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# From the Field to the Laboratory: Air Pollutant-Induced Genomic Effects in Lung Cells



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## Supplementary Issue: Ambient Air Quality (B)

**ABSTRACT:** Current *in vitro* studies do not typically assess cellular impacts in relation to real-world atmospheric mixtures of gases. In this study, we set out to examine the feasibility of measuring biological responses at the level of gene expression in human lung cells upon direct exposures to air in the field. This study describes the successful deployment of lung cells in the heavily industrialized Houston Ship Channel. By examining messenger RNA (mRNA) levels from exposed lung cells, we identified changes in genes that play a role as inflammatory responders in the cell. The results show anticipated responses from negative and positive controls, confirming the integrity of the experimental protocol and the successful deployment of the *in vitro* instrument. Furthermore, exposures to ambient conditions displayed robust changes in gene expression. These results demonstrate a methodology that can produce gas-phase toxicity data in the field.

**KEYWORDS:** *in vitro*, air pollutants, field campaign, gene expressions, hazardous air pollutants, epithelial lung cell, air-liquid interface

**SUPPLEMENT:** Ambient Air Quality (B)

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## Introduction

Current *in vitro* studies do not typically assess cellular impacts in relation to real-world atmospheric mixture of gases. This atmospheric mixture may include some of the 187 federally regulated toxic compounds classified as hazardous air pollutants (HAPs).<sup>1,2</sup> Among these HAPs, the largest ambient exposures are from benzene, formaldehyde, and toluene.<sup>3–5</sup> These three HAPs have been associated with cancer risk, respiratory disease, and numerous other adverse health effects.<sup>4,6,7</sup> Once in the atmosphere, HAPs undergo oxidation that transforms them into different gas species. For example, due to atmospheric oxidation, the average lifetime of toluene in the atmosphere is ~26 hours before it is transformed into an array of secondary products.<sup>8</sup> Thus, understanding the health risks of real-world exposures requires exposure studies on mixtures of fresh and oxidized gases.

All gas pollutants when emitted into the atmosphere are transformed into new secondary pollutants. These secondary pollutants have been shown to increase *in vitro* cellular responses in epithelial lung cells when compared to freshly emitted pollutants.<sup>9–17</sup> These laboratory-based studies relied on

an *in vitro* technology called the Gas *In Vitro* Exposure System (GIVES) that exposed human cultured epithelial lung cells at the air-liquid interface (ALI).<sup>9–17</sup> Data from the GIVES showed that secondary pollutants from photochemical transformations caused a 22-fold increase in the expression of interleukin 8 (IL-8) messenger RNA (mRNA) relative to control.<sup>9,10</sup> Furthermore, a genome-wide transcriptional assay found that photochemically altered air pollutants induce a robust transcriptional response in lung cells.<sup>13</sup> This included the alteration of the expression of genes that play a role in the inflammatory response and genes that are involved in cell cycle control. While data from these *in vitro* laboratory-based toxicological studies have shown the importance of photochemistry, there have been no studies conducted in the field to confirm these findings.

Exposures to ambient air are needed to confirm results observed in the laboratory and also to guide new experiments. Bringing an *in vitro* instrument into the field introduces several challenges. First, there is the difficulty in safely transporting biological samples to the field site. Second, the field site must be located near a suitable laboratory with appropriate tissue



culture equipment. Finally, sufficient baseline and field data must be provided to convince the scientific community that results are trustworthy.

In this article, we describe the successful deployment of the GIVES in the field, used to expose human lung cells to ambient air found near industrial facilities. These field exposures were part of the February 2015 Benzene and other Toxics Exposure (BEE-TEX) study campaign that focused on pollution sources from the Houston Ship Channel. The GIVES deployment generated the necessary baseline data from negative and positive controls to ensure the quality of results generated in the field. This study demonstrated a real-world test of experimental protocols and exposure technology and generated the data needed to justify further analyses and future deployments.

## Methods

**Cell culture.** The human epithelial lung A549 cell line was used for the field deployment.<sup>18</sup> The A549 cell line is a human pulmonary type II epithelial-like cell line derived from human alveolar cell carcinoma of the lung. The A549 cells are reproducible, culture well on membranes, and provide a robust genomic signal measured in previous laboratory-based pollutant exposures. Therefore, these cells were ideally suited for this study as it allows for a reliable replication of prior experiments. A549 cells were shipped to Baylor College of Medicine (BCM) in Houston, TX. Cells were thawed and transferred into a T75 flask with 20 mL of culture medium (F-12K, fetal bovine serum, and penicillin/streptomycin) and cultured at 37 °C under 5% CO<sub>2</sub>. The cells were between 73 and 78 passages for the experiments described. For all exposure experiments, 1.2 mL of Bovine Serum Albumin (BSA) starvation medium was used under the basolateral surface of the cells. At confluence (80%), the cells were split into a new T75 flask at a density of  $1.8 \times 10^6$  cells/mL.

