Notes to Editor:

1. We have highlighted in red all of the changes made for the final submission throughout the main text. These include the changes suggested by the Reviewers.
2. Below we provide a point-by-point response to the Editor and the reviewers.

Anonymous Referee #1

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The health effects of ambient particulate matter, including SOA components from natural source, is an important scientific concern. Focusing on this issue, this study examined the toxicity of isoprene-derived SOA (generated in an outdoor chamber) on the expression of two inflammation associated genes with an in vitro model of human lung cell line. A novel direct deposition exposure method was applied, and the result was verified with a classical method of resuspended particle exposure. In general, this study was well designed (mainly for the chamber experiment) and has certain scientific significance, therefore it could be considered by the journal of ACP.

A major suggestion is on the discussion section. Obviously, the discussion section was neither in-depth nor penetrating enough, especially for the subsection of "biological implications". In this subsection, it provided only some comparison between this study and others. There is no further discussion on the mechanism between PM exposure and the expression of two inflammation genes, nor any discussion between specific SOA components and gene expression. In addition, why chose mRNA instead of inflammatory factors as the indicator of effects? Increase of gene expression (i.e., mRNA) doesn't always suggest the enhancement of corresponding functional proteins.

Some further discussion about mechanism between exposure and expression of the two inflammation genes has been added on page 17-19, lines 389-423 as follows:

"IL-8 and COX-2 are both linked to inflammation and oxidative stress (Kunkel et al., 1991; Uchida, 2008). IL-8 is a potent neutrophil chemotactic factor in the lung and its expression by various cells plays a crucial role in neutrophil recruitment leading to lung inflammation (Kunkel et al., 1991). COX-2 is the inducible form of the cyclooxygenase enzyme, regulated by cytokines and mitogens, and is responsible for prostaglandin synthesis associated with inflammation (FitzGerald, 2003). Consistent with the reports that IL-8 and COX-2 play important roles in lung inflammation (Li et al., 2013; Nocker et al., 1996), in vivo studies have shown that isoprene oxidation products cause airflow limitation and sensory irritation in mice (Rohr et al., 2003). In humans, the role of IL-8 and COX-2 in lung inflammation can be associated with diseases such as chronic obstructive pulmonary disease and asthma (Fong et al., 2000; Nocker et al., 1996; Peng et al., 2008).

The mechanism by which isoprene-SOA causes elevation of the inflammatory markers IL-8 and COX-2 is not yet fully understood. However, recent work from our laboratory using the acellular dithiothreitol (DTT) assay demonstrated that isoprene-derived SOA have equal or greater ROS generation potential than diesel exhaust PM (Kramer et al., 2016; Rattanavaraha et al., 2011). High levels of ROS in cells can overwhelm the antioxidant defense and lead to cellular oxidative stress (Bowler and Crapo, 2002; Li et al., 2003; Sies, 1991). Following the discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016) showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative stress-associated genes, including COX-2 in human lung cells. Oxidative stress caused by ROS plays a major role in lung inflammation and the induction of oxidative stress can lead to

IL-8 expression (Tao et al., 2003; Yan et al., 2015). Pathway analysis showed that gene expression of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was induced in cells exposed to isoprene-SOA (Lin et al., 2016) which has been reported to alter the expression of IL-8 through mRNA stabilization (Zhang et al., 2005). Therefore, isoprene-SOA may cause increases in both IL-8 and COX-2 primarily through an oxidative stress response. Additionally, the relationship between IL-8 and COX-2 can also explain the observed increase in IL-8 gene expression as the production of IL-8 can be stimulated through a COX-2 dependent mechanism in airway epithelial cells (Peng et al., 2008).

In vitro studies such as this one using a direct deposition model cannot fully elucidate mechanisms of lung inflammation and potential pathogenesis but serve as a necessary part of hazard characterization, particularly for a complex air mixture that has not been fully studied (Hayashi, 2005; Paur et al., 2011). Therefore, further in vitro studies exploring the health implication of the elevation of IL-8 and COX-2 due specifically to isoprene-SOA exposure are necessary and may in turn justify further extension to in vivo work."

The following are some specific comments:

Line 82: This abbreviation should be "VOCs".

This has been corrected in the text on page 4, line 82.

Lines 99-102: The reason for the selection of these two genes was too simple. Suggest the authors to provide some molecular mechanisms between these two genes and oxidative stress and inflammation. Furthermore, this information could also be discussed in the section of results and discussion.

