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Research Article

Ozone Responsive Gene Expression as a Model for Describing Repeat Exposure Response Trajectories and Interindividual Toxicodynamic Variability *In Vitro*

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ABSTRACT

Inhaled chemical/material exposures are a ubiquitous part of daily life around the world. There is a need to evaluate potential adverse effects of both single and repeat exposures for thousands of chemicals and an exponentially larger number of exposure scenarios (eg, repeated exposures). Meeting this challenge will require the development and use of *in vitro* new approach methodologies (NAMs); however, 2 major challenges face the deployment of NAMs in risk assessment are (1) characterizing what apical outcome(s) acute assays inform regarding the trajectory to long-term events, especially under repeated exposure conditions, and (2) capturing interindividual variability as it informs considerations of potentially susceptible and/or vulnerable populations. To address these questions, we used a primary human bronchial epithelial cell air-liquid interface model exposed to ozone (O_3), a model oxidant and ubiquitous environmental chemical. Here we report that O_3-induced proinflammatory gene induction is attenuated in repeated exposures thus demonstrating that single acute exposure outcomes do not reliably represent the trajectory of responses after repeated or chronic exposures. Further, we observed 10.1-, 10.3-, 14.2-, and 7-fold ranges of induction of interleukin (IL)-8, IL-6, heme oxygenase 1, and cyclooxygenase 2 transcripts, respectively, within in our population of 25 unique donors. Calculation of sample size estimates that indicated that 27, 24, 299, and 13 donors would be required to significantly power similar *in vitro* studies to identify a 2-fold change in IL-8, IL-6, HMOX1, and cyclooxygenase 2 transcript induction, respectively, to inform considerations of the uncertainty

factors to reflect variability within the human population for *in vitro* studies.

Key words: *in vitro* to *in vivo* extrapolation; interindividual variability; new approach methods (NAMs); inhalation risk assessment; ozone; air-liquid interface (ALI).

Single and repeated exposure to inhaled chemicals from consumer products, commercial/industrial processes, and environmental pollutants impacts human health around the world. The utility of traditional *in vivo* animal approaches for acute chemical exposure testing is limited by the large number of chemicals to be tested, as well as biological, financial, and ethical concerns regarding the use of *in vivo* animal models (Collins *et al.*, 2008; Kleinstreuer *et al.*, 2014; National Research Council, 2007). These challenges are amplified when contemplated in the context of comparing the effects of single and repeated exposure scenarios and consideration of susceptible and/or vulnerable subpopulations. The success of the broad integration of new approach methodologies (NAMs) into decision-making processes depends on building confidence that *in vitro* cell-based models and assays are reliable and sufficiently representative of *in vivo* outcomes (United States Environmental Protection Agency, 2020a). Accomplishing this requires that the relevance of *in vitro* models and endpoints be considered in the context of the analogous cell/tissue in the target organ and biological process (eg, proinflammation, inflammation, barrier function, etc.). Establishing the relevance of acute assays will need to address the same challenges posed to validation of clinical biomarkers, including an understanding of how the measured endpoint culminates in a disease, as well as sensitivity and specificity of each endpoint (Thurston *et al.*, 2017). It has been asserted that *in vitro* studies are more relevant than *in vivo* laboratory animal studies because they utilize human tissues; however, the toxicokinetic (TK) and toxicodynamic (TD) components of interindividual variability have yet to be well characterized, especially across different exposure systems and human cell sources (Clippinger *et al.*, 2018a; Interagency Coordinating Committee on the Validation of Alternative Methods, 2018).

Addressing these requirements for validation and an understanding of whether *in vitro* systems can accurately recapitulate interindividual variability poses several unique challenges including constraints posed by the limited amount of *in vivo* human data for most inhaled chemicals. Conducting human exposure studies is not feasible for most inhaled chemicals for a variety of reasons; however, there is a large body of existing *in vivo* human data regarding the effects of the inhaled air pollutant ozone (O_3) that can be used to develop and assess the relevance of NAMs for inhaled chemical research and testing. O_3 is one of the U.S. Environmental Protection Agency's six National Ambient Air Quality Standards criteria air pollutants and exposure causes various adverse cardiopulmonary outcomes including airway inflammation, which results from the production of proinflammatory cytokines/chemokines by the bronchial epithelium (reviewed in United States Environmental Protection Agency, 2020b). The proinflammatory and inflammatory effects of acute O_3 exposure exhibit interindividual variability and are reproducible across exposures both *in vivo* and *in vitro* (Holz *et al.*, 1999; Bowers *et al.*, 2018). Additionally, *in vivo* controlled human exposure studies have demonstrated that the effects of single and repeated exposures differ in that airway proinflammation and inflammation resulting from acute O_3 exposure are attenuated following repeated exposures (Christian *et al.*, 1998; Devlin *et al.*, 1997).

Primary human bronchial epithelial cell (pHBEC) cultures that have been differentiated at air-liquid interface (ALI) culture conditions are often used to evaluate the potential effects of inhaled chemical exposures *in vitro* due to their ability to recapitulate the *in vivo* bronchial epithelial barrier *in vitro*. The use of *in vitro* models such as these in chemical testing often relies on exposure-induced changes in gene transcription to identify potential adverse effects (Harrill *et al.*, 2019; Judson *et al.*, 2011); however, the influence of intraindividual variability as well as the relationship between the effects of single and repeated inhaled chemical exposures in these *in vitro* systems is relatively unstudied and thus poorly understood. Therefore, we hypothesized that the attenuation of proinflammatory gene expression observed *in vivo* could be recapitulated in an *in vitro* chemical testing-type approach using an ALI exposure model utilizing differentiated pHBEC (pHBEC-ALI). To test this hypothesis, we compared the induction of the O_3-responsive gene transcripts for *interleukin* (*IL*)*-8*, *IL-6*, *cyclooxygenase 2* (*COX2*), and *heme oxygenase 1* (*HMOX1*) following matched single and repeated exposures of pHBEC-ALI cultures derived from 25 young healthy donors. In addition to providing insight on the comparability of single/acute and repeated exposure outcomes, we leveraged the size of our pHBEC donor group to evaluate interindividual variability in the proinflammatory response to O_3 and estimate the sample sizes required to reflect the interindividual variability in the induction of common toxicant-responsive gene transcription.

MATERIALS AND METHODS

Cell culture and O_3 exposure. pHBECs were obtained via bronchial brushing from healthy, nonsmoking donors ages 18–40 with no more than a 1-pack year of lifetime smoking history. Donors gave their informed consent after being informed of risks and procedures. The consent and collection protocol were approved by the UNC School of Medicine Committee on the Protection of the Rights of Human Subjects and by the U.S. EPA. pHBEC isolated from brush biopsy were plated ("passage 0"), expanded to passage 3, and differentiated for 24 days at ALI culture on 24 mm Transwell inserts with 0.4 μm pore polyester membranes (Corning No. CLS3450). Detailed descriptions of the techniques, reagents, and materials used for the culture/expansion of pHBEC and differentiation of pHBEC at ALI used in this study are available as open access methods documents (Dailey and McCullough, 2021a,b). Day 24 ALI cultures were visually evaluated for the presence of beating cilia and the production of mucus as indicators of differentiation status to qualify their use in experiments. Donor demographics are listed in Table 1 and Supplementary Table 1. For all experiments, exposures began on ALI day 24 and consisted of 2 h of exposures each day. Filtered air (FA) control samples were exposed to FA on each of the 4 days, single O_3 exposure ($1XO_3$) samples were exposed to FA for the first 3 days followed by O_3 exposure on the fourth day, and repeated O_3 ($4XO_3$) samples were exposed to O_3 for each of the 4 days (Figure 1). Two hours prior to each daily exposure, the basolateral medium was replaced, and the apical surface was washed with 500 μl of prewarmed (37°C) Dulbecco's PBS (DPBS, ThermoFisher No. 14190-144). Cultures were then

Table 1. Summary of Primary Human Bronchial Epithelial Cell Donor Demographics

Characteristic	Value
Number of donors (*n*)	25
Age (years)	29.0 ± 5.1
Sex	
Male	20 (80.0)
Female	5 (20.0)
Ethnicity (self-identified)	
Caucasian	18 (72.0)
Black	4 (16.0)
Hispanic	1 (4.0)
Asian	2 (8.0)

Values are presented as mean ± SD or *n* (%). These values do not include the 2 donors that were excluded from data analysis. Characteristics for all individual donors can be found in Supplementary Table 1.

