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Acute air pollution exposure alters neutrophils in never-smokers and at-risk humans

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Social media take-home message: Acute human exposure to traffic-related air pollution leads lower airway neutrophils to release neutrophil extracellular traps, linked to COPD severity and airflow limitation. Ex-smokers and COPD patients mount a greater neutrophil response to exposure.

ABSTRACT

Outdoor air pollution exposure increases COPD hospitalizations, and may contribute to COPD development. The mechanisms of harm, and the extent to which at-risk populations are more susceptible, are not fully understood. Neutrophils are recruited to the lung following diesel exhaust (DE) exposure, a model of traffic-related air pollution (TRAP), but their functional role in this response is unknown. The purpose of this controlled human exposure crossover study was to assess the effects of acute DE exposure on neutrophil function in never-smokers and at-risk populations, with support from additional *in vitro* studies. 18 participants, including never-smokers (N=7), ex-smokers (N=4), and mild-moderate COPD patients (N=7), were exposed to DE and filtered air (FA) for 2 hours on separate occasions, and neutrophil function in blood (0h and 24h post) and bronchoalveolar lavage (24h post) was assessed. Compared to FA, DE exposure reduced the proportion of circulating band cells at 0h, which was exaggerated in COPD patients. DE increased the amount of neutrophil extracellular traps (NETs) in the lung across participants. COPD patients had increased peripheral neutrophil activation following DE. *In vitro,* suspended diesel exhaust particles also increased the amount of NETs measured in isolated neutrophils. We propose NET formation as a possible mechanism through which TRAP exposure affects airway pathophysiology. In addition, COPD patients may be more prone to an activated inflammatory state following exposure. This is the first controlled human TRAP exposure study directly comparing at-risk phenotypes (COPD and ex-smoker) with lower-risk (never-smokers), elucidating the human susceptibility spectrum.

Significance

Exposure to traffic-related air pollution is a significant contributor to respiratory morbidity and mortality. Epidemiologically, these exposures tend to impact susceptible populations, such as those living with chronic obstructive respiratory disease (COPD) disproportionately, through unknown mechanisms. In this controlled human study of exposure to diesel exhaust, we determined that neutrophils, our immune system's first-responders, release neutrophil extracellular traps (NETs) following exposure. NET release is known to be related to COPD severity and worsening of lung function. We also showed that blood neutrophils of susceptible individuals, such as people with COPD, elicited a greater response to diesel exhaust than never-smokers. This study identifies new mechanisms of potential harm and susceptibility to this ubiquitous pollutant.

INTRODUCTION

Outdoor air pollution is a significant contributor to global morbidity and is responsible for 3.3 million yearly premature deaths worldwide [1, 2]. Traffic-related air pollution (TRAP) is one of the most significant sources of outdoor air pollution exposure in developed countries [1, 2]. Chronic obstructive pulmonary disease (COPD) is the 3rd leading cause of global years of life lost to non-communicable disease [3]. Epidemiological studies consistently demonstrate a strong association between airborne pollutant levels and COPD exacerbations requiring hospitalization [4-8], indicating that COPD patients may be more susceptible to the harmful effects of pollutant exposure than the general population. In addition, populations susceptible to the harmful effects of air pollution exposure may extend beyond those living with disease, including ex-smokers without COPD, as well as children and adolescents [9].

The mechanisms through which air pollution exerts it harmful effects, and how these may differ in susceptible populations, are not completely understood. While a number of controlled human exposure studies to diesel exhaust (DE), a model of TRAP, described inflammation and oxidative stress as key mechanisms [10-12], no studies of this kind have ever included at-risk groups such as COPD patients and ex-smokers to delineate the underlying mechanisms of their potential susceptibility to these exposures.

Neutrophils are recruited to the lung following acute controlled human exposure to DE [12-15]. However, very little is known about their functional role in this response. Neutrophils play a key role in protection through their antimicrobial defenses [16] ,but a high level of neutrophil activation is a hallmark of COPD correlating with disease severity [17]. Neutrophil extracellular

traps (NETs) consist of neutrophil-derived DNA that is released from neutrophils in chromatin filaments forming web-like structures coated with granular histone protein [18]. NETs mediate a wide range of inflammatory diseases including asthma [19] and COPD, where they are found in high concentrations in the airways and correlate with airflow limitation [20-22].

The primary purpose of this controlled human exposure crossover study was to determine the effect of acute TRAP (DE) exposure, on neutrophil function in the blood and lower airways in humans. Second, we aimed to assess whether at-risk populations, namely ex-smokers and COPD patients, experienced greater effects of DE exposure relative to never-smokers. We hypothesized that DE increases neutrophil activation and NET formation, and decreases peripheral band cell levels and these effects are enhanced in ex-smokers and those with COPD.