We thank the reviewer for the comment. We believe investigating more genes and molecular mechanisms are the next steps in examining the biological effects of isoprene-SOA. The purpose of this particular study was to serve as an initial step in a long planned analysis of the biological impacts of SOA exposure on lung cells and we anticipate that work on health implications of isoprene-SOA exposure will continue through further *in vitro* and *in vivo* studies. We included statements about the role of *IL-8* and *COX-2* in inflammation and diseases such as chronic obstructive pulmonary disease and asthma but did not want to make overreaching statements about what the elevations of *IL-8* and *COX-2* from our study mean in terms of human health. However, we have included further discussion about the link between the two genes investigated and oxidative stress and inflammation on page 17-19, lines 389-423 as stated in a previous comment.

Additionally, we included citations to other air pollution mixture studies that used *IL-8* as the only gene expression biomarker on page 5, lines 107-110:

"Other studies on air pollution mixtures have also examined IL-8 as a biological endpoint due to its involvement with inflammation (Doyle et al., 2004; Doyle et al., 2007; Ebersviller et al., 2012a, b; Zavala et al., 2014)."

Lines 121 and 129: Many factor could influence the photochemical reactions, for example, temperature. What's the temperature (or range) of these sunny days?

The temperature ranged from 24.9°*C* to 26.8°*C* at time of isoprene injection on the sunny days. Particles from all experiments were collected onto filters and analyzed to ensure all the same isoprene SOA tracers were measured in all experiments

We have added on page 6, lines 135-137 the temperature range for these photochemical experiments as follows:

"This chamber experiment was replicated on three separate sunny days with temperatures ranging from 24.9°C to 26.8°C with a relative humidity of approximately 70% in the chamber."

Lines 134 and 135: Could NO3 radical trigger the formation of SOA at nighttime? Moreover, this statement sounds too assertive, and how about the temperature of the chamber? There must be some difference between nighttime and daytime.

If any NO_3 radicals were present, they were likely present at very low mixing ratios as we did not observe any organic particle growth during our nighttime experiments. Additionally, the GC/MS and UPLC/ESI-HR-QTOFMS showed that there were no measurable isoprene-derived SOA tracers (or any other OA species) in the collected filters as shown in Figure 2. We have clarified this in the text on page 13 line 300 as follows:

"No isoprene-SOA tracers were observed in the filters collected from dark control experiments."

The reviewer is correct that there are differences in nighttime and daytime temperatures. There were many variables to control when exposing cells using a direct deposition device paired with an atmospheric chamber. To control the temperature issue, the EAVES and incoming air lines were housed in an incubator kept at 37°C as stated in the text on page 8 line 171.

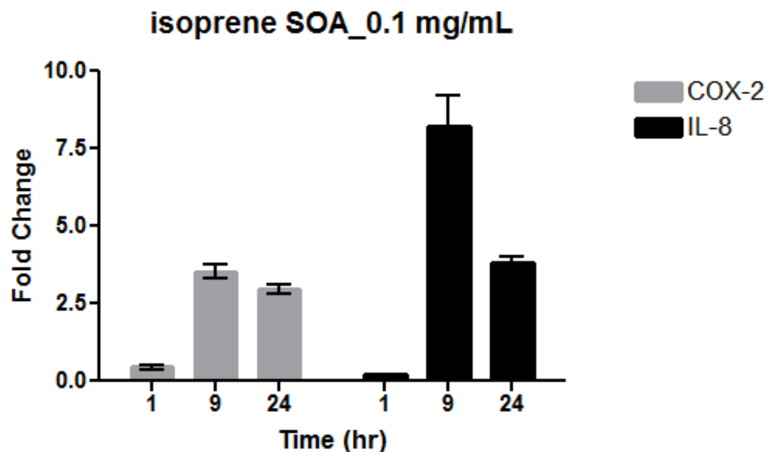
Line 151: There is the symbol of "-" between number and unit. Please unify this expression in lines 174 and 201.

This has been corrected in the text on page 7, lines 160. Also the expressions in line 174 and 201 have been unified and line 201 has been revised to the following on page 9, lines 205-207:

"Following a 9-hour exposure, extracellular medium was collected and total RNA was isolated using Trizol (Life Technologies) and stored alongside samples from direct deposition exposures until further analysis."

Line 169: Why choose nine hours as exposure time? Was there any temporal variation during the nine hours?