	Day 1	Day 2	Day 3	Day 4
4X FA	FA	FA	FA	FA
1X O_3	FA	FA	FA	O_3
4X O_3	O_3	O_3	O_3	O_3
				Harvest

Figure 1. Schematic of single and repeated ozone (O_3) exposure scenarios used in this study. Cultures were exposed to either filtered air or O_3 as indicated for 2 h each day. Samples were collected 2 h following the day 4 exposure.

exposed for 2 h to either FA control or 0.5 ppm O_3 in a 5% CO_2 atmosphere with ≥85% relative humidity according to Figure 1 using the EPA Human Studies Facility *in vitro* exposure apparatus which has previously been described by Hatch *et al.* (2014). Immediately following completion of exposures, cells were transferred to a cell culture incubator until the next day's exposure or harvest as indicated below. To avoid any confounding effects resulting from different durations of pre-exposure ALI culture or other aspects of exposure preparations, single O_3 exposed cultures ($1XO_3$) were exposed to FA on each of the three days immediately preceding the $1XO_3$ (Figure 1). Three inserts were collected separately for each exposure condition as independent technical replicates for each donor.

Rationale for O_3 dosing. When considered in the context of quantifying dose as the product of concentration and exposure time (ie, C × T), the dose used in this study is comparable with *in vivo* human studies evaluating the effects of repeated O_3 exposure on proinflammatory endpoints (Christian *et al.*, 1998; Devlin *et al.*, 1997; Jörres *et al.*, 2000). The dose used here is also similar to, or less than, previously published *in vitro* (Bowers *et al.*, 2018; Devlin *et al.*, 1994; McCullough *et al.*, 2014, 2016; Wang *et al.*, 2018; Wu *et al.*, 2011) and *in vivo* (Devlin *et al.*, 1996; Hernandez *et al.*, 2010; Koren *et al.*, 1989) human acute exposure studies. While none of the O_3 concentrations used in these studies reflect "real world" exposure conditions at a given time, these exposure strategies are designed to represent the cumulative dosing that occurs during high ambient O_3 days, while working outdoors (increased ventilation resulting in greater tissue dose), and/or multi-hour exposures. For example, an O_3 exposure at the current National Ambient Air Quality Standard (NAAQS; ie, 70 ppb for 8 h) would result in 560 ppb*h. If adjusted to account for the increase between ventilation that occurs during rest (approximately 7.5 l/min) and light physical activity (approximately 15 l/min) then the resulting cumulative dose at the NAAQS regulatory limit would be reasonably represented by the cumulative dosing used in this study. Further, studies evaluating the effects of inhaled gases within the respiratory tract typically assume uniform deposition within a given region of the respiratory tract; however, the dosimetry of test agents within the airway is nonuniform and depends on complex interactions of several parameters. *In vivo* O_3 exposure causes focal injuries in the respiratory tract with increased injury occurring immediately downstream of airway bifurcations (Postlethwait *et al.*, 2000). These observations were supported by subsequent 3D computational fluid dynamics modeling indicating that O_3 deposition was enhanced in the region surrounding the carinal ridge by up to 60- and 25-fold relative to other areas during inspiratory and expiratory flow, respectively (Taylor *et al.*, 2007).

Transepithelial electrical resistance. Transepithelial electrical resistance (TEER), a measure of epithelial monolayer integrity, was assessed using an epithelial volt/ohm meter (World Precision Instruments) with chopstick electrode 2 h postexposure prior to RNA harvest. The following exposure as indicated in Figure 1, 1 ml of DPBS was added to the apical surface and electrical resistance was measured between the apical and basolateral compartments using a probe tip. Each condition had a total of 9 readings (3 readings from 3 inserts per donor per treatment). Final TEER was calculated by subtracting the resistance of an empty 24 mm Transwell insert (with DPBS in the apical compartment and exposure medium in the well) from the reading of each culture-containing insert and then multiplying by the area of the insert (4.67 cm^2). The statistical significance of differences in TEER readings between FA, $1XO_3$, and $4XO_3$ exposed cells was determined using a 1-way ANOVA with Holm-Sidak's multiple comparison test using GraphPad Prism version 9.2.0.

RNA isolation and gene expression analysis. Cultures were lysed in RNA lysis buffer (component of PureLink RNA Mini Kit; ThermoFisher No. 12183025) 2 h following completion of the final exposure according to the manufacturer's protocol. Lysates were stored at −80°C until ready for extraction. RNA extraction was conducted according to the manufacturer's protocol and was quantified using a NanoDrop ND1000. One microgram of RNA was then used to synthesize complimentary DNA (cDNA) using iScript Reverse Transcription Kits (BioRad No. 1708891) according to the manufacturer's protocol. Fold change in target transcript abundance was assessed using multiplexed primers and hydrolysis probes (sequences listed in Supplementary Table 2) in technical triplicates on a CFX96 Touch (Bio-Rad). Both $1XO_3$ and $4XO_3$ treatments were expressed as fold changes relative to 4XFA treatment and target gene C_q values were normalized to matched β-actin C_q values using the Pfaffl method (Pfaffl, 2001).

Statistical analysis. All statistical analyses were conducted using GraphPad Prism 9.0.1 (San Diego, California) unless otherwise noted. The attenuation of target gene induction was determined by subtraction of the $\log_2$ fold change observed for the repeated exposure treatment from that observed for the matched single exposure treatment (1X–4X). Gene expression data were evaluated for outliers using the Grubbs test ($\alpha = 0.01$). Two donors were identified to have outlying values across several gene expression measures. To avoid having these data points significantly impact the conclusions regarding gene expression changes with respect to repeat exposure in the donor

population, we excluded these donors from all analyses brining the n to 25; however, as a sensitivity analysis for the sample size estimates, results including the outliers are reported in Supplementary Materials.

First, differences between mean induction of target genes in single and repeated exposure treatments for each target gene were assessed using a 2-tailed paired t test and considered statistically significant if the Bonferroni-corrected $p \leq .05$ ($p \leq .0125$). Then, the correlation between single exposure gene induction and attenuation effect was assessed using a 2-tailed Pearson correlation analysis with a 95% confidence interval and considered statistically significant if $p \leq .05$. Finally, the relationship between single exposure gene induction and attenuation effect was also assessed by regression analysis. Correlations between both single exposure induction and attenuation effect for target genes were determined using 2-tailed Pearson correlation analysis with a 95% confidence interval and considered statistically significant if the Bonferroni-corrected $p \leq .05$ ($p \leq .008$). Spearman correlation coefficients were also calculated as a sensitivity analysis.

In addition to the direct analysis of the effect of exposure and attenuation in the pHBEC-ALI model, we sought to characterize variance observed in a normal health human population to empower future studies that seek to utilize this pHBEC-ALI exposure model. Therefore, the variance of single and repeated O_3 exposure-mediated target gene transcript induction was assessed by F test in R 4.0.4. Variances were considered significantly different at $p \leq .05$. Additionally, sample sizes to assess differences in gene expression across the indicator genes used in this study were estimated using pwr in R (Champely *et al.*, 2020).

RESULTS

Repeated Exposure Does Not Alter the Impact of O_3 Exposure on TEER

Acute O_3 exposure reduces epithelial barrier integrity *in vitro* and *in vivo* (Aris *et al.*, 1993; Bayram et al. 2002; Koren *et al.*, 1989; Van Bree *et al.*, 2002). Here, we evaluated whether single and repeated exposure resulted in different effects on bronchial epithelial barrier integrity as determined by TEER. pHBEC-ALI cultures exhibited TEER values (mean ± SD) of 304.4 (±20.6), 120.5 (±29.2), and 129.5 (±37.6) Ω^*cm^2 following FA, $1XO_3$, and $4XO_3$ exposures, respectively (Figure 2A). The effects of both the $1XO_3$ and $4XO_3$ exposures were significantly different from FA exposures ($p = 3.33 \times 10^{-5}$, and 1.35×10^{-4}, respectively), but not significantly different from each other ($p = .58$). The relationship between the FA, $1XO_3$, and $4XO_3$ data from each of the donor's pHBEC-ALI cultures evaluated is shown in Figure 2B.

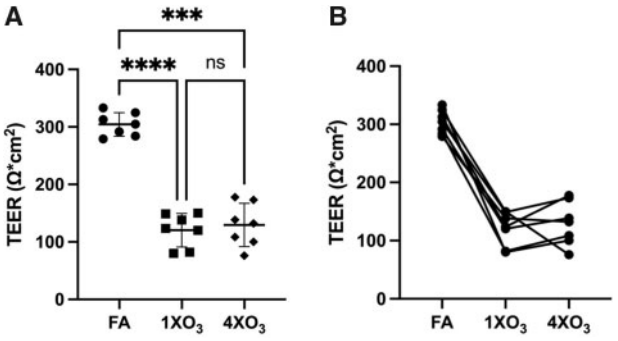

Figure 2. TEER is decreased following both single and repeated ozone (O_3) exposures. A, Significant reductions in TEER occurred following both single (1X) and repeated (4X) O_3 exposure scenarios compared with filtered air (4XFA) control. TEER values observed in single and repeated exposure scenarios were not significantly different. B, Before and after plots linking TEER values observed following each exposure condition for each donor's primary human bronchial epithelial cell (pHBEC)-air-liquid interface (ALI) culture. Values from each donor's pHBEC-ALI culture are shown as individual data points with the bar representing the group mean and error bars indicating SD are shown in (A). ***, ****, and ns indicate $p \leq .001$, $p \leq .0001$, and not significant as determined with 1-way ANOVA with Holm-Sidak's multiple comparisons test, respectively.

