue its function. Endotoxin has also been shown to trigger the NF- $\kappa$ B pathway and downstream pro-IL-1 $\beta$  production via TLR4 (Schins et al. , Bauernfeind et al. 2009), suggesting that ambient PM-induced adverse health outcomes may be heavily influenced by the PM<sub>c</sub> fraction.

A tenet of toxicology is that exposure to environmental toxicants will likely occur as a mixture, with the outcome being a function of the joint effects of this mixture. A measure of how a mixture can maintain, lower, or increase a toxic outcome is termed additivity. For example, laboratory studies exposing humans to both carbon black particles and O<sub>3</sub> resulted in greater lung inflammation and suppression of alveolar macrophage phagocytosis than would be predicted using the sum of both toxicities alone (Jakab and Hemenway 1994). These results suggest a *synergism* in the lung, or a *greater than additive* toxicity between carbon black and O<sub>3</sub>.

Toxic consequences from mixture exposures have two other outcomes other than synergism. An inverse relationship to synergism may also be observed where *antagonism* takes place, i.e. a decrease in toxic potential, *less than additive*. Finally, when two toxicants are introduced in a mixture and if one toxic does not affect the other, and visa versa, the mixture effect is termed *additive*.

Although multiple studies have been conducted linking either PM<sub>2.5</sub> or PM<sub>10</sub> to respiratory disease throughout the world, little research has been conducted investigating the role of these two PM size fractions in mixture, specifically how the PM<sub>c</sub> size class influences overall PM<sub>10</sub> inducible toxicity. In the real world, exposure to PM<sub>10</sub> occurs as a function of PM<sub>c</sub> and PM<sub>2.5</sub> mass ratios. These mixtures can change as a function of geography, temporality, and seasonal conditions (e.g. wildfires, winter inversions, etc.). In this manuscript, we describe the results of studies that evaluated the additive effect of ambient PM<sub>2.5</sub> and PM<sub>c</sub> mixtures. Following the harvesting of ambient PM using a particle concentrator, an *in vitro* model of inflammation was used to determine the contributions of PM<sub>c</sub> within a mixture. The overall goal of these studies was to not only investigate the inflammation potential of PM<sub>c</sub> compared to PM<sub>2.5</sub>, but also to elucidate the relative contributions of these two size fractions to observed health effects.

## Methods

### Collection of Ambient Particles

Seasonal PM was collected in Missoula, Montana, a valley airshed located in west-central Montana. With a population of 70 000, Missoula is the 2nd largest city in the state of Montana. Particles were collected using a versatile aerosol concentration enrichment system particle concentrator (VACES-PC, University of Southern California). The concentrator has three parallel sampling lines that simultaneously collect PM<sub>2.5</sub> and PM<sub>c</sub>, respectively, at a set flow rate of 110 L/min (Kim et al. 2001a, Kim et al. 2001b). Following each sample run, the product (i.e. particles in ultrapure water) was stored at -20° and later lyophilized to concentrate particles for further studies. The particle concentrator is housed on the roof of a four story building on the University of Montana campus. Particles were harvested during the winter (February 2012), spring (May 2012), and summer months (July and August 2012). These collection periods yielded from 100 µg to about 1 mg of each PM type, depending on conditions.

### Ambient PM Levels in Missoula

Although PM<sub>c</sub> sampling is not currently conducted in Missoula, continuous PM<sub>2.5</sub> and PM<sub>10</sub> are routinely measured as part of Missoula's EPA compliance monitoring program. This program uses these continuous PM data to calculate 24-hour ambient PM averages, which are then reported. Using these continuous PM<sub>2.5</sub> and PM<sub>10</sub> data (collected with a MetOne BAM at the Missoula Boyd Park site), we were able to calculate, by difference, the seasonal ambient PM<sub>c</sub> mass concentrations during 2012 ( $[PM_{10}] - [PM_{2.5}] = [PM_c]$ ). We also calculated the 24-hour average mass concentrations on days when PM was collected with the particle concentrator and used in this work. The unique seasonal patterns in Missoula, MT are accounted for in this study, as high latitudes and high elevations stimulate longer cold seasons. For the purpose of this manuscript, winter months are categorized as the months of November, December, January, and February; spring are the months of March, April, and May; summer months are the months of June, July, and August; and autumn is September and October.