For all exposures, a six-well (800 mL) cell culture plate (CORNING, Life Sciences) was used. Cells were plated onto Millicell cell culture inserts with a 30 mm diameter, 5 mm wall height, and a Polytetrafluoroethylene (PTFE) membrane with 0.4 µm pore diameter (PICM0RG50, EMD Millipore Corporation). The Millicell inserts were placed into the plate wells, which allowed the medium to maintain the culture from beneath while the cells were exposed directly to air above the membrane maintaining the ALI. At least 24 hours prior to exposures,  $8.5 \times 10^5$  cells/mL were placed in each well of the culture plate. Four hours prior to exposure, the culture medium was replaced with the starving medium (F-12K, BSA, and penicillin/streptomycin) and then transported from the BCM laboratory to the field site using an insulated cooler to minimize temperature variation and a bubble balance to maintain level. For all exposures, two identical six-well cell culture plates were used. One plate was placed in an incubator and exposed to clean air, while the second plate was placed in the GIVES for exposures to clean air, ozone, or the ambient air. All exposures with the GIVES were reported in comparison with the separate matching set of lung cells retained in the incubator.

**Air pollutant exposure site, equipment, and protocol.** The exposure equipment needed for the field exposures consisted of: GIVES instrument, humidification system, positive/negative control generators, CO<sub>2</sub> supply, and a temperature-controlled incubator. This equipment was housed in a secure temperature-controlled portable field laboratory building bought for the BEE-TEX study, which was situated at 9700 Manchester Street (29°43'00.18"N, 95°15'21.83"W). To the north and northeast of the field site is the Valero Houston petroleum refinery; 450 m to the northeast of the field site is the Buffalo Bayou Park channel, and 545 m to the southeast of the field site is the Sims Bayou channel. Additional air pollution sources include the 610 East Loop Freeway 1.2 km to the east of the field site and a major railway connection 600 m from the field site.

At least four hours prior to exposure, the GIVES instrument was cleaned with ethanol, and the heating system was initiated until temperatures stabilized at 37 °C. At this time, the humidification system was also started and monitored until relative humidity was in the range of 60–90%. A flow rate of 1 L/minute was verified using a Gilibrator flowmeter, and CO<sub>2</sub> was also supplied to the instrument at 0.05 L/minute. We have demonstrated in our previous experiments that the GIVES had no biological response in expression of IL-8 mRNA relative to control when sampling clean air with particles.<sup>9</sup> Thus, a filter was not used to remove particles from the sample line. After exposures, the plates of A549 cells from both the *in vitro* instrument and the incubator were covered and placed in an insulated box and transported immediately back to the BCM laboratory incubator. Since the cells in the GIVES were exposed to an unknown mixture of pollutants, the optimal post-exposure time to lyse the cells could not be determined. We have also demonstrated in our previous experiments that a post-exposure time of 9–24 hours was sufficient to produce a genomic response.<sup>13</sup> Thus, with 24 hours as an upper limit, a 16-hour post-exposure time was used to facilitate logistical demands. After 16 hours, A549 cells from both plates were collected in TRIzol, and the supernatant was collected separately. Cell exposure samples were frozen at –20 °C during the field campaign and then driven back to University of North Carolina – Chapel Hill (UNC) on dry ice (–80 °C). Once the cells arrived at UNC, they were immediately stored at –80 °C in a laboratory freezer.

**Cytotoxicity measurements.** The cellular supernatant was analyzed for the enzyme lactate dehydrogenase (LDH) using a Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific). Lung cells in the incubator and also from the GIVES were analyzed in triplicate and scanned for measuring the absorbance at 492 and 690 nm wavelengths using Thermo/LabSystems 352 Multiskan MS Microplate Reader. The outliers were identified as those with <5% probability of occurrence relative to a normal distribution.<sup>19</sup> LDH data were determined for each of the six wells in the exposed plate from GIVES and for the six wells in the plate in the incubator exposed to clean air. Fold change in LDH was calculated by

dividing the six well mean values of the exposed samples in the GIVES by the six well mean values of the samples from the incubator. Percent death was calculated by normalizing to 100% cytotoxic conditions of a 7.5-fold change.<sup>20</sup> Data were analyzed using an unpaired Student's *t*-test where differences were considered statistically significant if  $P \leq 0.05$ .

**RNA extraction.** Total RNA, including small RNAs with  $\geq 18$  nucleotides, was extracted from exposed cells using a modified QIAGEN's miRNeasy protocol. Briefly, the cells stored in TRIzol were allowed to thaw at room temperature and were homogenized using a QIAshredder. The homogenate was incubated at room temperature for five minutes, after which 200  $\mu$ L of chloroform was added and the mixture was again incubated at room temperature for three minutes, followed by centrifugation at  $12,000 \times g$  for 15 minutes at 4 °C. The aqueous phase was transferred to a new tube containing 750  $\mu$ L of 100% molecular grade ethanol and mixed thoroughly. The sample was then added to a QIAGEN's miRNeasy spin column, and the remaining steps were followed as per the manufacturer's instructions (QIAGEN).