METHODS

Human exposure study design

In this randomized double-blind, controlled human exposure crossover study, 18 participants were exposed for 2h on separate occasions to diluted diesel exhaust (DE) and filtered air (FA) (**Figure 1**, Clinical Trial ID: NCT02236039). Exposures were randomized to order by an automated algorithm, with a minimum 4-wk washout period, and took place at the Air Pollution Exposure Laboratory at Vancouver General Hospital in Vancouver, Canada, between August 2017 and May 2019. Participant and investigator were blind to the exposure. Participants abstained from any short- or long-acting bronchodilator use, inhaled corticosteroid use and nutritional supplements for 48h, and caffeine for 12h prior to exposures, and visits were rescheduled if participants exhibited signs of upper respiratory tract infection (Common Cold Questionnaire). Sample size was determined from previous controlled human exposure study data demonstrating a change in blood neutrophil counts after DE exposure with 15 participants [13]. Added detail for the methods described below, including detailed exclusion criteria, antibody details, flow cytometry controls, and methods for quality control in cell counting, is available in the online supplement.

Participants

Participants aged 40-80 were recruited via local online classified advertisements, flyers, clinical referrals and newspaper advertisements. An initial telephone screening identified eligible participants from three phenotypes: never-smokers, ex-smokers without COPD (minimum 10 pack-years smoking history and quit >6 months prior), and mild-moderate COPD patients (quit smoking >6 months prior). Individuals then visited the lab for an in-person screening visit to

assess baseline spirometry, confirm participant phenotype (COPD if $FEV_1/FVC < 0.7$, $FEV_1 \geq$ 50% predicted) and study enrolment if eligible **(Table 1)**. All participants self-reported a least 6 months of smoking cessation prior to study enrolment, which was continued throughout the study. Serum cotinine was measured at each exposure visit to verify abstinence from smoking, which remained <10ng/mL for all participants (Cotinine ELISA Kit, OriGene Technologies, Inc, catalog# EA100901).

Table 1. Participant characteristics

Group	Sex	Age	Pack-yrs smoking history	Post-bronchodilator FEV_1/FVC
Never-smoker	M	63	-	78
Never-smoker	M	58	-	75
Never-smoker	F	48	-	81
Never-smoker	F	72	-	80
Never-smoker	M	50	-	79
Never-smoker	F	42	-	86
Never-smoker	F	56	-	81
Ex-smoker	M	73	30	91
Ex-smoker	M	70	90	72
Ex-smoker	M	63	47	70
Ex-smoker	M	56	50	74
COPD	M	80	10	54
COPD	F	66	24	54
COPD	F	70	12	48
COPD	M	67	52.5	49
COPD	M	65	45	41
COPD	M	75	7	67
COPD	M	70	19.5	64

Exposures

Exposures were 2h in duration and followed a previously described protocol with endotoxin levels below a limit of detection of 0.52 EU/m^3 **(Table 2)** [23]. DE is a robust model of TRAP,

and a dose approximating 300μg/m^3 of particulate matter sized 2.5 microns or less ($PM_{2.5}$) was used to represent exposure levels documented in polluted mega-cities and occupational exposures [23, 24]. Participants cycled on a stationary bike for 2 bouts of 15m at 30% maximal oxygen uptake (VO_2-max) to simulate a breathing pattern of typical outdoor daily activity such as walking.

Table 2. Exposure characteristics

	$PM_{2.5}$ (μg/m^3)	NO (ppb)	NO_2 (ppb)	NO_x (ppb)	CO (ppm)	CO_2 (ppm)	TVOC (ppb)
DE	260 ± 55	3340 ± 2592	114 ± 104	3450 ± 2597	13 ± 3	1963 ± 974	1407 ± 788
FA	4 ± 3	211 ± 173	20 ± 16	230 ± 184	2 ± 1	2147 ± 2476	158 ± 110

Sampling

Blood samples were collected into EDTA tubes, placed on ice then processed immediately. A bronchoscopy was performed at 24h, and bronchoalveolar lavage (BAL) from the upper lobe was obtained with 2x50mL or 2x60mL instillations of saline, obtained from the opposite lobe with consistent volume in each of the two exposures. Samples were kept on ice and processed immediately. BAL was spun and cells were resuspended in RPMI medium for the assays described below.