### Mice and Bone Marrow-Derived Macrophages

A major component of respiratory immunity is the pulmonary macrophage, which plays a key role in the regulation of inflammation as well as subsequent adaptive responses. Pulmonary macrophages are perpetual residents of the lung and act as sentinels or first responders for particle uptake. These macrophages have the necessary receptors for particle uptake/phagocytosis (Hirayama et al. 2011, Geiser 2010, Goodridge et al. 2012, Underhill and Goodridge 2012). Our laboratory has published multiple *in vivo* particulate exposure studies using the Balb/c mouse model (Migliaccio et al. 2005, Migliaccio et al. 2008, Lacher et al. 2010, Migliaccio et al. 2013). In addition to these studies, our laboratory has also utilized an *in vitro* macrophage system using bone marrow-derived macrophages (BMdMs) to evaluate the effects of particles on cell functions (Migliaccio et al. 2005, Migliaccio et al. 2008). Therefore, by exposing mouse BMdMs to PM, an inflammatory response can be measured.

A variety of studies have described the key role of the pulmonary macrophage in particle clearance (Geiser 2010), and its link between the innate and adaptive immune systems (Gordon 2003, Mosser 2003). Bone marrow-derived macrophages utilized in this study to evaluate the inflammatory response of relevant doses of different PM fractions (PM<sub>2.5</sub>, PM<sub>10</sub>, and PM<sub>c</sub>). Methods for BMdM cultures are previously described (Pfau et al. 2004, Migliaccio et al. 2008). Briefly, hind legs of euthanized Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were flushed with media (RPMI 1640 with 10% FBS) to release the

marrow. After overnight stromal elimination, cells were stimulated with M-CSF for an additional 7-10 days of culturing. On day 10, cells were aliquoted ( $10^5$  cells/well) in 96-well plates for culturing with particles. All animal procedures were approved by the University of Montana Institutional Animal Care and Use Committee (Missoula, MT, USA). Method of euthanasia was by lethal dose of sodium pentobarbital via interperitoneal injection.

### **PM<sub>c</sub> vs. PM<sub>2.5</sub> Comparison Studies**

During the summer (July, 2011) and winter (February, 2012) months, PM<sub>2.5</sub> and PM<sub>c</sub>, were simultaneously harvested in Missoula using our VACES-PC. Stock solutions of PM were made by diluting pre-determined masses of PM into a small volume of PBS (< 0.5 mL) followed by sonication. The volume of PBS necessary is dependent on the amount of PM harvested. Cells were incubated with particles for 1 hour (mixing) in 1.5 mL eppendorf tubes, then aliquoted to 96-well plates and cultured overnight. Final cultures contained 150  $\mu$ L per well of media, with each condition in triplicate exposures at 100 000 cells/well. A media negative control was also used that contained no particles. Particle exposure levels included varying masses of PM<sub>2.5</sub> or PM<sub>c</sub> at 5  $\mu$ g, 20  $\mu$ g, and 40  $\mu$ g/well. Collection of supernatants occurred after 24 hours.

### **PM Mixture Studies**

PM<sub>2.5</sub> and PM<sub>c</sub> used in the mixture exposures were harvested during May 2012. BMdMs were exposed in a similar fashion as the comparison studies described above, but at one level of exposure at 20  $\mu$ g/well. To represent the varying conditions of PM<sub>10</sub>, 20  $\mu$ g mixtures of PM<sub>2.5</sub> and PM<sub>c</sub> varied from 0%, 20%, 40%, 60%, 80%, and 100% PM<sub>c</sub> by mass. For these studies, 20  $\mu$ g was used, as it was the lowest mass of exposure that induced the most significant differences between PM size and season with both IL-1 $\beta$  and TNF- $\alpha$ . In similar studies using *in vivo* models, particulate exposures ranged from 5-50  $\mu$ g of particles per instillation (Porter et al. 2013, Stoeger et al. 2006). Considering that there are roughly 200-300 thousand alveolar macrophages in the mouse lung, if 50  $\mu$ g is distributed evenly, each cell will theoretically receive approximately 0.17-0.25 ng of particles. In our model using 20  $\mu$ g and 100 000 cells, if particles were distributed evenly, each cell would receive roughly 0.2 ng of particles. Therefore, the levels of particulates used in this research are representative of *in vivo* studies.

Collection of supernatants occurred after 24 hours of incubation. The resulting inflammatory cytokine levels were used to determine the additivity potential of the mixtures. To ensure that these particles were undergoing phagocytosis, bright field illumination microscopy of BMdMs following 24-hours of exposure to PM<sub>10</sub>, was performed (Nikon Eclipse E800, Tokyo, Japan; Nuance Multispectral Imaging System, Hopkinton, Massachusetts).

### **Endotoxin Analysis**

Levels of endotoxin were evaluated using methods adapted by Thorne (Thorne 2000). Briefly, the harvested PM samples were analyzed for endotoxin levels using a kinetic chromogenic *Limulus* amoebocyte lysate (LAL) assay (Charles River Endosafe® - Endochrome-K™, Charles River Laboratories, Charleston, SC, USA), where a 12-point standard curve ranged from 50 to 0.005 EU/ml. The absorbance was measured at 405 nm every 30 seconds for 90 minutes.