**Gene expression/mRNA analysis.** A total of 50 ng of RNA extracted from A549 cells was evaluated for comparative expression of various gene targets using NanoString's nCounter Inflammation and PanCancer Pathway Panels representing 249 and 730 genes respectively (NanoString Technologies, Inc.). These genes were selected because they represent major biological response categories that we observed to be modified in expression in our previous genome-wide study in lung cells.<sup>13</sup> These panels also include 40 and 6 housekeeping genes. The NanoString Technologies, Inc., employs the specific binding of unique digital barcodes for each target of interest. Probes for each target include a visible reporter probe and a biotinylated capture probe, to which target mRNA is hybridized overnight at 65 °C. Excess probes and nontarget transcripts were washed away, and the remaining probe-bound target transcripts were immobilized on a streptavidin-treated cartridge. The molecules were aligned and fixed using an electrical field, and the cartridge was moved to the nCounter instrument where an epifluorescence microscopy and a Charge-coupled device (CCD) camera were used to capture the images of target–probe complexes. The digital images were processed within the nCounter instrument and counts were tabulated and reported.

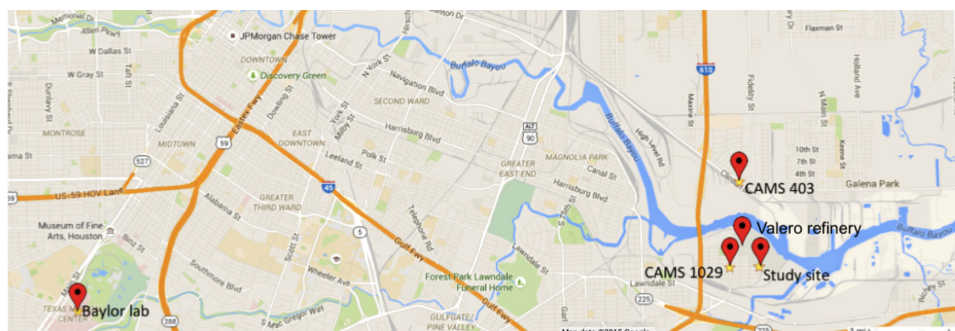
**mRNA normalization and analysis.** mRNA data were normalized and processed separately using the Partek Genomic Suite. Data were normalized in a two-step process as per the manufacturer's specifications.<sup>21</sup> First, positive control normalization was carried out followed by housekeeping gene normalization. The six housekeeping genes used for normalization of the Inflammation Panel were clathrin heavy chain (*CLTC*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-glucuronidase, (*GUSB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), phosphoglycerate kinase 1 (*PGK1*), and tubulin, beta class I (*TUBB*). The 40 housekeeping genes used for the normalization of the Cancer Pathway Panel

were acyl-CoA dehydrogenase family member 9 (*ACAD9*), acylglycerol kinase (*AGK*), AMME chromosomal region gene 1-like (*AMMECR1L*), chromosome 10 open reading frame 76 (*C10orf76*), coiled-coil and C2 domain containing 1B (*CC2D1B1*), CCR4-NOT transcription complex subunit 10 (*CNOT10*), CCR4-NOT transcription complex subunit 4 (*CNOT4*), component of oligomeric golgi complex 7 (*COG7*), DEAD (Asp-Glu-Ala-Asp) box polypeptide 50 (*DDX50*), EAH (Asp-Glu-Ala-His) box polypeptide 16 (*DHX16*), DnaJ (Hsp40) homolog, subfamily C, member 14 (*DNAJC14*), enhancer of mRNA decapping 3 (*EDC3*), eukaryotic translation initiation factor 2B, subunit 4 delta, 67 kDa (*EIF2B4*), excision repair cross-complementation group 3 (*ERCC3*), FCF1 rRNA-processing protein (*FCF1*), FtsJ RNA methyltransferase homolog 2 (*FTSJ2*), G-patch domain containing 3 (*GPATCH3*), histone deacetylase 3 (*HDAC3*), mitochondrial ribosomal protein S5 (*MRPS5*), myotubularin-related protein 14 (*MTMR14*), nucleolar protein 7 (*NOL7*), nucleotide-binding protein 1 (*NUBP1*), protein inhibitor of activated STAT1 (*PLI1*), phosphoinositide-3-kinase (*PIK3R4*), pre-mRNA processing factor 38A (*PRPF38A*), RNA-binding motif protein 45 (*RBM45*), Sin3A-associated protein (*SAP130*), splicing factor 3a, subunit 3 (*SF3A3*), solute carrier family 4 (anion exchanger), member 1, adaptor protein (*SLC4A1AP*), tousel-like kinase 2 (*TLK2*), transmembrane and ubiquitin-like domain containing 2 (*TMUB2*), tripartite motif containing 39 (*TRIM39*), tetratricopeptide repeat domain 31 (*TTC31*), ubiquitin-specific peptidase 39 (*USP39*), vacuolar protein sorting 33 homolog B (*VPS33B*), zinc finger CCCH-type containing 14 (*ZC3H14*), zinc finger with KRAB and SCAN domains 5 (*ZKSCAN5*), zinc finger protein 143 (*ZNF143*), zinc finger protein 346 (*ZNF346*), and zinc finger protein 384 (*ZNF384*). Together, these processes control the batch effect and artifact error. Differential expression was defined as an analysis of variance with  $P < 0.05$  and a fold change of  $>|1.5|$ , which provide false discovery corrected *P* values.