Comparison of Single and Repeated Exposure on O_3-Responsive Gene Induction

Acute exposure to O_3 causes airway neutrophilia accompanied by increases in proinflammatory cytokines in the respiratory tract (Devlin *et al.*, 1991; Kim *et al.*, 2011; Koren *et al.*, 1989); however, these effects are attenuated following 4–5 days of repeated exposures (Christian *et al.*, 1998; Devlin *et al.*, 1997). Bronchial epithelial cells upregulate prototypical proinflammatory (eg, *IL-8* and *IL-6*) and oxidative stress-responsive genes (eg, *HMOX1* and *COX2*) in response to acute O_3 exposures *in vitro* at both the transcript (McCullough *et al.*, 2014, 2016; Jaspers *et al.*, 1997, 1998) and protein levels (Bayram *et al.*, 2001; Devlin *et al.*, 1994; Wu *et al.*, 2011). Thus, we sought to determine whether this pattern of attenuation in O_3-induced proinflammatory gene expression *in vivo* could be recapitulated *in vitro* using a pHBEC-ALI model.

We observed significant differences in the induction of all 4 target gene transcripts between single and repeated O_3 exposure scenarios (Figure 3 and Table 2). This change was accompanied by a significant difference of the variance between single and repeated exposure induction of *HMOX1* ($p = 5.15 \times 10^{-9}$), with nearly significant differences in *IL-8* ($p = .07$), *IL-6* ($p = .08$), and *COX2* ($p = .08$) gene expression. Despite these observations in the study population taken as a whole, significant differences between single and repeated O_3 exposure outcomes were not observed in cultures derived from every donor. Cultures derived from 52% (13/25) of donors exhibited significant differences in the induction of one or more target genes between single and repeated exposure scenarios. Of these, cultures from 12 donors exhibited attenuation while repeated exposure resulted in greater induction in cultures derived from 1 donor (Figure 3).

Relationships Between Induction and Attenuation Effect in O_3-Responsive Gene Expression

After observing that repeated exposure resulted in attenuation of O_3-responsive gene expression, we sought to determine whether the magnitude of induction was related to the attenuation effect for each target gene. We observed significant positive linear correlations between the induction and attenuation effect for IL-8 ($r = 0.733$, $p = 3.07 \times 10^{-5}$), IL-6 ($r = 0.719$, $p = 5.09 \times 10^{-5}$), HMOX1 ($r = 0.972$, $p = 3.06 \times 10^{-15}$), and COX2 ($r = 0.728$, $p = 3.76 \times 10^{-5}$) transcripts (Figure 4). The induction of all 4 target gene transcripts was positively correlated with one another; however, only the association between IL-8/COX2 ($r = 0.672$, $p = 6.12 \times 10^{-4}$) remained significant after Bonferroni correction (Figure 5A and Supplementary Table 3). Spearman correlation coefficients were also calculated as a sensitivity analysis, which supported the significant association of IL-8/COX2 ($\rho = 0.567$, $p = 3.14 \times 10^{-3}$) and also indicated a significant association between HMOX1/COX2 ($\rho = 0.510$, $p = 4.74 \times 10^{-4}$) (Supplementary Figure 1 and Table 5).

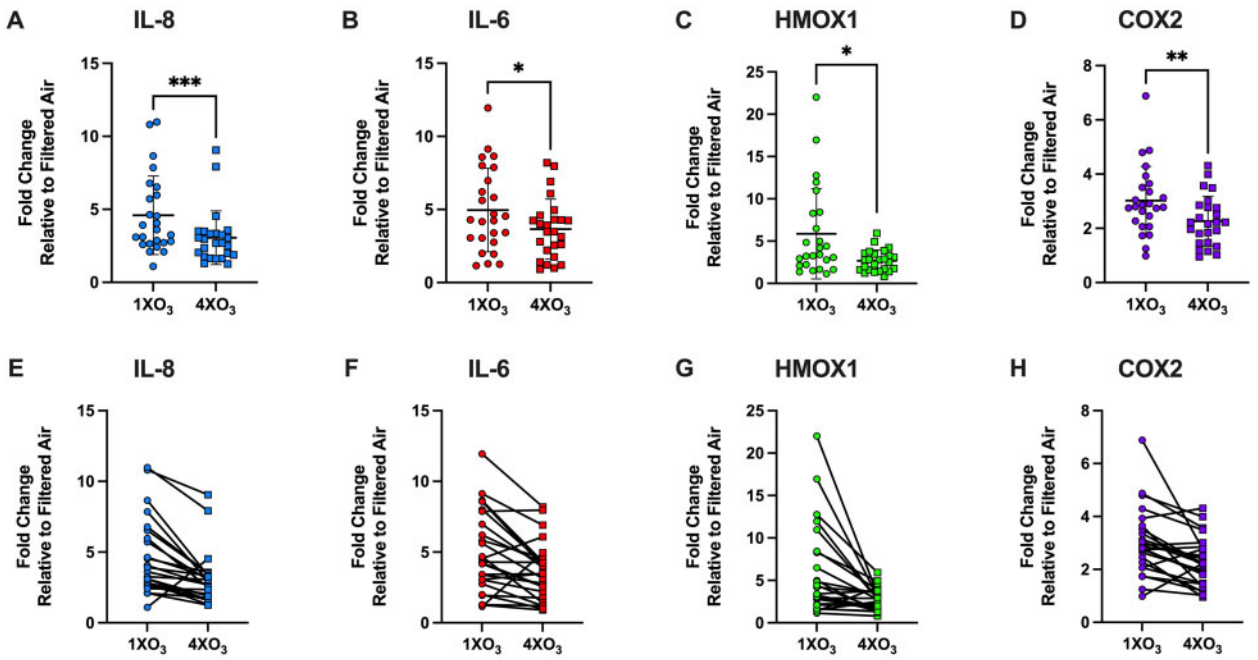

Figure 3. The induction of O_3-responsive gene expression is attenuated following repeated exposures. (A–D) Target O_3 responsive gene induction following single and repeated O_3 exposure scenarios. (E–H) Before and after plots linking the fold change in target gene expression following in single and repeated O_3 exposure scenarios for each donor's pHBEC culture used in this study. Fold change values are expressed relative to donor-matched filtered air (4XFA) treatment and normalized using β-actin as a reference gene. Group mean and standard deviation are shown in A–D. *, **, and *** indicate Bonferroni corrected $p \leq .05$, $p \leq .01$, and $p \leq .001$, respectively.

The attenuation of all 4 target transcripts was positively correlated with each other with all correlations being statistically significant after Bonferroni correction (Figure 5B and Supplementary Table 4) with the exception of IL-6/HMOX1. Spearman correlation coefficients were also calculated as a sensitivity analysis, which supported the collinearity of IL-8/HMOX1 ($\rho = 0.574$, $p = 2.71 \times 10^{-3}$) following Bonferroni correction (Supplementary Figure 1 and Table 6).

Assessment of Interindividual Variability in Single and Repeated Exposures

Across subjects, the induction (fold change relative to FA control) of each gene displayed substantial variation with the ranges of single exposure of 1.09–11.00 (IL-8), 1.16–12.00 (IL-6), 1.36–19.30 (HMOX1), and 0.98–6.88 (COX2; Table 2). The variation in the repeated (4X) exposures and attenuation effects (1X–4X) were significantly less variable than the initial exposures, suggesting repeated/subchronic/chronic exposures reduces the interindividual variability that would otherwise occur in individuals following a single exposure. In support of this, the standard deviation for repeated exposures and attenuation effects tended to be smaller than the initial exposure. From the measurements of variation (Table 2), power calculations were performed to provide insights into the number of donors required when designing future studies. In addition, sensitivity analyses were conducted using the full data set ($n = 27$) for all power calculations to determine the impact of outliers (Supplementary Table 7). Using the common base method for power calculation for qPCR (Ganger *et al.*, 2017), we calculated the number of donors required in both the cases of highest and lowest variation with standard type I (0.05) and type II (0.8) error rates assuming a 2.0-fold change would be deemed statistically significant (Table 3). For the least variable gene, COX2, a total of 13 subjects were estimated as necessary to detect a 2.0-fold change increase or decrease between FA control and $1XO_3$. For the most variable gene, HMOX1, 299 donors would be required to adequately assess changes between FA control and $1XO_3$. In support of the idea that the attenuation effect reduces interindividual variability, smaller required sample numbers were observed with 6–12 donors being required to detect a 2.0-fold change difference between $1XO_3$ and $4XO_3$ exposures. Sensitivity analyses demonstrated that representing the entire donor population in sample size estimates through the inclusion of statistical outliers resulted in larger sample size estimates for both 1X and 1X–4X exposure scenarios for all 4 target genes evaluated (Supplementary Table 7). These data demonstrate that variability of gene expression levels impacts the number of donors necessary to detect a significant change in gene expression when conducting a multi-donor study. Further, they support the idea that some genes will have reduced variability following multiple exposures while others may become more variable, depending on their role in the pathogenesis process.