### **Mixture Effect Calculation**

Additivity was modeled using equation 1, a modified version of the toxic unit model (PapeLindstrom and Lydy 1997). Modification was necessary, as endpoints were not based on concentration/dose response thresholds, but were based on the resulting levels of the

expressed cytokines from 20  $\mu\text{g}$  of PM exposure. Where additivity is  $> 1$ ,  $= 1$ , or  $< 1$  the effect of the mixture is deemed synergistic, additive, or antagonistic, respectively. The fraction of  $\text{PM}_{2.5}$  or  $\text{PM}_c$  in  $\text{PM}_{10}$  is represented with  $f$  and can range anywhere from 0 to 1. For example, if  $\text{PM}_{10}$  contained 25%  $\text{PM}_{2.5}$  and 75%  $\text{PM}_c$ ,  $f$  would equal 0.25 and 0.75, respectively.

$$f_{\text{PM}_{2.5}} \frac{[\text{PM}_{10}]}{[\text{PM}_{2.5}]} + f_{\text{PM}_c} \frac{[\text{PM}_{10}]}{[\text{PM}_c]} = \text{additivity} \quad (1)$$

As the ratios of  $\text{PM}_{2.5}$  and  $\text{PM}_c$  change within total  $\text{PM}_{10}$ , a departure from an effect level of 1 explains whether a larger burden of one PM size fraction is either affecting a synergistic (additivity  $> 1$ ) or an antagonistic (additivity  $< 1$ ) effect. One other alternative is that the mixture will not cause a departure from 1, and that the increased presence of one or another PM type will result in an additive response.

### Cytokine and Statistical Analysis

Collection of pro-inflammatory cytokines was completed following the 24-hour exposure. The collected media/supernatant was then stored at  $-20^\circ\text{C}$  prior to analysis via enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). Each well's concentration was measured in duplicate. Levels of IL-1 $\beta$  and TNF- $\alpha$  released from BMDMs were then plotted using Prism, version 5.0a. Student t-tests were also performed in Prism to determine significant differences between two PM types within each mass of exposure (5  $\mu\text{g}$ , 20  $\mu\text{g}$ , and 40  $\mu\text{g}$ ).

## Results

### Ambient air data in Missoula, MT

Table 1 summarizes the average concentrations of  $\text{PM}_{2.5}$ ,  $\text{PM}_c$ , and  $\text{PM}_{10}$  measured throughout 2012, illustrating that  $\text{PM}_c$  is not only a substantial component of  $\text{PM}_{10}$  throughout the year, but also is present in elevated concentrations (compared to  $\text{PM}_{2.5}$ ) throughout all four seasons in this northern Rocky Mountain airshed. The data presented in Table 1 indicate that ambient PM concentrations ( $\text{PM}_{2.5}$ ,  $\text{PM}_c$ , and  $\text{PM}_{10}$ ) vary as a function of season. Throughout the year, seasonal  $\text{PM}_c$  concentrations measured in Missoula, MT were consistently higher than  $\text{PM}_{2.5}$ , even in winter when there were more combustion-related particles in the Missoula airshed due to residential wood stove use (Ward and Lange 2010). Unlike  $\text{PM}_{2.5}$ ,  $\text{PM}_c$  average concentrations were fairly consistent through winter and spring (9.3-9.6  $\mu\text{g}/\text{m}^3$ ), with a marked increase in  $\text{PM}_c$  from summer to fall.  $\text{PM}_{2.5}$  concentrations in fall were at least twice as high as all other seasons.

PM concentrations on days that ambient particles were harvested for the controlled laboratory exposure studies ( $\text{PM}_c$  vs.  $\text{PM}_{2.5}$  comparison and mixture studies) are presented in Table 2. During the days that PM was harvested during the winter months, the  $\text{PM}_c$  concentrations were more than twice as high compared to the winter-long average presented in Table 1, though  $\text{PM}_{2.5}$  concentrations were similar. The authors are unsure why  $\text{PM}_c$  was elevated on these sample days. When comparing  $\text{PM}_c$  concentrations measured during harvest days compared to overall summer averages, concentrations are comparable. The same is true for collected Spring PM. Also during these collection days (Table 2),  $\text{PM}_{2.5}$  concentrations were comparable to the winter, spring, and summer averages presented in Table 1.