## Results

**Exposure characterization.** To characterize the ambient exposure to the A549 lung cells, meteorological data and ambient measurements were collected from nearby Continuous Ambient Monitoring Stations (CAMS) 1029 and 403.<sup>22,23</sup> The Texas Commission on Environmental Quality operates these monitors, and their locations relative to the field site are shown in Figure 1. Both monitors report hourly average data and collect pollutant concentrations and meteorological data. Ambient measurements were also collected at the field site by the University of Houston (UH) with their Mobile Atmospheric Laboratory for real-time monitoring of ambient pollution. In addition to ozone, NO, NO<sub>2</sub>, and SO<sub>2</sub> measurements, the mobile lab operated a proton-transfer-reaction mass spectrometer (IONICON) and provided measurements of methanol, acetonitrile, acetaldehyde, acetone, benzene, toluene, C2-benzenes, C3-benzenes, styrene, and methyl ethyl ketone. The pollutant concentrations were recorded





**Figure 1.** Map showing the location of the CAMS 1029 and CAMS 403 monitors. Also shown are the field site, the Valero refinery, and the Baylor College of Medicine laboratory where the human lung cells were prepared.

every three to five seconds, and the data were averaged into hourly values. Whenever possible, the UH data were used since it was the closest to the field site otherwise data were supplemented by the CAMS.

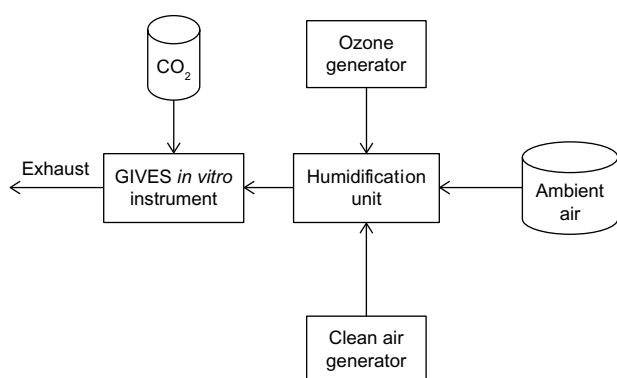
The GIVES sampled ambient air for the three days, February 18, 24, and 26, 2015. For each day, the pollutant concentration mean, standard deviation, and median were calculated using hourly average data that coincided with the

**Table 1.** Ambient measurements from the CAMS 403 monitor and the University of Houston field site (bold).

SPECIES	18-FEB-15	24-FEB-15	26-FEB-15
<b>Ozone (ppb)</b>	40 ± 39.9 (3.1)	18.5 ± 18.5 (3.5)	36 ± 37.2 (3.7)
<b>NO (ppb)</b>	5.8 ± 5.7 (2.6)	8.9 ± 10 (2.3)	4.6 ± 3.7 (2.1)
<b>NO<sub>2</sub> (ppb)</b>	68.6 ± 7.2 (136.8)	17.6 ± 19.3 (7)	11.4 ± 8 (8)
<b>Sulfur dioxide (ppb)</b>	0.1 ± 0.2 (0.1)	0.4 ± 0.2 (0.3)	0.2 ± 0 (0.2)
Carbon monoxide (ppm)	0.2 ± 0 (0.2)	0.3 ± 0 (0.3)	0.2 ± 0 (0.2)
Total non-methane organic compounds	22.8 ± 1.9 (22.8)	15.7 ± 5.9 (17.9)	20 ± 7.3 (22.6)
Ethane	16.8 ± 1.4 (17.4)	13.8 ± 0.8 (14)	21.6 ± 2 (21.2)
Propane	13.5 ± 1.8 (12.9)	11.1 ± 0.6 (11.1)	16.2 ± 1.2 (16.2)
n-Butane	9.2 ± 0.8 (9.2)	8 ± 1.2 (8)	8.4 ± 0.8 (8.8)
<b>Acetone</b>	3.9 ± 0.9 (4.2)	4.8 ± 1.8 (5.1)	6.6 ± 1.2 (7.2)
Isopentane	3.8 ± 0.5 (4)	4.15 ± 0.5 (4)	3.2 ± 0.5 (3)
Isobutane	3.2 ± 0.4 (3.2)	3.2 ± 0.4 (3.2)	3.2 ± 0.4 (3.2)
<b>C2-benzenes</b>	2.4 ± 1.6 (1.6)	3.2 ± 0.8 (3.2)	2.4 ± 1.6 (1.6)
<b>C3-benzenes</b>	3.6 ± 2.7 (2.7)	1.8 ± 0 (1.8)	1.8 ± 0.9 (1.8)
n-Pentane	2.8 ± 0.5 (2.5)	2.75 ± 0.5 (2.5)	2.65 ± 0.5 (2.5)
<b>Toluene</b>	2.1 ± 1.4 (0.7)	3.5 ± 0.7 (4.2)	2.1 ± 0.7 (1.4)
<b>Methanol</b>	1.9 ± 0.2 (2)	0.7 ± 0.2 (0.7)	2.2 ± 1.1 (2.5)
<b>Benzene</b>	1.2 ± 0.6 (1.2)	1.8 ± 0.6 (2.4)	1.2 ± 0.6 (1.2)
<b>Acetaldehyde</b>	1.6 ± 0.4 (1.6)	1.2 ± 0.2 (1.2)	1.8 ± 0.4 (2.2)
Ethylene	1.2 ± 0.4 (1)	2 ± 0.2 (1.8)	1 ± 0.2 (0.8)
<b>Methyl ethyl ketone</b>	0.8 ± 0.16 (0.8)	0.8 ± 0.04 (0.8)	1.6 ± 0.6 (1.6)
n-Hexane	1.2 ± 0 (1.2)	1.2 ± 0 (1.2)	1.2 ± 0 (1.2)
Acetylene	0.8 ± 0.2 (0.8)	1.2 ± 0 (1.2)	1 ± 0.2 (1)
p-Xylene + m-Xylene	0.88 ± 0.24 (0.8)	0.96 ± 0.32 (1.04)	0.64 ± 0.08 (0.56)
Propylene	0.6 ± 0 (0.6)	0.6 ± 0 (0.6)	0.3 ± 0 (0.6)
<b>Styrene</b>	0.8 ± 0.16 (0.8)	0.8 ± 0.08 (0.8)	0.8 ± 0.32 (0)
<b>Acetonitrile</b>	0.14 ± 0.04 (0.16)	0.26 ± 0.06 (0.28)	0.28 ± 0.08 (0.32)