Peripheral blood and BAL neutrophil flow cytometry

Blood and BAL were stained for CD45, CD16, CD66b, Activated CD11b (Mac-1) and CXCR2. In blood and BAL [25], live neutrophils were identified as CD45+, CD16+, CD66b+, LIVE/DEAD- events, which were assessed for their expression of CD16, CD66b and CXCR2,

quantified as the change from baseline median fluorescence intensity (MFI) across the exposure. The percentage of neutrophils expressing an activated form of CD11b (Mac-1; CBRM 1/5 clone), which may by expressed only after a signal is received from a stimulus such as a chemoattractant, was also quantified.

Peripheral blood neutrophil and band cell differential

500 leukocytes per time point were identified in blood smears by a skilled observer (quality control details in supplement) as mature polymorphonuclear cells (PMN), band cells, eosinophils, or others.

BAL neutrophil extracellular traps

150,000 BAL cells were seeded onto uncoated round coverglass, fixed, blocked, and stained for the hallmark NETs markers H3 citrullinated histones (H3cit), neutrophil elastase (NE) and Hoechst 33342 DNA stain. A 4x4 grid of photos for each slide was acquired at 60X magnification and stitched into one large image (EVOS FL Auto Imaging System). NETs were defined as structures meeting these three criteria: 1) clear extracellular fibrous citrullinated histone strands, 2) positive DNA stain, and 3) positive NE stain. NETs were quantified as NET-covered area (by tracing) normalized to total nuclear area (by automatic quantification on binary image) and expressed as a % of total nuclear area.

In vitro study design

Neutrophils were isolated from peripheral blood of four consenting male volunteers (N=2 healthy never-smokers, N=2 mild asthmatics) using magnetic negative selection (StemCell EasySep direct human neutrophil isolation kit) then resuspended in RPMI medium supplemented with 2% fetal bovine serum (FBS). Cells were stimulated *in vitro* with diesel exhaust particles (DEPs) obtained from the diesel engine exhaust outlet of the Air Pollution Exposure Laboratory described above, after suspending in the same media and sonicating to prevent agglomeration.

Peripheral blood neutrophil extracellular traps by flow cytometry and immunocytochemistry

For flow cytometric quantification of NETs stimulated *in vitro,* $1x10^6$ isolated neutrophils were stimulated for 2h *in vitro* with 100μg/mL DEPs, 4μM ionomycin (positive control) or media alone (negative control). Using a previously described flow cytometry assay [26], NETs were quantified as the percentage of cells staining triple-positive, without a permeabilization step, for myeloperoxidase (MPO), H3Cit and DAPI DNA stain (all antibody details are found in supplement). To visualize NETs by immunocytochemistry,120,000 isolated neutrophils were seeded for 1h in an incubator onto uncoated, round coverglass, then stimulated for 2h with 100μg/mL DEPs, 4μM ionomycin, DEPs + ionomycin at these same concentrations, or media. Cells were fixed, blocked, and stained in the same manner as the BAL NETs assay, except neutrophil elastase was used at the protein marker instead of MPO. NETs were assessed qualitatively with confocal microscopy (Zeiss LSM780 laser scanning confocal microscope).

Statistics

Human exposure study data was analyzed using mixed effects models (nlme package, version 3.1-131) in RStudio (Version 1.1.383). The primary outcome was to assess the fixed effect of exposure (DE vs. FA) on each endpoint, with participant ID as the random effect. The secondary outcome was to assess whether participant phenotype (never-smoker, ex-smoker, COPD) modified the effect of exposure (exposure x phenotype interaction), with participant ID as the random effect. The potential for order effect was assessed, with order (FA first vs. DE first) as the fixed effect and participant ID as the random effect. P-values <0.05 were considered statistically significant. *In vitro* data and baseline neutrophil counts were analyzed in Prism 6 (GraphPad Software Inc.) using a repeated measures one-way ANOVA and Sidak's multiple comparisons test.

Study approval

This study was approved by the University of British Columbia Clinical Research Ethics Board (H14-00821) and Vancouver Coastal Health Research Institute (V14-00821). All participants provided written informed consent prior to inclusion.

RESULTS

Peripheral blood neutrophil surface marker activation was greater in COPD after DE

Cell surface expression was quantified as median fluorescence intensity (MFI) and data are presented as mean effect, 95% CI, p-value for interaction effect, where MFI values represent deltas (post-exposure minus pre-exposure for DE minus FA). Patient phenotype significantly modified the effect of DE exposure, such that individuals with COPD had increased peripheral neutrophil CD16 (MFI +262, 113 to 414, p=0.006) and CXCR2 expression (MFI +292, 150 to 434, p = 0.002), as well increased % activated CD11b-expressing neutrophils (+12.2%, -152 to 22, p=0.046) relative to never-smokers 24h after DE **(Figure 2)**. There was no main effect of DE and no modifying effect of order, on activation marker expression in peripheral blood or BAL neutrophils, and patient phenotype did not significantly modify the effect of DE on neutrophil surface markers in the BAL.