## PM non-mixture exposures, in vitro

In side-by-side controlled exposures within the laboratory, winter PM<sub>C</sub> induced the highest levels of IL-1 $\beta$  and TNF- $\alpha$  at all three doses (Figure 1), supporting our hypothesis that PM<sub>C</sub> is more biologically active compared to PM<sub>2.5</sub>. Summer PM<sub>C</sub> was the second most bioactive PM, followed by summer PM<sub>2.5</sub> and winter PM<sub>2.5</sub>. In each season (winter and summer), the most bioactive size fraction of PM was PM<sub>C</sub>, with winter PM<sub>C</sub> inducing more than twice the levels of IL-1 $\beta$  than summer PM<sub>C</sub>. Similar results were observed for TNF- $\alpha$ . IL-1 $\beta$  increased in a linear fashion in response to both winter and summer PM<sub>C</sub>. However, IL-1 $\beta$  from winter and summer PM<sub>2.5</sub> increased logarithmically, and leveled out at around 25 pg/mL. This logarithmic growth was not observed for TNF- $\alpha$ , as all four PM types generated linear increases in cytokine expression.

The data on the significant differences between PM<sub>C</sub> and PM<sub>2.5</sub> are listed in Table 3. Two-tailed, student *t*-tests were used to determine if the two PM size fractions induced significantly different responses at a single dose. Both summer and winter PM<sub>C</sub> induced significantly higher IL-1 $\beta$  levels compared to their PM<sub>2.5</sub> counterparts (at 20 $\mu$ g, IL-1 $\beta$  between summer PM<sub>C</sub> v. PM<sub>2.5</sub> p=0.02, and winter PM<sub>C</sub> v. PM<sub>2.5</sub> p=0.03). TNF- $\alpha$  levels were only significantly different between PM<sub>C</sub> and PM<sub>2.5</sub> for winter, but not for summer (at 20 $\mu$ g, TNF- $\alpha$  between summer PM<sub>C</sub> v. PM<sub>2.5</sub> p=0.09, and winter PM<sub>C</sub> v. PM<sub>2.5</sub> p=0.0002). IL-1 $\beta$  levels from winter PM<sub>C</sub> were triple that of summer PM<sub>C</sub>. Differences in IL-1 $\beta$  levels were mostly non-significant when comparing low doses of PM exposure, except between both summer and winter PM<sub>2.5</sub> where there was no significant differences. These results suggest that PM<sub>C</sub> is an important mediator in inducing an inflammatory response.

## Cellular uptake of ambient PM<sub>10</sub>

Most of the particles used in these exposures were phagocytosed by the BMdMs, as the majority of the particles were intracellular (Figure 2). Using BFI microscopy, we determined that the cells exposed to PM<sub>10</sub> had phagocytosed the majority of the particles in solution. In other images where PM<sub>C</sub> and PM<sub>2.5</sub> burden in PM<sub>10</sub> varied, similar uptake was observed (images not shown). Particle size (both PM<sub>2.5</sub> and PM<sub>C</sub>) did not seem to play a major role in the uptake of these particles. Small particles and larger sized particulates, such as the fungi, *Torula herbarum* (Figure 2), with diameters of approximately 3-4  $\mu$ m, appear to be equally phagocytosed. This confirms that both PM<sub>C</sub> and PM<sub>2.5</sub> are being taken up by the BMdMs.

## PM endotoxin burden

Endotoxin levels were determined as a function of PM mass (Figure 3). Results show that overall endotoxin concentrations were highest during the summer months compared to the winter months for both fractions. As predicted, there was more endotoxin in the winter PM<sub>C</sub> compared to the winter PM<sub>2.5</sub>. Endotoxin concentrations during the summer were comparable between the two size fractions. These results suggest that endotoxin concentrations composing ambient PM can vary as a function of PM size and season, and not primarily found in the coarse fraction (compared to the fine fraction).

## Mixture effects

As noted earlier, PM<sub>2.5</sub> and PM<sub>C</sub> used in the mixture exposures were harvested during May 2012. Results from the mixture studies show that varying the combinations of PM<sub>2.5</sub> and PM<sub>C</sub> in PM<sub>10</sub> influence cytokine output, with Figure 4 illustrating the effect of PM<sub>C</sub> in a PM<sub>10</sub> mixture. All cytokine concentrations (i.e. [IL-1 $\beta$ ] and [TNF- $\alpha$ ]) varied as a function of exposure to 20  $\mu$ g of each PM type (PM<sub>10</sub>, PM<sub>2.5</sub>, or PM<sub>C</sub>). Shown in Figure 4, as PM<sub>10</sub> gains a greater proportion of PM<sub>C</sub>, a synergistic release of IL-1 $\beta$  and TNF- $\alpha$  occurs. Conversely, as the fraction of PM<sub>10</sub> contains more PM<sub>2.5</sub> than PM<sub>C</sub>, antagonistic effects are

observed. These results support our hypothesis that the  $PM_c$  fraction is an important mediator in inducing an inflammatory response with  $PM_{10}$  exposure.