**Notes:** Total nonmethane organic compound measurements are from the CAMS 1029 monitor. All units are ppbC unless otherwise noted. The mean and standard deviation are given, and the median is in parenthesis. All metrics were based on hourly averaged data from 12 to 4 pm CST. C2-Benzenes = Sum of p-Xylene, m-Xylene, and ethyl benzene, C3-Benzenes = Sum of all benzene with 3 carbon groups.

exposure period from 12 to 4 pm central standard time (CST). As shown in Table 1, each of these days consisted of a mixture of fresh and oxidized pollutants. February 18 and 26 show the largest measurements of ozone concentrations with a mean of 40 and 36 ppb. February 18 also had a mean of 68.6 ppb of NO<sub>2</sub>, a concentration more than four times higher than that on the other days. These data suggest that all these days consisted of exposures to an air mass that has gone through significant photochemical aging. Freshly emitted pollutants included a number of aromatics and light alkenes with concentrations similar for all three days. The measured total sum of aromatics had a mean of ~10 ppb and measurements of propylene of ~0.5 ppb and ethylene of ~1 ppb.



**Figure 2.** Schematic of the sample airflow from sources (clean air, ozone, and ambient air) through the humidification unit and into the GIVES exposure instrument housing the lung cells.

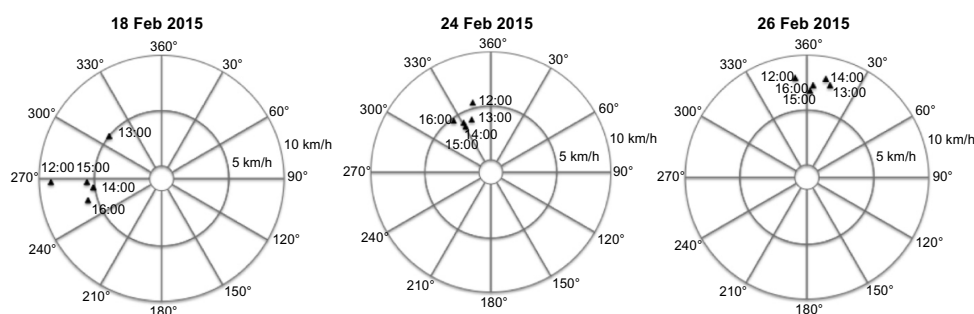
Figure 2 shows the wind speed and direction for all the three days. February 18 shows a wind from the west, which suggests the 610 East Loop Freeway as a possible source of the elevated NO<sub>2</sub> concentrations. The other two exposure days show winds from the north, which could be impacted by the Valero refinery. Wind speeds were relatively calm with February 24 having the slowest speeds. Table 2 provides additional meteorological parameters from the exposure period. There was no precipitation on these days, and the relative humidity ranged from a mean of 28.7% on February 18 to a mean of 73.8% on February 24. February 24 also had the lowest solar radiation among the three exposure days.

**Negative and positive control exposures.** The negative control exposures confirm the integrity of the exposure equipment and protocols used in the field. For the negative control exposure, the GIVES was connected at the field site to medical grade clean air as illustrated in Figure 3, and a total of three clean air experiments were completed. For each negative control exposure, a second six-well plate of lung cells was placed in an incubator and exposed to clean air. The LDH assay was used to quantify the results of the three negative control experiments and reported as fold change over the lung cells housed in the incubator. All data were then normalized to a maximum cytotoxicity of a 7.5-fold change.<sup>20</sup> In Figure 4, LDH expression is presented as the mean of the three positive control experiments and two negative control experiments normalized to LDH released under conditions of maximum cytotoxicity. The data show that the clean air exposures did not induce a significant increase in LDH.