DISCUSSION

The development and application of *in vitro* approaches that represent the influence of repeated exposures and interindividual variability on susceptibility and exposure effects to recapitulate *in vivo* human exposure outcomes are essential for assessing the effects of inhaled chemical exposures and protecting public health. The bronchial epithelium plays a central role in O_3-induced airway inflammation through the induction of proinflammatory cytokines (Devlin *et al.*, 1994; McCullough *et al.*, 2014; Bowers *et al.*, 2018; Jaspers *et al.*, 1997, 1998). We used a differentiated pHBEC-ALI model to determine whether the

Table 2. Descriptive Statistics of Target Gene Transcript Induction Following Single and Repeated Exposure Scenarios

	IL-8			IL-6			HMOX1			COX2		
	1X O_3	4X O_3	1X-4X	1X O_3	4X O_3	1X-4X	1X O_3	4X O_3	1X-4X	1X O_3	4X O_3	1X-4X
Mean	4.39	2.91	1.49	4.65	3.44	1.21	5.35	2.51	2.84	2.94	2.18	0.76
SD	2.76	1.90	1.73	2.73	1.90	1.89	4.95	1.28	4.51	1.26	0.87	1.04
%RSD	22.78%	65.29%		58.71%	55.23%		92.52%	51.00%		42.86%	39.91%	
Range	1.09–11.0	0.93–9.05	−3.44 to 5.31	1.16–12.00	0.98–8.06	−3.80 to 4.57	1.36–19.30	0.77–5.93	−2.89 to 16.50	0.98–6.88	1.02–2.54	−1.25 to 4.12
IQR	3.59	1.48	1.48	4.25	2.67	2.73	5.25	1.93	3.81	1.36	1.66	0.78
95% CI of mean	3.26–5.53	2.12–3.69	0.77–2.20	3.53–5.78	2.66–4.23	0.43–1.99	3.31–7.40	1.98–3.04	0.98–4.70	2.42–3.46	1.82–2.54	0.33–1.19
t test[a]	$p = .001$			$p = .015$			$p = .017$			$p = .005$		
Donors with significant difference 1X and 4X	28%			16%			28%			28%		
F test	$p = .073$			$p = .081$			$p = 5.15 \times 10^{-9}$			$p = .079$		

[a]Bonferroni corrected *p*-values. 1X–4X column indicates the values obtained by subtracting the 4X value from the corresponding 1X value.

attenuation of proinflammatory cytokine expression observed in controlled *in vivo* repeated exposure studies could be recapitulated *in vitro*. Further, we conducted these studies using a large *in vitro* study population ($n = 25$ pHBEC donors) to provide insight into the range of interindividual variability in the induction of several common toxicant-responsive genes. Here, we demonstrated that repeated exposure to the model oxidant O_3 caused attenuation of the induction of O_3-responsive proinflammatory and oxidative stress gene transcription in our differentiated pHBEC-ALI model. Our observations support the use of differentiated pHBEC-ALI cell culture models to examine the effects of repeated inhaled oxidant chemical exposures on the induction of proinflammation in the human tracheobronchial airways *in vitro*. Additionally, the results of our study demonstrate that the effects of a single inhaled chemical exposure cannot be assumed to represent those of repeated exposures. These findings provide insight on the ability of the outcomes of a single acute exposure to predict the trajectory of measured responses after repeated or chronic exposures, which are more relevant to "real world" human exposure scenarios. The analysis also provides a basis for the determination of sample size to ensure that *in vitro* primary cell-based models account for interindividual variability in exposure outcomes and can uniquely inform the magnitude of the TD component of the uncertainty factor for variability within the human population (UF_H) for *in vitro* studies.

Comparison of the Effects of Repeated Exposure In Vitro *with Existing* In Vivo *Data*

Demonstrating concordance between *in vitro* models and *in vivo* human outcomes is critical for demonstrating the biological relevance of these models and advancing the use of *in vitro* model-derived data in chemical risk assessment. One of the critical challenges is characterizing the disease dimension that acute assays may represent regarding the trajectory to long-term events, especially under repeated exposure conditions. Here, we are the first to report that the attenuation of O_3-responsive proinflammatory gene transcript induction following repeated daily O_3 exposure observed *in vivo* can be recapitulated *in vitro* using a differentiated pHBEC-ALI model.

Our observation of *IL-6* attenuation was consistent with differences observed in the abundance of IL-6 protein in bronchoalveolar lavage (BAL) between single and repeated controlled human and rodent exposures *in vivo* (Christian *et al.*, 1998; Devlin *et al.*, 1997; Van Bree *et al.*, 2002). Further, our observation of COX2 attenuation was also consistent with the lack of elevated PGE_2, which is increased following $1XO_3$ (Devlin *et al.*, 1991; Koren *et al.*, 1989; Seltzer *et al.*, 1986), in repeated exposure reported by Devlin *et al.*, (1997). Unfortunately, PGE_2 levels were not reported by either Christian *et al.*, (1998) or van Bree *et al.*, (2002). Christian *et al.* (1998) and Jörres et al. (2000) reported non-statistically significant 43.1% and 43.2% attenuation in BAL IL-8 following repeated exposure compared with single exposure, respectively, which is similar to the 33.3% attenuation that we observed in *IL-8* transcript induction. The pHBEC-ALI model used in this study was not designed to assess neutrophil recruitment directly; however, given the role of the bronchial epithelium in the production of proinflammatory mediators following O_3 exposure, our observations indicating attenuation of *IL-6*, *IL-8*, and *COX2* transcript induction are consistent with the attenuation of neutrophil recruitment observed *in vivo* in both humans and rats (Christian *et al.*, 1998; Devlin *et al.*, 1997; Van Bree *et al.*, 2002).

Table 3. *In Vitro* Study Donor Sample Sizes Required to Identify a Statistically Significant 2.0-Fold Change in the Expression of Genes Examined in This Study

	Sample Size Required	
	1X	1X–4X
IL-8	27	12
IL-6	24	8
HMOX1	299	60
COX2	13	6

The 1X column refers to identifying a change in the indicated target gene expression following a single exposure relative to FA control. The 1X–4X column refers to identifying a change between 1X and 4X exposure scenarios.

Correlations in Target Gene Induction and Attenuation

The significant positive correlation of the induction of IL-8/COX2 and HMOX1/COX2, but not IL-8/HMOX1 that we observed following $1XO_3$ (Figure 5A) may indicate that the regulation of COX2 transcript induction is driven by at least 2 discrete pathways with one being in common with IL-8 and another with HMOX1. This could be occurring through the ERK1/2-mediated regulation of IL-8 induction, which we have previously shown in pHBEC (Bowers *et al.*, 2018; McCullough *et al.*, 2014), and NRF2-mediated regulation of HMOX1 and COX2 (Alam and Cook, 2007; Jessen *et al.*, 2020; Knörr-Wittmann *et al.*, 2005). Additionally, we have previously reported that the induction of both HMOX1 and COX2, but not IL-8 and IL-6, in pHBEC following O_3 exposure is correlated with pre-exposure chromatin modification states in target gene promoters (McCullough *et al.*, 2016). Although these suppositions may account for the correlations in gene expression that we observed, the underlying mechanisms are likely more complex and require more thorough investigation. For example, the expression of IL-6 has been shown to be regulated through both the ERK1/2 and p38 MAPK, and the NRF2 pathways by us (McCullough *et al.*, 2014), and others (Wruck *et al.*, 2011), respectively.

The magnitude of induction was significantly positively correlated with the attenuation effect (1X–4X) for each of the target genes in this study (Figure 4). The presence of additional correlations in the attenuation effects observed between our target genes suggests that the processes involved in attenuation are even more complex and likely involve some level of global mechanism that may induce a hyporesponsive state (Figure 5B). Others have demonstrated that repeated O_3 exposures lead to increased antioxidant capacity in the lung in rodents (Wiester *et al.*, 2000, 1996; Rahman *et al.*, 1991), which may account for the attenuation that we observed here; however, the role of antioxidants could be supplemented or supplanted by a myriad of other possible changes in cellular physiology.