## Discussion

Understanding the mass ratio mixtures of ambient  $PM_{2.5}$  and  $PM_c$  composing  $PM_{10}$  can help determine additivity of the inflammatory response. As shown in our controlled laboratory studies,  $PM_c$  was constantly more bioactive than  $PM_{2.5}$  throughout each side-by-side exposure in comparing both summer and winter months, and with its increased burden in  $PM_{10}$ . We were successful in showing that there are certainly additive effects from these different mass ratio exposures. Furthermore, our results suggest that the outcome from exposure to  $PM_{10}$  can be partly understood as a function of  $PM_{2.5}$  and  $PM_c$  burden in  $PM_{10}$ .

A source apportionment study has yet to be completed for  $PM_c$  in Missoula, MT. However,  $PM_{2.5}$  source apportionment modeling has shown that the largest  $PM_{2.5}$  source throughout the year is wood combustion, contributing an average of 41% to the fine fraction. This includes residential wood combustion during the winter, slash and prescribed burns from the surrounding areas during the spring and fall, and smoke from wildfires during the summer months. Other sources of  $PM_{2.5}$  throughout the year include diesel exhaust (19%), secondary ammonium nitrate (17%), local industry (20%), and street sand (5%) (Ward and Smith 2005, Ward and Lange 2010). Though these studies did not focus specifically on apportioning the sources of  $PM_c$ , they do provide an idea of the types of exposure conditions that exist throughout the year in Missoula, MT.

The present study is the first attempt at determining ambient concentrations of  $PM_c$  in the state of Montana within the last 15 years. As illustrated in Table 1, ambient concentrations of  $PM_c$  were consistently greater than  $PM_{2.5}$  throughout the year, with the exception of the winter months where the average  $PM_c$  and  $PM_{2.5}$  concentrations were not significantly different. It should be noted, however, that harvested particles used in our laboratory studies were collected on days when the average  $PM_c$  mass concentrations were about 2 - 10  $\mu\text{g}/\text{m}^3$  greater than  $PM_{2.5}$ . In addition, particles were harvested during sample days in which there was higher winter  $PM_c$  concentrations ( $22.6 \pm 9.2 \mu\text{g}/\text{m}^3$ ) compared to the winter 2012 average  $PM_c$  concentrations presented in Table 1 ( $9.3 \pm 7.7 \mu\text{g}/\text{m}^3$ ).  $PM_{2.5}$  concentrations (both throughout the winter of 2012 and during the dates of sample collection) were consistent ( $9.1 \pm 4.9 \mu\text{g}/\text{m}^3$  vs.  $12.3 \pm 3.0 \mu\text{g}/\text{m}^3$ ). During the  *$PM_c$  vs.  $PM_{2.5}$  Comparison studies*, particle exposure levels ( $PM_{2.5}$  and  $PM_c$ ) were normalized at 5  $\mu\text{g}$ , 20  $\mu\text{g}$ , and 40  $\mu\text{g}/\text{well}$  in the 96-well plates. Therefore, it is unknown how the overall ambient concentrations during the days of particle harvesting influenced the results of the comparison studies (if at all). Additional studies within the laboratory across multiple seasons/years will be able to address this issue.

As evidenced in Figure 3, the differences in endotoxin content on the PM cannot explain the differences in the resulting IL-1 $\beta$  expression. Something other than endotoxin is potentially stimulating the production of pro-IL-1 $\beta$  upon exposure to  $PM_c$ , as the levels of IL-1 $\beta$  do not seem to be limited with an increase in  $PM_c$  exposure. In addition, increasing concentrations of  $PM_{2.5}$  did not induce a linear increase in IL-1 $\beta$  expression (Figure 1). Further studies can evaluate the levels of pro-IL-1 $\beta$  in cells, via western blot, to determine if a decreased IL-1 $\beta$  release is due to a lowered level of expression of the pro form of IL-1 $\beta$  or if the inflammasome is simply not active. A large pool of pro-IL-1 $\beta$  remaining in the cells would suggest that there is likely a decrease in inflammasome activation, which catalyzes the maturation of pro-IL-1 $\beta$  to its active IL-1 $\beta$  form (Tschopp 2008).