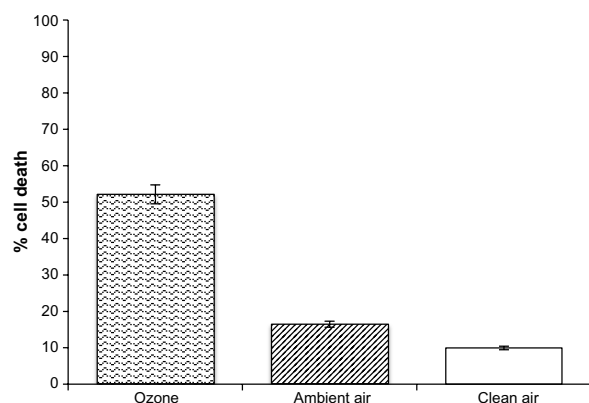
**Table 2.** Meteorological measurements from the CAMS 403 monitor and the University of Houston field site (bold).

	18-FEB-15	24-FEB-15	26-FEB-15
<b>Outdoor temperature (°C)</b>	16.6 ± 17 (1.5)	5.1 ± 5.4 (0.8)	11.3 ± 12.5 (2.1)
Dew point temperature (°C)	-2 ± 0.6 (-2)	0.3 ± 0.4 (0.3)	0.7 ± 0.4 (0.7)
<b>Relative humidity (%)</b>	28.7 ± 27.9 (4.2)	73.8 ± 73.9 (1.7)	50.5 ± 47.7 (6.3)
Solar radiation (Langley/min)	0.8 ± 0.3 (0.9)	0.2 ± 0.1 (0.2)	0.8 ± 0.3 (0.9)
Precipitation (cm)	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)

**Notes:** The mean and standard deviation are given, and the median is in parenthesis. All metrics were based on hourly averaged data from 12 to 4 pm CST.



**Figure 3.** Hourly averaged wind speed and resultant wind direction for the three ambient exposures (February 17, 22, and 27, 2015) where the dot represents the tail of the wind vector.



**Figure 4.** LDH expression and standard deviation calculated as a fold change when compared with lung cells in an incubator exposed to clean air normalized to maximum cytotoxicity. A 7.5-fold change represents maximum cytotoxicity. The positive control exposure ( $O_3$ ) results are the mean of three experiments, and the negative control exposure (clean) results are the mean of two experiments. The ambient air exposure (Ambient) results are the mean of three experiments (February 17, 22, and 27, 2015).

The field site also contained an ozone generator (Dynamic  $NO-NO_2-O_3$  Calibrator Series 101, Thermo Electron Corporation) that was connected to the GIVES for two positive control exposures at concentrations of 400 ppb. For each positive control exposure, a second six-well plate of lung cells was placed in an incubator and exposed to clean air. The LDH results of ozone exposures were compared with the lung cells housed in the incubator and normalized to maximum cytotoxicity. Figure 4 shows a 53% response confirming that the exposure concentration of ozone utilized in this study did produce a significant ( $P = 0.005$ ) cytotoxic response. In addition to the LDH analysis, we determined whether ozone and clean air exposures altered the expression levels of mRNA that encode for inflammation and cancer-related proteins when compared to the lung cells housed in the incubator. As shown in Figure 5, the clean air resulted in a decreased expression of *DEFA1*. For the ozone exposure, both the *IL12B* and *IL11* had increased expression.

Both the clean air and ozone exposures replicated responses seen in the laboratory.<sup>10</sup> This suggests that the instrument itself and the field deployment did not have an adverse impact on the cells. Thus, any cellular responses are solely the result of the ambient exposure.

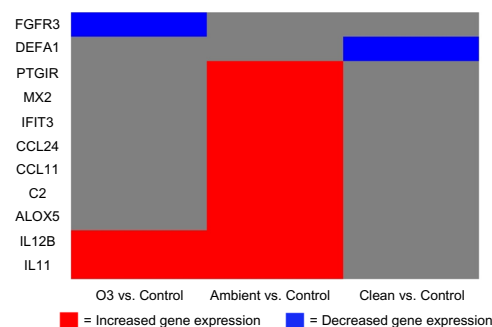
**Ambient exposures alter immune-related gene expression in lung cells.** Three days of ambient exposures were completed on February 18, 24, and 26, 2015. For these ambient exposures, the *in vitro* exposure instrument sample inlet was connected to a Teflon (fluorinated ethylene propylene [FEP], ¼ in. outer diameter [OD]) sample tube mounted on a 7 ft long mast on top of the field laboratory. The end of the ambient sample line at the top of the mast was protected with a funnel to prevent rain droplets and insects from touching the tube inlet. Although particles are permitted to enter the

GIVES instrument, there is minimal interaction of particles with the A549 cells. All ambient exposures started at ~12 pm CST and lasted for four hours. LDH results were compared with the incubator controls and normalized to maximum cytotoxicity. Figure 4 shows a 16% increase in cell death from the incubator, and the difference was not statistically significant ( $P = 0.5$ ). For each exposure, the expression levels of mRNA that encode for inflammation and cancer-related proteins were assessed in A549 cells. Of the 249 immune-related genes and 730 cancer-related genes that were measured, 11 genes showed significant ( $P < 0.05$ ) differential expression in response to at least one of the conditions (Fig. 5 and Table 3). Nine of these were modulated in relation to ambient exposure, three were altered in relationship to ozone exposures, and only one was changed in response to clean air (Table 3).