Future studies are required to assess the role of changes in cellular signaling pathway activation and the time course of antioxidant production in observed responses to both single and

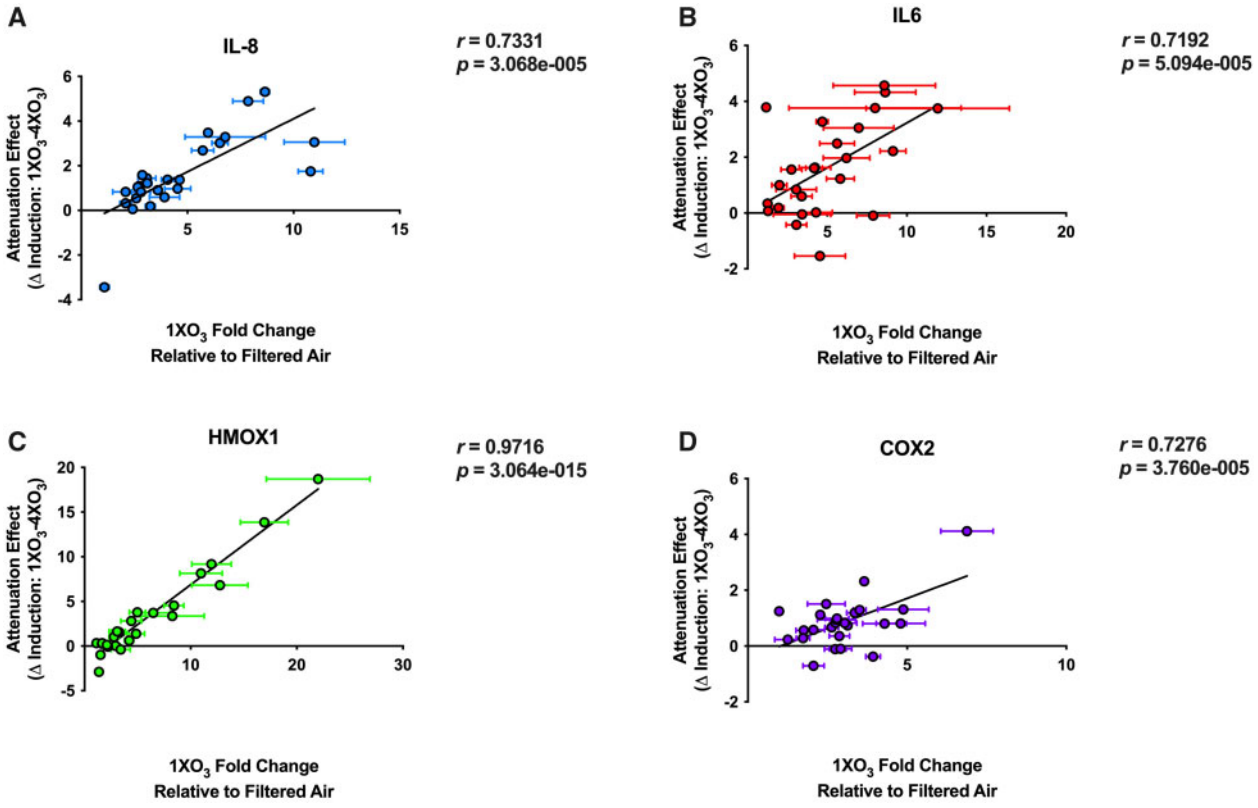

Figure 4. Ozone (O_3)-responsive gene induction is positively correlated to the attenuation effect following repeated exposures. A–D, Correlations between target gene induction and attenuation effect following single and repeated O_3 exposures, respectively. Target gene induction fold change and attenuation effect were assessed by Pearson regression analysis. Pearson correlation coefficient (r) and p-values are shown for the comparison for each target gene. Error bars represent the SEM of the single O_3 exposure induction of target gene expression from triplicate wells from each donor.

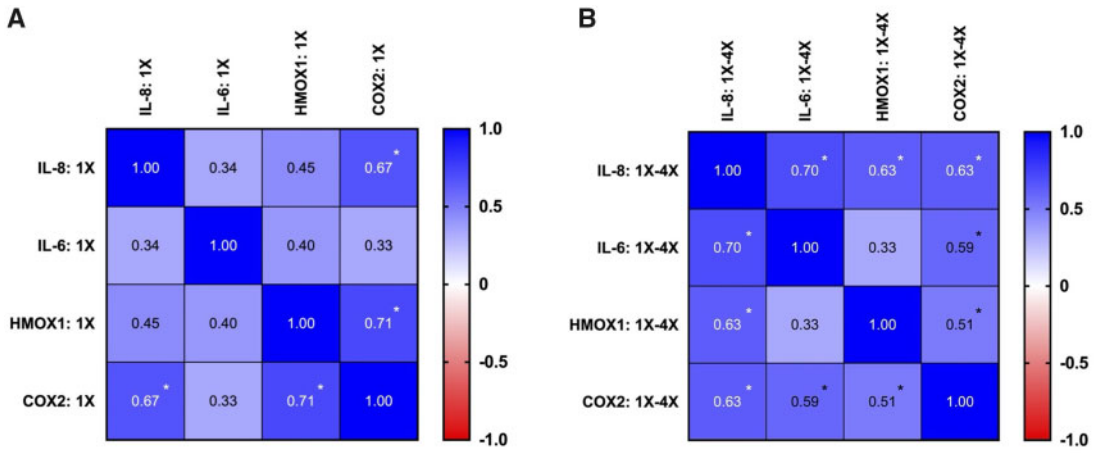

Figure 5. Correlations between ozone (O_3)-responsive target gene expression. Pearson correlation coefficients between the (A) induction and (B) attenuation of all 4 O_3-responsive target genes. * indicates Bonferroni corrected $p \leq .05$ ($p \leq .008$). Individual p-values for induction and attenuation comparisons are shown in Supplementary Tables 3 and 4, respectively.

repeated exposure outcomes. Overall, our observations suggest that the crosstalk between cellular signaling pathways in the regulation of exposure responsive genes is highly complex and augmented by repeated exposures. Understanding these intricate relationships is critical to our ability to elucidate mechanisms of both susceptibility and interindividual variability. The development of computational virtual tissue models that incorporate cellular and molecular dynamics will further support predictive toxicology to increase the throughput, accuracy, and biological relevance of chemical testing.

Interindividual Variability in the pHBEC-ALI Model

The effects of O_3 exposure on lung proinflammation and inflammation have been studied in *in vivo*-controlled human exposure studies for decades, thus making these O_3 exposure outcomes an *in vivo* human data-rich context for comparison, development, and assessment of *in vitro* inhalation models, assays, and other NAMs. The severity of O_3-induced airway inflammation is highly variable among young healthy volunteers in controlled human exposure studies, which is reflected by the differences that we observed in the induction of proinflammatory cytokines between pHBEC-ALI cultures derived from different donors (Figure 3). Although it is possible that our observations could be the result of interexperimental variability instead of interindividual variability, both individual proinflammatory/inflammatory responses are known to be reproducible over time *in vivo* and *in vitro* (Bowers *et al.*, 2018; Holz *et al.*, 1999). Nonetheless, additional studies are required to gain greater insight into the magnitude of interexperimental variability within pHBEC-ALI cultures derived from individual donors and interisolation variability that may occur when primary cells are isolated from volunteers at different points over time. Primary cell-based *in vitro* systems are more controlled in terms of exposure while also being reflective of genetic and epigenetic variation observed in human populations. Importantly, this variation contributes to an increase in the variability of biological outcomes, such as gene expression, levels of secreted proteins, and even macroscale phenotypic outcomes (Bisogno *et al.*, 2020; Grimm *et al.*, 2018; Mackey *et al.*, 2018). In this way, primary cell studies are likely to be more reflective of the normal range of human variation in chemical exposure outcomes than studies utilizing cell lines or inbred rodent strains due to their genetic homogeneity.

Therefore, to capture the effect of exposure on a genetically variable background, the use of 3 biological replicates is likely to be insufficient. As the goal of NAMs is to use *in vitro* models for public health applications (eg, chemical toxicity testing, drug development, and precision medicine/environmental health), the identification and incorporation of a normal range of interindividual variability for endpoints of interest is critical to ensuring adequate reflection of the range of the effects of tested materials on the target cell/tissue type within the population. Minimally, an understanding of the range of interindividual variability for different assays and how they relate to key events of pathogenesis or in adverse outcome pathways (AOPs), or to more traditional health endpoints of interest such as histopathology, will allow for replacement of the specific TD component of the UF_H used in risk assessment with data-driven estimations; while improvements offered by emerging computational approaches to *in vitro* to *in vivo* extrapolation will help inform the TK component (Clippinger *et al.*, 2018b). Thus, when designing *in vitro* studies with the intention of representing interindividual variability in a target population (eg, total population, young healthy individuals, susceptible populations, etc.), such as chemical toxicity/efficacy testing, sample size should be determined empirically rather than relying on the traditional 3 biological replicates.