Figure 1 supports the hypothesis that ambient  $PM_c$  is more bioactive than  $PM_{2.5}$ . However, these exposures were done on a side-by-side comparison using controlled laboratory conditions, and did not take into account that real-world exposures to these two size fractions occur concomitantly. The mixture exposures take into account possible mixing conditions of  $PM_{2.5}$  and  $PM_c$  in  $PM_{10}$ . The results of the *in vitro* mixture exposures showed that  $PM_{2.5}$  is not as potent an activator of the inflammasome as  $PM_c$ , even in the presence of another PM type. In order to stimulate these levels of IL-1 $\beta$  production, the inflammasome complex must be activated by a receptor (e.g. binding of receptor to PAMP) or lysosomal stress (e.g. the release of cathepsin-B) (Tschopp 2008, Yazdi et al. 2010). Potentially, there are components in  $PM_c$  that are responsible for priming the formation of the inflammasome, such as endotoxin, an activator of the NF- $\kappa$ B pathway via TLR4, and another component affecting lysosomal membrane integrity. NF- $\kappa$ B activation is a precursor to transcription induction of pro-IL-1 $\beta$  (Yazdi et al. 2010, De Nardo and Latz 2011). Activation of the TLR or priming of the NF- $\kappa$ B pathway has been shown to stimulate the production of pro-IL-1 $\beta$  and further production of NLRP3 inflammasome precursors (De Nardo and Latz 2011). It is likely that not only is the inflammasome being formed, but also more pro-IL-1 $\beta$  is being produced following  $PM_c$  exposure. Also, reactive oxygen species (ROS) may play a significant role in inflammation as generation of ROS from particulates has been shown to exacerbate the inflammatory response (Fubini and Hubbard 2003). Furthermore, exposure to biogenic substances, such as pollen, largely found in  $PM_c$ , can increase ROS in the lung (Boldogh et al. 2005).

## Conclusion

In this study, we utilized harvested ambient particles and biological *in vitro* models to investigate potential adverse health effects following exposures to different size fractions found in the Rocky Mountains in North America. The results of these studies conducted under controlled laboratory conditions suggest that there are properties (chemical, physical, or biological) of  $PM_c$  that are responsible for elevated levels of inflammation activity compared to ambient  $PM_{2.5}$ . An important step to further this research would be to investigate  $PM_c$  harvested throughout the year. For example, winter  $PM_c$  contained about two-thirds the endotoxin burden as compared to summer  $PM_c$ , yet winter  $PM_c$  induced more than twice the levels of IL-1  $\beta$  than summer  $PM_c$ . Other methods of *in vitro* exposure could also be employed, such as an air-liquid interface model. Also, an alternative method of collecting particles that does not include the removal of water (via lyophilization) could be explored to minimize the loss of volatile and semi-volatile compounds. Such methods could include employing the use of a cyclone or another dry method of harvesting ambient air particles.

The mixture results presented here suggest that if the percentage of  $PM_c$  is known, the relative bioactivity of the ambient  $PM_{10}$  might be predicted. This study supports our hypothesis that  $PM_c$  adversely affects macrophage activity with increasing ratios of  $PM_c:PM_{2.5}$  in  $PM_{10}$ . It is also important to note that there are currently no National Ambient Air Quality Standards (NAAQS) for the coarse fraction, though our results suggest that a new  $PM_c$  ambient standard should perhaps be considered in addition to the existing daily and annual  $PM_{2.5}$  NAAQS. Including additional chemical and biogenic characterizations will facilitate our future source apportionment studies for different PM fractions. Also, evaluation of reactive oxygen species activity in both  $PM_c$  and  $PM_{2.5}$  should be considered in future research. Further seasonal and dose-response relationship studies will aid in determining real-world mixture effects, as well as provide an understanding of the causal health effects from exposure to  $PM_c$ .

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### Declaration of Interest

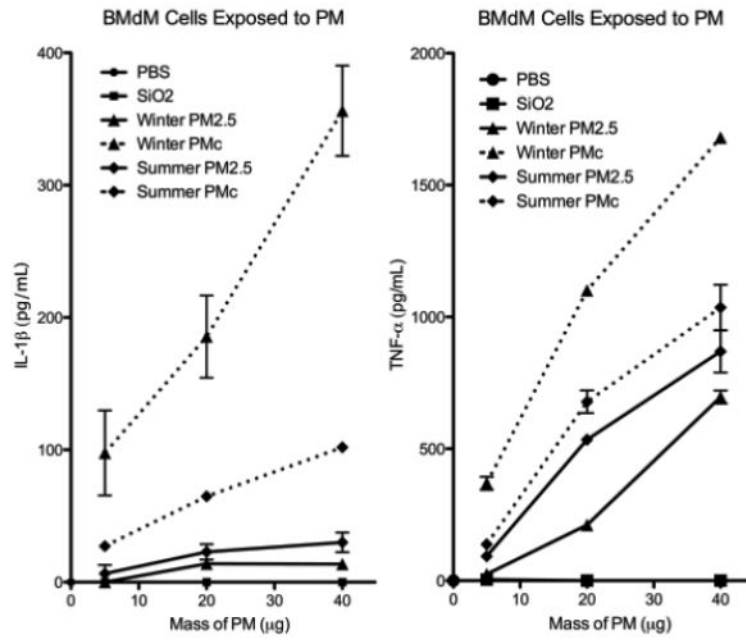
The authors have no financial or other conflicts of interest.