The nine genes that showed significant changes in gene expression in response to ambient air were associated with immune and inflammatory responses including arachidonate 5-lipoxygenase (*ALOX5*), complement component 2 (C2), chemokine (C-C motif) ligand 11 (*CCL11*), chemokine (C-C Motif) ligand 24 (*CCL24*), interferon-induced protein with tetratricopeptide repeats 3 (*IFIT3*), interleukin 11 (*IL11*), interleukin 12B (*IL12B*), MX dynamin-like GTPase 2 (*MX2*), and prostaglandin I2 (prostaglandin) receptor (*PTGIR*). Two of these genes were also modulated in response to ozone exposure, namely *IL12B* and *IL11*. Fibroblast growth factor receptor 3 (*FGFR3*) showed decreased expression in relation to ozone exposure and is involved in mitogenesis and differentiation, specifically as related to bone development and maintenance. Only one gene changed in response to clear air, namely defensin, alpha 1 (*DEFA1*).

## Discussion

The current paradigm for the identification of the *in vitro* biological effects of air pollutants on human lung cells is based on the laboratory setting. Due to the complexity of the experimental design, these studies do not typically assess the biological endpoints upon exposure to true atmospheric mixture of gases that are constantly undergoing chemical transformations. Furthermore, there are several challenges



**Figure 5.** Heatmap displaying the 11 differentially expressed genes in association with the exposures.

**Table 3.** Differentially expressed genes in response to air toxics exposures.

GENE NAME	FULL GENE NAME	BIOLOGICAL FUNCTION	EXPOSURE (P-VALUE, Q-VALUE)/FOLD CHANGE	KNOWN RESPONSE TO AIR TOXIC
<i>ALOX5</i>	Arachidonate 5-Lipoxygenase	Important mediator of inflammatory and allergic conditions.	Ambient (0.009, 0.123)/1.845	Single nucleotide polymorphism (SNP) involved in benzene-induced hematotoxicity. <sup>35</sup>
<i>C2</i>	Complement component 2	Deficiency in C2 reported to be associated with certain autoimmune diseases.	Ambient (0.005, 0.123)/1.677	Upregulated in rats exposed to particulate matter. <sup>36</sup>
<i>CCL11</i>	Chemokine (C-C Motif) Ligand 11	An antimicrobial chemokine from a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes, asthma, and parasitic infections.	Ambient (0.009, 0.123)/2.487	Elevated in response to ozone and nitrogen dioxide in mice. <sup>27</sup> Induced in response to ozone in adult and newborn mice. <sup>37</sup> Secretion of <i>CCL11</i> increased in individuals with asthma. <sup>25</sup>
<i>CCL24</i>	Chemokine (C-C Motif) Ligand 24	A cytokine, a family of secreted proteins involved in immunoregulatory and inflammatory processes.	Ambient (0.007, 0.123)/2.347	Increased in monkeys exposed to house dust mites and ozone. <sup>28</sup> Second hand smoke exposure in mice results in increased <i>CCL24</i> . <sup>32</sup>
<i>DEFA1</i>	Defensin, Alpha 1	Defensins are a family of proteins involved in host defense.	Clean (0.007, 0.994)/-2.241	Hypermethylated in cord blood of infants born to mothers with asthma. <sup>26</sup>
<i>FGFR3</i>	Fibroblast growth factor receptor 3	Member of the fibroblast growth factor receptor (FGFR) family, <i>FGFR3</i> is a transmembrane protein. Plays a role in bone development and maintenance. Mutations in this gene lead to craniosynostosis and multiple types of skeletal dysplasia.	Ozone (0.022, 0.275)/-2.059	Increased in expression in smokers who acquired lung cancer as compared to smokers who did not. <sup>31</sup>
<i>IFIT3</i>	Interferon-induced protein with tetra-ricopeptide repeats 3	<i>IFIT3</i> is a novel antiviral gene; the protein that it codes for is an extracellular protein.	Ambient (0.003, 0.123)/1.977	Decreased in response to ozone exposure in rats. <sup>29</sup>
<i>IL11</i>	Interleukin 11	The protein encoded by this gene is a part of a cytokine family that drives the assembly of multi-subunit receptor complexes involved in transmembrane signaling receptor.	Ozone (0.008, 0.992)/2.047 Ambient (0.026, 0.153)/1.819	Exposure to formaldehyde upregulates IL-11. <sup>33</sup>
<i>IL12B</i>	Interleukin 12B	<i>IL12B</i> encodes a subunit of interleukin 12, a cytokine that primarily acts on T and natural killer cells, and serves as an essential inducer of Th1 cell development.	Ozone (0.033, 0.992)/1.626 Ambient (0.002, 0.123)/2.050	
<i>MX2</i>	MX Dynamin-Like GTPase 2	<i>MX2</i> protein is upregulated by interferon-alpha but does not contain the antiviral activity of a similar myxovirus resistance protein 1.	Ambient (0.004, 0.123)/1.869	
<i>PTGIR</i>	Prostaglandin I2 (Prostacyclin) receptor	The protein encoded by <i>PTGIR</i> is a member of the G-protein coupled receptor family 1 and has been shown to be a receptor for prostacyclin.	Ambient (0.007, 0.123)/2.151	