Here, we had the opportunity to estimate variation across an *in vitro* study primary cell donor population ($n = 25$) that was considerably greater than nearly all previous primary cell-based studies. The findings of this study show that even for the least variably expressed gene, *COX2*, 13 donors would be necessary to detect substantial changes in gene expression (ie, a 2.0-fold change in single and repeated exposure conditions). Additionally, this study is likely underpowered to detect differences among the most variable gene measured *HMOX1*. The estimated variation from this sample population suggests a minimum of 299 donors should be used to detect a significant 2.0-fold difference between FA and $1XO_3$ in a single exposure scenario, a likely overestimate related to the variability of the gene and sample size of this donor cohort. In contrast to this, based on the sensitivity analysis conducted in which outliers

were included in the power and sample size calculations, it is possible that the number of donors could be even higher than initially estimated. Future research with larger donor populations will be needed to fully address the true variability of *HMOX1* and other highly variable exposure-responsive genes in pHBEC, as well as other lung cell types, to better estimate the number of donors required for *in vitro* studies to reflect interindividual variability. Further work will also be required to develop statistical methodologies for the analysis of primary cell-based *in vitro* data based on Bayesian or nonparametric methods that do not rely on an assumption of a normally distributed population. Additionally, applications of hierarchical Bayesian methods that utilize probability of health outcomes based upon the prior occurrence of key molecular events will be important for development *of in vitro* NAMs applications. It is important to note that these sample size estimates apply to *in vitro*, not *in vivo*, studies and that they should be considered in the context of the exposure conditions, endpoints, and timing of sample collection used in this study. Nonetheless, our observations suggest that the use of a small numbers of donors (eg, $n = 3$) in *in vitro* cell-based chemical toxicity/efficacy studies is not likely to be adequate to reflect interindividual variability in exposure-associated effects on key endpoints. Thus, the use of underpowered studies may underpredict exposure effects on susceptible populations or overpredict effects on nonsusceptible populations.

Finally, this analysis provides insights on the potential adequacy or magnitude needed for the TD component of the UF_H. Concerns for interindividual variability are typically addressed in most risk assessment approaches based on *in vivo* studies by the application of an UF_H that assumes that the variation of the magnitude of effects resulting from an exposure within the population, including susceptible subpopulations, is a lognormal distribution and within a factor of 10-fold or less. The UF_H is the product (ie, $UF_H = TK \times TD$) of explicit components reflecting the contributions of variation in TK (ie, $\sqrt[2]{10}$ or 3.16-fold) and TD (another 3.16-fold) processes in exposure outcomes (Bogdanffy and Jarabek, 1995). After both the single (1X) and repeated (4X) exposures were evaluated, the range in gene expression for all transcripts was closer to the full magnitude of a 10-fold UF_H rather than the typical 3.16-fold factor ascribed to the TD component of the UF_H alone with HMOX1 being 1.5-fold higher than the factor of 10 typically used for the entire UF_H (Table 2). It should also be considered that transcript induction is a relatively early key event in AOPs and variability observed at the transcript level may be enhanced or mitigated by the interindividual variation in subsequent key events leading to an apical outcome; however, accounting for differences between default TD uncertainty and actual variability at the transcript level is of particular importance given the increasing emphasis on the use of transcriptomic data for chemical testing. Additional studies are required to improve our understanding of, and accounting for, potential variability in specific response measures and how they can help develop data-driven values for the TD component of UF_H using NAMs.

Study Limitations and Implications on Future Studies

The study reported here is an early step into evaluating interindividual variability and the relationship between single and repeated exposure outcomes in *in vitro* chemical testing approaches. Thus, it has several limitations with implications on future studies that should be noted. First, this study was designed to assess the interindividual variability on O_3-responsive gene expression in differentiated pHBEC and evaluate the relationship between the effects of single and repeated O_3 exposures using an *in vitro* testing-type approach. Previous studies have demonstrated that *in vitro* O_3 exposure causes the release of proinflammatory mediators evaluated in this study at the protein level in human bronchial epithelial cells (Devlin *et al.*, 1994; Jaspers *et al.*, 1997; Wu *et al.*, 2011); however, the effect of single and repeated exposures on target genes at the protein level was not evaluated in this study. Additionally, our evaluation of a single timepoint does not represent the impact of interindividual variation on the temporality of either transcript induction or translation of transcript into protein and thus may under-represent the total effect of interindividual variation on target gene expression. Future studies are required to evaluate the impact of interindividual variation on the temporality of target gene transcript induction and translation.

Second, despite the large number of pHBEC donors used, relative to other *in vitro* studies, the study population was not adequately powered to assess the effect of sex on exposure outcomes ($n = 20$ males and 5 females), thus we did not conduct any analyses that included sex as a variable. Additional studies are required to assess the effects of sex on both exposure outcomes and the utility of *in vitro* models in representing these variables in inhaled chemical research and testing.

Third, bronchial epithelial cells play an important role in the induction of proinflammatory and oxidative stress-responsive gene expression in response to O_3; however, these are not their only role in these exposures. Comparing the effects of single and repeated exposures, on other key events in an AOP or endpoints (eg, changes in relative numbers of different epithelial cell types, ciliary beat frequency, total viability, and barrier function) was beyond the scope of this study. Although not quantified directly in this study, the ozone dose used in this study is not known to be cytotoxic and higher doses have relatively small effects on viability (Devlin *et al.*, 1994; Hatch *et al.*, 2014; Wu *et al.*, 2011). Further, we examined cultures before and after exposures for indicators of cell death including loss of ciliary beating, fluid accumulation on the apical side of the insert (indicator of loss of epithelial barrier function), and loss of barrier continuity (formation of "holes" in the epithelial layer), which were not observed following either single or repeated exposures. Additionally, TEER values were not significantly different following single and repeated O_3 exposures, suggesting that there was no overt loss of viability in the repeated exposure condition. Incorporating these additional endpoints into future studies will be important to understanding the role of these variables on exposure outcomes and the utility of *in vitro* models in inhaled chemical testing and research.

Fourth, the ranges for the various measurements in this study suggest that the interindividual variability captured by *in vitro* studies may be more than the typical factor ascribed to the TD component of the UF_H. Because these results are restricted to the domain of the pHBEC-ALI model, the TD variability demonstrated should be explored in other human cell exposure systems. In particular, the role of the exposure system (eg, ALI or submerged culture) and dosimetry determinants such as physicochemical properties that alter the TK component should be explored for their impacts on the apparent-demonstrated variability in the TD component.

Fifth, while the *in vitro* differentiated pHBEC-ALI model contains key constituents of the pseudostratified bronchial epithelial barrier layer (ie, basal epithelial cells, ciliated epithelial cells, and goblet cells), the model used here does not include other biologically relevant factors that exist *in vivo* (eg, stromal cells/cellular microenvironment). We have recently demonstrated that

the effects of inhaled pollutant exposures in the lung extend beyond the bronchial epithelial barrier (Faber *et al.*, 2020). Given the function of airway stromal cells in maintaining lung homeostasis, considering their role as targets and mediators of inhaled chemical exposures through incorporation of these cell types in multicellular coculture models into future studies will be critical to the biological relevance and fidelity of *in vitro* inhaled chemical testing and research. As the biological complexity and thus relevance of *in vitro* models increases they will have greater utility in the assessment of whether attenuation of toxicant-induced effects occurs in multiple cell types, as well as what roles other cell types play in variability, susceptibility, and attenuation.