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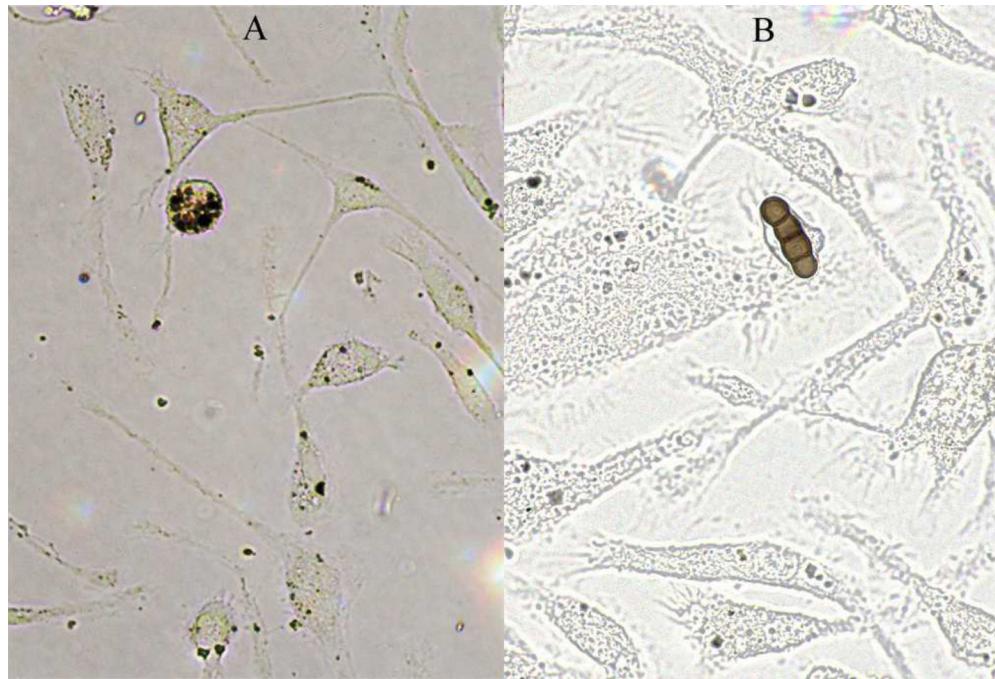
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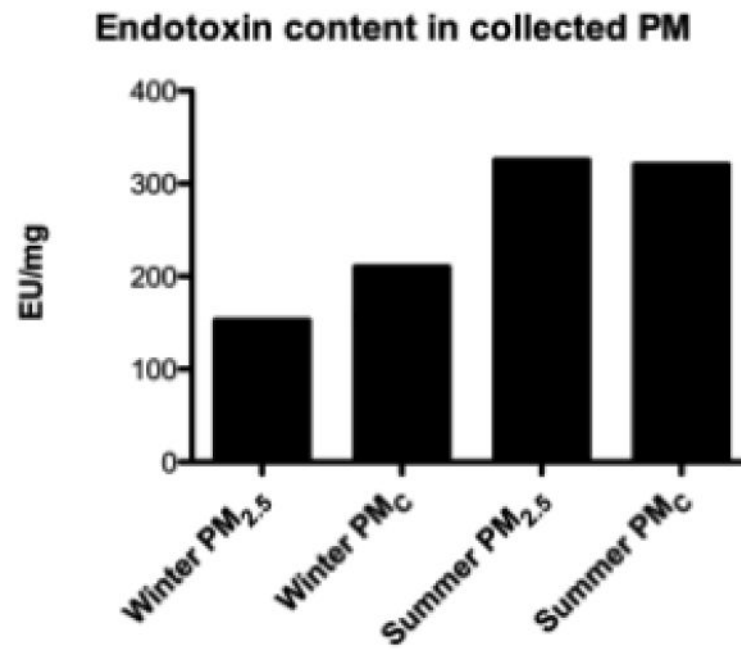
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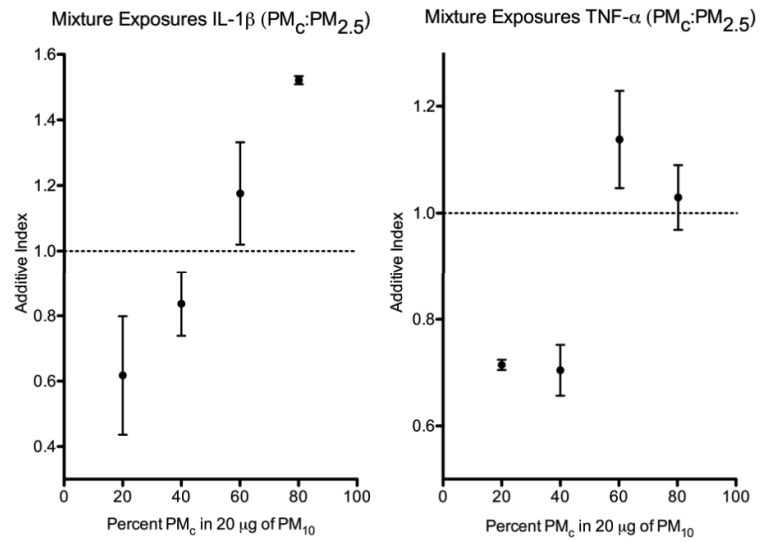
**Figure 1.** Levels of cytokine production by mass of PM added per well (containing 100 000 BMdMs). The left and right graphs are IL-1 $\beta$  and TNF- $\alpha$  levels, respectively, in response to PM and controls.