for the *in vitro* sampling of and exposure to ambient air. In this study, we set out to determine the feasibility of deploying lung cells in the field and whether a genomic response could be quantified. Specifically, we measured transcript levels for a targeted set of genes upon exposure to ambient air pollutant mixtures, controlled ozone, and clean air. We demonstrated the achievability of measurement of cellular responses, specifically measurements of mRNA, to exposures to ambient

air and identified changes in a set of genes that play a role in inflammatory response in the cell. These data provide the first evidence that this system can be used for field-deployed measurements of toxicological response in lung cells exposed to ambient air.

Evidence for the success of the field deployment of the *in vitro* instrument includes high-quality data collected from the A549 lung cells. High-quality RNA was collected from





field-deployed cells and analyzed for mRNA changes related to the ambient exposures. These results highlight that cells exposed to clean air at the field site had minimal cellular change, while, as anticipated, cells exposed to the ambient conditions displayed greater transcriptional changes, specifically in nine genes. The present study builds upon our previous work where we used laboratory-based *in vitro* techniques to identify pollutants that are important drivers to the overall biological response in lung cells. This study provides support for the application of a field-testing system for air pollutant responses in human lung cells directly exposed to ambient air at realistic concentrations.

Changes in the expression levels of genes known to play a role in inflammatory response were identified in response to ambient air exposure. These included asthma-associated genes: *ALOX5*,<sup>24</sup> *CCL11*,<sup>25</sup> and *DEFA1*.<sup>26</sup> Additionally, some of the identified genes are involved in response to airway injury. Both *CCL11* and *CCL24* have previously been shown to be upregulated in response to ozone previously.<sup>27,28</sup> *IFIT3* has been shown to decrease in response to ozone exposure in rats.<sup>29</sup> Other genes have been previously associated with other air pollutants: *C2* is upregulated in response to particulate matter,<sup>30</sup> *FGFR3*<sup>31</sup> and *CCL24*<sup>32</sup> have been shown to be upregulated in smokers, and *IL11* has been found to increase in response to formaldehyde exposure.<sup>33</sup> With the exception of *FGFR3* that showed decreased expression in response to ambient exposure, the gene expression changes of all the other genes followed the anticipated expression patterns reported in previous studies. Taken together, changes in the expression levels of these genes are likely related to the presence of specific air pollutants and provide insight into the cellular response to complex air pollutants.

While our data highlight the feasibility of *in vitro* field deployment for toxicological measurements, it is not without limitations. In this study, we have analyzed the ambient measurements as a group to demonstrate feasibility, while realistically the differences in chemical composition will greatly impact the specific cellular response. This study was limited only to afternoon exposures where photochemical processes produced oxidized pollutants that would decrease during the night. Future experiments exposing the cells at night would be useful to compare and determine the relative importance of these oxidized pollutants. Additionally, we have selected a set of genes involved in inflammation and cancer based on the a priori interest in these pathways; however, other genes may be altered upon exposure to the air pollutants if analyzed using a genome-wide approach.

## Conclusion

This study provides the first test of a field-deployed lung cell experiment to examine the genomic response of lung cells to complex mixtures of air pollutants, including HAPs. Future research can expand upon this study to examine other biological responses to measured atmospheric pollutants. The

potential to produce *in vitro* field data would allow researchers to determine which pollutants or combination of pollutants are most important to overall toxicity. These data could be used to confirm laboratory findings on our understanding of how mixtures of fresh and aged pollutants impact public health. This aligns with the EPA Strategic Plan to develop cutting-edge scientific tools, specifically to address the impacts on human health due to exposures to mixtures of air pollutants.<sup>34</sup> This technology can be used in conjunction with chemical characterization capabilities to discover causal associations and identify critical pollutants. This identification process could guide the focus of scarce ambient monitoring resources onto gas-phase HAPs that are most relevant.

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## Author contributions

Conceived and designed the experiments: WV, KS, RCF, BL. Analyzed the data: WV, KS, HN, LS, JHF, SA, MHE, RCF, BL. Wrote the first draft of the article: WV, KS, HN, RCF, BL. Contributed to the writing of the article: WV, KS, HN, LS, KMA, CS, JHF, SA, MHE, RCF, BL. Agreed with the article results and conclusion: WV, KS, HN, LS, KMA, CS, JHF, SA, MHE, RCF, BL. Jointly developed the structure and arguments for the article: WV, KS, HN, LS, KMA, CS, JHF, SA, MHE, RF, BL. Made critical revisions and approved the final version: WV, KS, HN, LS, KMA, CS, JHF, SA, MHE, RCF, BL. All authors reviewed and approved the final article.

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