CONCLUSIONS

Examining the effects of repeated daily exposure to inhaled chemicals with *in vitro* models is critical to characterizing the effects of "real world" inhaled chemical exposure scenarios. Our observations in this study highlight several important considerations for inhaled chemical research and testing with *in vitro* models. First, differentiated pHBEC-ALI culture models recapitulate targeted single and short-term repeated exposure outcomes when used to represent the bronchial epithelium *in vivo*. Second, single and short-term repeated exposures lead to different outcomes in some, but not all, endpoints involved in the response to toxicant exposures. Here, we observed that there was not a significant difference in the effect of single and repeated O_3 exposures on TEER, but there were significant differences in the induction of O_3-responsive gene transcripts following single and repeated exposures. This study was limited to the comparison of a single day and 4 days of O_3 exposure; however, the degree to which a single exposure is representative of multiple exposure outcomes should also be considered in context with respect to specific chemicals and physicochemical properties, exposure systems, cell models, and evaluated endpoints or key events of pathogenesis. Other single and repeated exposure outcomes may not be similar, especially if the TK processes inherent in the exposure system, dosimetry mechanisms, or key events of pathogenesis due to physicochemical properties are in a different domain than O_3 as an oxidant gas. Further, endpoints such as TEER do not appear to be reflective of transcriptional differences that occur between single and repeated exposure scenarios. Ultimately, additional studies are required to determine which endpoints have similar and divergent outcomes in single and repeated exposure scenarios within physicochemical categories and whether those relationships are affected by the number of repeated exposures. Third, while the traditional approach of using 3 biological replicates may be suitable for observation of biological phenomena in an isogenic background, larger numbers of biological replicates are required to represent the normal range of interindividual variability. For example, using the data collected in this study we determined that 13–27 primary cell donors would be required to provide sufficient power to identify statistically significant differences in a 2.0-fold change in the 1X condition for most exposure-response genes, with about half as many being required to assess attenuation effects. Further, the donor population used for this study was relatively homogenous (ie, young, healthy, nonsmoking donors) and the magnitude of variability and attenuation effect would differ in cultures derived from a more diverse donor population. Fourth, our findings suggest that the use of the default value for the TD component of the UF_H (ie, 3.16-fold) may be underestimating interindividual variability and highlight a unique opportunity to use primary cell-based *in vitro* models to empirically refine UF_H in chemical risk assessment. Overall, the observations reported here highlight the need and the value in improving our understanding of the role, and underlying mechanisms, of interindividual variability and impact of repeated exposures on the use of data derived from *in vitro* NAMs in risk assessment.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

AUTHOR CONTRIBUTIONS

L.A.D. cultured primary cell cells until they were ready for experimental use. D.S.M., S.D.M., L.A.D., and E.C.B. conducted exposures, cell harvests, processed samples, and performed all other experiments. E.C.B., D.D.S., and S.D.M. designed the study. S.D.M. and E.M.M. conducted the data analysis. S.D.M., E.M.M., and A.M.J. wrote the article. All authors reviewed the article.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

DISCLAIMER

The research described in this article has been reviewed by the U.S. Environmental Protection Agency and approved for publication. The contents of this article do not necessarily represent Agency policy, nor does mention of trade names or commercial products constitute endorsement or recommendations for use.

REFERENCES

Alam, J., and Cook, J. L. (2007). How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am. J. Respir. Cell Mol. Biol.* **36**, 166–174.

Aris, R. M., Christian, D., Hearne, P. Q., Kerr, K., Finkbeiner, W. E., and Balmes, J. R. (1993). Ozone-induced airway inflammation in human subjects as determined by airway lavage and biopsy. *Am. Rev. Respir. Dis.* **148**, 1363–1372.

Bayram, H., Rusznak, C., Khair, O. A., Sapsford, R. J., and Abdelaziz, M. M. (2002) Effect of ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures of non-asthmatic and asthmatic subjects. *Clin. Exp. Allergy* **32**, 1285–1292.

Bayram, H., Sapsford, R. J., Abdelaziz, M. M., and Khair, O. A. (2001). Effect of ozone and nitrogen dioxide on the release of proinflammatory mediators from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients in vitro. *J. Allergy Clin. Immunol.* **107**, 287–294.

Bisogno, L. S., Yang, J., Bennett, B. D., Ward, J. M., MacKey, L. C., Annab, L. A., Bushel, P. R., Singhal, S., Schurman, S. H., Byun, J. S., *et al.* (2020). Ancestry-dependent gene expression correlates with reprogramming to pluripotency and multiple dynamic biological processes. *Sci. Adv.* **6**, 1–14.

Bogdanffy, M., and Jarabek, A. (1995). Understanding mechanisms of inhaled toxicants: Implications for replacing default factors with chemical-specific data. *Toxicol. Lett.* **82–83**, 919–932.

Bowers, E. C., McCullough, S. D., Morgan, D. S., Dailey, L. A., and Diaz-Sanchez, D. (2018). ERK1/2 and p38 regulate interindividual variability in ozone-mediated IL-8 gene expression in primary human bronchial epithelial cells. *Sci. Rep.* **8**, 1–11.

Champely, A., Ekstrom, C., Dalgaard, P., Gill, J., Weibelzahl, S., Anandkumar, A., Ford, C., Volcic, R., and De Rosario, H. (2020) pwr. Available at: https://nyuscholars.nyu.edu/en/publications/pwr-basic-functions-for-power-analysis. Accessed November 1, 2021.

Christian, D. L., Chen, L. L., Scannell, C. H., Ferrando, R. E., Welch, B. S., and Balmes, J. R. (1998). Ozone-induced inflammation is attenuated with multiday exposure. *Am. J. Crit. Care Med.* **158**, 532–537.

Clippinger, A. J., Allen, D., Behrsing, H., BéruBé, K. A., Bolger, M. B., Casey, W., DeLorme, M., Gaça, M., Gehen, S. C., Glover, K., *et al.* (2018a). Pathway-based predictive approaches for non-animal assessment of acute inhalation toxicity. *Toxicol. In Vitro* **52**, 131–145.

Clippinger, A. J., Allen, D., Jarabek, A. M., Corvaro, M., Gaça, M., Gehen, S., Hotchkiss, J. A., Patlewicz, G., Melbourne, J., Hinderliter, P., *et al.* (2018b). Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements: An international workshop report. *Toxicol. In Vitro* **48**, 53–70.

Collins, F. S., Gray, G. M., and Bucher, J. R. (2008). Transforming environmental health protection. *Science*, **319**, 906–907.

Dailey, L., and McCullough, S. (2021a). Culture of primary human tracheobronchial epithelial cells. *Protoc. Exch.* doi: 10.21203/rs.3.pex-1379/v1.

Dailey, L., and McCullough, S. (2021b). Establishing differentiated air-liquid interface primary human bronchial epithelial cell cultures. *Protoc. Exch.* doi: 10.21203/rs.3.pex-1380/v1.

Devlin, R. B., Folinsbee, L. J., Biscardi, F., Hatch, G., Becker, S., Madden, M. C., Robbins, M., and Koren, H. S. (1997). Inflammation and cell damage induced by repeated exposure of humans to ozone. *Inhal. Toxicol.* **9**, 211–235.

Devlin, R. B., McDonnell, W. F., Becker, S., Madden, M. C., McGee, M. P., Perez, R., Hatch, G., House, D. E., and Koren, H. S. (1996). Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4ppm ozone for 2hr: A comparison of mediators found in broncoalveolar lavage fluid 1 and 18 hr after exposure. *Toxicol. Appl. Pharmacol.* **138**, 176–185.

Devlin, R. B., McDonnell, W. F., Mann, R., Becker, S., House, D. E., Schreinemachers, D., and Koren, H. S. (1991). Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am. J. Resp. Cell Mol. Biol.* **4**, 72–81.

Devlin, R. B., McKinnon, K. P., Noah, T., Becker, S., and Koren, H. S. (1994). Ozone-induced release of cytokines and fibronectin by alveolar macrophages and airway epithelial cells. *Am. J. Physiol.* **266**, 612–619.

Faber, S. C., McNabb, N. A., Ariel, P., Aungst, E. R., and McCullough, S. D. (2020). Exposure effects beyond the epithelial barrier: Transepithelial induction of oxidative stress by diesel exhaust particulates in lung fibroblasts in an organotypic human airway model. *Toxicol. Sci.* **177**, 140–155.

Ganger, M. T., Dietz, G. D., and Ewing, S. J. (2017). A common base method for analysis of qPCR data and the application of simple blocking in qPCR experiments. *BMC Bioinformatics* **18**, 11.

Grimm, F. A., Blanchette, A., House, J. S., Ferguson, K., Hsieh, N. H., Dalaijamts, C., Wright, A. A., Anson, B., Wright, F. A., Chiu, W. A., *et al.* (2018). A human population-based organotypic in vitro model for cardiotoxicity screening. *ALTEX* **35**, 441–452.

Harrill, J., Shah, I., Setzer, R. W., Haggard, D., Auerbach, S., Judson, R., and Thomas, R. S. (2019) Considerations for strategic use of high-throughput transcriptomics chemical screening data in regulatory decisions. *Curr. Opin. Toxicol.* **15**, 64–75.

Hatch, G. E., Duncan, K. E., Diaz-Sanchez, D., Schmitt, M. T., Ghio, A. J., Carraway, M. S., Mckee, J., Dailey, L. A., Berntsen, J., and Devlin, R. B. (2014). Progress in assessing air pollutant risks from in vitro exposures: Matching ozone dose and effect in human airway cells. *Toxicol. Sci.* **141**, 198–205.