For the purpose of this study, nine hour post-collection time point was chosen to be consistent with Lichtveld et al. (2012) who used the same exposure device on this outdoor chamber facility. There will be changes to the levels of mRNA expressed at any given point of collection and gene expression does have a time profile. Because of the nature of these chamber exposures, one post-collection time point was chosen and the isoprene-SOA exposures were compared to the dark control exposure for that specific time. However, a time course analysis was conducted using resuspension techniques to show that *COX-2* and *IL-8* are maximized at 9 hours as shown in the graph below.



Line 171: Was there any preliminary experiment to show this storage did not change the extracted mRNA?

We have assessed the integrity of extracted RNA samples after 12 months of storage at -80°C using Nanodrop and Bioanalyzer. We did not observe changes in RNA quality and concentration.

To address this question, we have added this information on page 8, lines 181-183, as follows:

"For quality assurance purposes, the RNA concentration and integrity were assessed using Nanodrop and Bioanalyzer over the period of storage. No changes were observed under the given storage conditions."

Line 176: Typo of "resupsension".

This has been corrected in the text on page 8, lines 187.

Lines 197 236: There are two subsection numbers of "2.6".

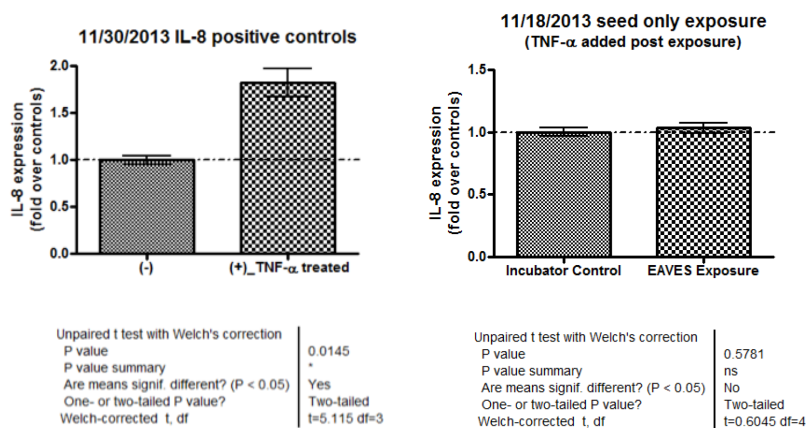
We have corrected the subsection numbers as the Reviewer pointed out.

Line 247: Why not measure the inflammatory factors release in the cell culture medium to verify the changes of mRNA?

We agree that measuring protein release in the cell culture medium to verify the changes of mRNA would be ideal. However, with the direct deposition exposure method, the particles deposit directly onto the cells and interfere with cytokine measurements as found in Seagraves (2008) so measurements of mRNA was chosen over measurement of inflammatory factors. This information has been added on page 12 line 258-263 as follows:

"We chose to measure the levels of the inflammation-related mRNA in the BEAS-2B cells exposed to isoprene-derived SOA generated in our outdoor chamber because various particle types are capable of sequestering cytokines (Seagrave, 2008). Other direct deposition studies have also used mRNA transcripts as a proxy for cytokine production (Hawley et al., 2014a; Hawley et al., 2014b; Hawley and Volckens, 2013; Volckens et al., 2009; Lichtveld et al., 2012)."

To verify if our particles of interest also interfered with cytokine measurements, we first confirmed through an ELISA assay that our cells were capable of releasing IL-8 by spiking them with TNF-α as shown in the left graph below. We then exposed cells using the EAVES to our acidified sulfate seed aerosol and spiked them with TNF-α post-exposure and found that no IL-8 could be measured through the ELISA assay as shown in the right graph below. This information supported our decision to measure mRNA levels as a proxy for cytokine production.



Line 258: Please define the abbreviation of SEM here.

SEM has now been defined: standard error of the mean (SEM) in the text on page 12, line 275.

Lines 290 to 292: Were there any particular data to support this statement?

Filters collected in Yorkville, GA were analyzed using GC/EI-MS and UPLC/ESI-HR-QTOFMS, as shown in Figure 2. Standards were used to identify the isoprene-derived SOA markers. The chamber-generated SOA were collected onto filters and analyzed in the exact same manner and the same pattern of isoprene-derived SOA markers were identified as shown in Figure 2.

We have revised this sentence on page 14, lines 309-312, as follows:

"As demonstrated in Figure 2, all the same particle-phase products are measured in the $PM_{2.5}$ sample collected in Yorkville, GA (a typical low-NO region), demonstrating that the composition of the chamber-generated SOA is atmospherically relevant"