**Figure 2.** Cellular uptake of ambient PM<sub>10</sub>. (A) 40X magnification of BMdMs containing PM<sub>10</sub>, (B) Oil immersion 100X magnification showing fungi (spp. *Torula herbarum*) inside macrophage.



**Figure 3.**  
PM Endotoxin content from winter and summer collected ambient particles.



**Figure 4.** Additivity values from different ratios of spring PM<sub>2.5</sub> and PM<sub>c</sub> mixtures. The left and right graphs are additivity values calculated from IL- 1 β and TNF- α levels, respectively.



**Table 1**

Average and  $\pm$  standard deviation of ambient PM concentrations ( $\mu\text{g}/\text{m}^3$ ) in 2012, Missoula, MT.

	<b>Winter</b>	<b>Spring</b>	<b>Summer</b>	<b>Fall</b>
	<b>Nov-Feb</b>	<b>Mar-May</b>	<b>June-Aug</b>	<b>Sept-Oct</b>
<b>PM<sub>C</sub></b>	9.3 $\pm$ 7.7	9.6 $\pm$ 7.7	13.8 $\pm$ 10.7	25.0 $\pm$ 30.4
<b>PM<sub>2.5</sub></b>	9.1 $\pm$ 4.9	5.9 $\pm$ 2.6	7.8 $\pm$ 6.4	18.1 $\pm$ 18.5
<b>PM<sub>10</sub></b>	18.4 $\pm$ 10.0	15.5 $\pm$ 9.8	21.6 $\pm$ 12.4	43.1 $\pm$ 37.0

**Table 2**

Average and  $\pm$  standard deviation concentrations ( $\mu\text{g}/\text{m}^3$ ) during winter and summer days when ambient particles were harvested.

	<b>Winter</b>	<b>Spring</b>	<b>Summer</b>
	<b>Feb, 2012</b>	<b>May, 2012</b>	<b>July, 2011</b>
<b>PM<sub>C</sub></b>	22.6 $\pm$ 9.2	7.4 $\pm$ 1.9	9.7 $\pm$ 4.4
<b>PM<sub>2.5</sub></b>	12.3 $\pm$ 3.0	5.3 $\pm$ 1.3	7.4 $\pm$ 2.1
<b>PM<sub>10</sub></b>	34.9 $\pm$ 8.5	12.7 $\pm$ 3.2	17.1 $\pm$ 6.5

**Table 3**

P-values comparing cytokine production of IL-1 $\beta$  or TNF- $\alpha$  between two PM exposure types at 5,20, or 40  $\mu$ g.

	<b>Winter PM<sub>2.5</sub></b>	<b>Winter PM<sub>c</sub></b>	<b>Summer PM<sub>2.5</sub></b>	<b>Summer PM<sub>c</sub></b>
Winter PM <sub>2.5</sub>	-----	<i>*</i> , 0.03, 0.01	<i>*</i> , 0.27, 0.16	<i>*</i> , 0.001, 0.003
Winter PM <sub>c</sub>	<i>0.005, 0.0002, 0.0007</i>	-----	0.11, 0.04, 0.01	0.16, 0.06, 0.02
Summer PM <sub>2.5</sub>	<i>0.009, 0.002, 0.17</i>	<i>0.008, 0.001, 0.001</i>	-----	0.10, 0.02, 0.01
Summer PM <sub>c</sub>	<i>0.008, 0.008, 0.06</i>	<i>0.01, 0.01, 0.02</i>	<i>0.06, 0.09, 0.29</i>	-----

Each of the grouped (three) reported p-values from left to right are from 5, 20, and 40  $\mu$ g exposures, respectively. *Italicized* = TNF- $\alpha$ , normal type = IL-1 $\beta$ .

*\** = cytokines not detected, cannot compare. Null hypothesis = Cytokines levels are the same between exposure treatments.