Hernandez, M. L., Lay, J. C., Harris, B., Esther, C. R. J., Brickey, W. J., Bromberg, P. A., Diaz-sanchez, D., Devlin, R. B., Kleeberger, S. R., Alexis, N. E., *et al.* (2010). Atopic asthmatic subjects but not atopic subjects without asthma have enhanced inflammatory response to ozone. *J. Allergy. Clin. Immunol.* **126**, 537–544.

Holz, O., Jörres, R., Timm, P., Mücke, M., Richter, K., Koschyk, S., and Magnussen, H. (1999). Ozone-induced airway inflammatory changes differ between individuals and are reproducible. *Am. J. Resp. Crit. Care Med.* **159**, 776–784.

Interagency Coordinating Committee on the Validation of Alternative Methods. (2018) A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States. doi: 10.22427/NTP-ICCVAM-ROADMAP2018.

Jaspers, I., Chen, L. C., and Flescher, E. (1998). Induction of interleukin-8 by ozone is mediated by tyrosine kinase and protein kinase A, but not by protein kinase C. *J. Cell. Physiol.* **177**, 313–323.

Jaspers, I., Flescher, E., and Chen, L. C. (1997). Ozone-induced IL-8 expression and transcription cells factor binding in respiratory epithelial cells. *Am. J. Physiol.* **272**, L504–11.

Jessen, C., Kreß, J. K. C., Baluapuri, A., Hufnagel, A., Schmitz, W., Kneitz, S., Roth, S., Marquardt, A., Appenzeller, S., Ade, C. P.,

et al. (2020). The transcription factor NRF2 enhances melanoma malignancy by blocking differentiation and inducing COX2 expression. *Oncogene* **39**, 6841–6855.

Jörres, R. A., Holz, O., Zachgo, W., Timm, P., Koschyk, S., Müller, B., Grimminger, F., Seeger, W., Kelly, F. J., Dunster, C., *et al.* (2000). The effect of repeated ozone exposures on inflammatory markers in bronchoalveolar lavage fluid and mucosal biopsies. *Am. J. Respir. Crit. Care Med.* **161**, 1855–1861.

Judson, R. S., Kavlock, R. J., Setzer, R. W., Hubal, E. A. C., Martin, M. T., Knudsen, T. B., Houck, K. A., Thomas, R. S., Wetmore, B. A., and Dix, D. J. (2011). Estimating toxicity-related biological pathway altering doses for high-throughput chemical risk assessment. *Chem. Res. Toxicol.* **24**, 451–462.

Kim, C. S., Alexis, N. E., Rappold, A. G., Kehrl, H., Hazucha, M. J., Lay, J. C., Schmitt, M. T., Case, M., Devlin, R. B., Peden, D. B., *et al.* (2011). Lung function and inflammatory responses in healthy young adults exposed to 0.06 ppm ozone for 6.6 hours. *Am. J. Respir. Crit. Care Med.* **183**, 1215–1221.

Kleinstreuer, N. C., Yang, J., Berg, E. L., Knudsen, T. B., Richard, A. M., Martin, M. T., Reif, D. M., Judson, R. S., Polokoff, M., Dix, D. J., *et al.* (2014). Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nat. Biotechnol.* **32**, 583–591.

Knörr-Wittmann, C., Hengstermann, A., Gebel, S., Alam, J., and Müller, T. (2005). Characterization of Nrf2 activation and heme oxygenase-1 expression in NIH3T3 cells exposed to aqueous extracts of cigarette smoke. *Free Radic. Biol. Med.* **39**, 1438–1448.

Koren, H. S., Devlin, R. B., Graham, D. E., Mann, R., McGee, M. P., Horstman, D. H., Kozumbo, W. J., Becker, S., House, D. E., and McDonnell, W. F. (1989). Ozone-induced inflammation in the lower airways of human subjects. *Am. Rev. Respir. Dis.* **139**, 407–415.

Mackey, L. C., Annab, L. A., Yang, J., Rao, B., Kissling, G. E., Schurman, S. H., Dixon, D., and Archer, T. K. (2018). Epigenetic enzymes, age, and ancestry regulate the efficiency of human iPSC reprogramming. *Stem Cells* **36**, 1697–1708.

McCullough, S., Duncan, K., Swanton, S., Dailey, L., Diaz-Sanchez, D., and Devlin, R. (2014). Ozone induces a pro-inflammatory response in primary human bronchial epithelial cells through MAP kinase activation without NF-κB activation. *Am. J. Respir. Cell Mol. Biol.* **51**, 1–45.

McCullough, S. D., Bowers, E. C., On, D. M., Morgan, D. S., Dailey, L. A., Hines, R. N., Devlin, R. B., and Diaz-Sanchez, D. (2016). Baseline chromatin modification levels may predict interindividual variability in ozone-induced gene expression. *Toxicol. Sci.* **150**, 216–224.

National Research Council. (2007). *Toxicity Testing in the 21st Century: A Vision and a Strategy*. National Research Council of the National Academies, Washington, DC.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Postlethwait, E. M., Joad, J. P., Hyde, D. M., Schelegle, E. S., Bric, J. M., Weir, A. J., Putney, L. F., Wong, V. J., Velsor, L. W., and Plopper, C. G. (2000). Three-dimensional mapping of ozone-induced acute cytotoxicity in tracheobronchial airways of isolated perfused rat lung. *Am. J. Respir. Cell Mol. Biol.* **22**, 191–199.

Rahman, I., Clerch, L. B., and Massaro, D. (1991). Rat lung antioxidant enzyme induction by ozone. *Am. J. Physiol.* **260**, 412–418.

Seltzer, J., Bigby, B. G., Stulbarg, M., Holtzman, M. J., Nadel, J. A. Y. A., Ueki, I. F., Leikauf, D., Goetzl, E. J., and Boushey, H. A. (1986). O3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J. Appl. Physiol.* **60**, 1321–1326.

Taylor, A. B., Borhan, A., and Ultman, J. S. (2007). Three-dimensional simulations of reactive gas uptake in single airway bifurcations. *Ann. Biomed. Eng.* **35**, 235–249.

Thurston, G., Kipen, H., Annesi-Maesano, I., Brook, R., Cromar, K., Matteis, S., De Forastiere, F., Forsberg, B., Frampton, M., Grigg, J., *et al.* (2017). A joint ERS/ATS policy statement: What constitutes an adverse health effect of air pollution? An analytical framework. *Eur. Respir. J.* **49**, 1–34.

United States Environmental Protection Agency. (2020a) EPA New approach methods work plan: Reducing use of animals in chemical testing. United States Environmental Protection Agency, Washington, DC, EPA 615B2001.

United States Environmental Protection Agency (2020b) Integrated Science Assessment (ISA) for Ozone and Related Photochemical Oxidants. U.S. Environmental Protection Agency, Washington, DC, EPA/600/R-20/012, 2020.

Van Bree, L., Dormans, J. A. M. A., Koren, H. S., Devlin, R. B., and Rombout, P. J. A. (2002). Attenuation and recovery of pulmonary injury in rats following short-term, repeated daily exposure to ozone. *Inhal. Toxicol.* **14**, 883–900.

Wang, Y., Tan, M., Ouyang, H., and Deng, L. (2018). Effects of ozone stimulation of bronchial epithelial cells on proliferation and collagen synthesis of co-cultured lung fibroblasts. *Exp. Ther. Med.* **15**, 5314–5322.

Wiester, M. J., Tepper, J. S., Winsett, D. W., Crissman, K. M., Richards, J. H., and Costa, D. L. (1996). Adaptation to ozone in rats and its association with ascorbic acid in the lung. *Fundam. Appl. Toxicol.* **31**, 56–64.

Wiester, M. J., Winsett, D. W., Richards, J. H., Jackson, M. C., Crissman, K. M., and Costa, D. L. (2000). Ozone adaptation in mice and its association with ascorbic acid in the lung. *Inhal. Toxicol.* **12**, 577–590.

Wruck, C. J., Streetz, K., Pavic, G., Götz, M. E., Tohidnezhad, M., Brandenburg, L. O., Varoga, D., Eickelberg, O., Herdegen, T., Trautwein, C., *et al.* (2011). Nrf2 induces interleukin-6 (IL-6) expression via an antioxidant response element within the IL-6 promoter. *J. Biol. Chem.* **286**, 4493–4499.

Wu, W., Doreswamy, V., Diaz-sanchez, D., Samet, J. M., Kesic, M., Dailey, L., Zhang, W., Jaspers, I., and Peden, D. B. (2011). GSTM1 modulation of IL-8 expression in human bronchial epithelial cells exposed to ozone. *Free Radic. Biol. Med.* **51**, 522–529.