Peripheral blood band cell and neutrophil counts decreased after DE exposure

Data are presented as mean values (%) and 95% CI where percentages represent the change in deltas (DE [post minus pre] vs. FA [post minus pre]). Circulating band cells decreased immediately following DE exposure, when expressed as a percentage of total neutrophils (mature + band), (-2.1%, -0.3 to -4.0, p = 0.04) **(Figure 3)**. At 24 hours, DE decreased the percentage of total neutrophils (-6.2%, -0.82 to -11.6, p = 0.04) but not band cells. Furthermore, the reduction in peripheral band cells immediately following exposure was modified by patient phenotype, showing a greater reduction in band cell percentage in COPD patients, when measured as a percentage of total cells (-2.8, -5.0 to -0.42, p=0.04), relative to never-smokers. There was no effect modification by order.

DE exposure increased the amount of NETs detectable in the lower airways

DE exposure increased the amount of NETs in BAL **(Figure 4)**, when measured as the proportion (%) of DNA-covered area that was associated with markers of NETs (+7.5%, 0.6 to 14.3, p = 0.04). There was no modifying effect by patient phenotype or order.

Baseline inflammatory state did not predict high and low responders

For outcomes where the effect of DE was modified by patient phenotype (peripheral blood band cell counts and peripheral blood CXCR2 expression), or where there was a clear high-responding group (BAL NETs), we sought to assess whether this was attributable to an elevated baseline inflammatory state (defined by baseline peripheral blood neutrophil counts). However, there was no significant difference between the three patient phenotypes in baseline neutrophil counts (**Supplemental Figure S1**), and baseline blood neutrophil counts did not modify the effect of DE on the outcomes described above when assessed as an interaction term in our mixed effects model.

DEPs induce NET formation *in vitro*

Given inherent challenges in accurately assessing NETs [27], multiple methods were used to assess the presence of NETs after *in vitro* stimulation with DEPs. For quantitative analyses, flow cytometry was employed [26], permitting rapid and robust quantification of *in vitro* samples independent of observer bias. In addition, qualitative visual assessment of NET-like morphology and the presence of NETs markers was confirmed using immunocytochemistry. There was a significant increase in the percentage of neutrophils forming NETs measured by flow cytometry

after *in vitro* stimulation of peripheral neutrophils with DEPs (37%, 19 to 55, $p<0.01$), ionomycin (26%, 13 to 39, $p<0.05$) and the combination of DEPs and ionomycin (51%, 20 to 83, $p<0.001$) **(Figure 5).** Combined stimulation with DEPs and ionomycin augmented NET formation relative to ionomycin alone (26%, 2 to 50, $p<0.05$). NET-like structures from these samples could also be visualized in immunocytochemistry images.

DISCUSSION

This is the first controlled human TRAP exposure study to examine at-risk COPD and ex-smoker phenotypes, along with healthy never-smokers, allowing for a direct comparison of exposure responses across the human susceptibility spectrum. Additionally, this study examined the functional role of neutrophils as a key cell type mediating innate immune responses in the circulation and airways following TRAP exposure. Indeed, previous studies have demonstrated that neutrophils migrate to the lungs following TRAP exposure [12-15, 28] and there is a significant body of evidence demonstrating that neutrophilic inflammation contributes to lung tissue damage [29], disease severity [17], and chronic inflammatory signalling [16].

Our data demonstrate that acute DE exposure led to a more activated peripheral neutrophil phenotype (greater CD16, CXCR2, and activated CD11b expression) in COPD and ex-smokers compared to never-smokers. CD16 is a marker of neutrophil degranulation and activation [30], while CXCR2, the GRO-α and IL-8 receptor, is responsible for neutrophil trafficking to the lung [31] and is involved in some pathways of NETs formation [32]. Evidence shows that CXCR2 plays an essential role in regulating NETosis in this population [33]. Therefore, elevated CXCR2 expression due to DE exposure in those with COPD relative to never-smokers could increase neutrophil trafficking, NET formation and neutrophil responsiveness. Increased CD11b on neutrophil surfaces of COPD patients relative to never-smokers after DE is also relevant, as this marker correlates with secreted proteases like MMP-8 and MMP-9 in a cigarette smoke extract model [34], both of which contribute to emphysematous tissue damage and correlate with severity of emphysema [35]. In addition, the activated form of this protein, which we demonstrate is increased in COPD patients exposed to DE, makes up only a small portion of total

CD11b on neutrophil surfaces but is believed to be the most important functionally for its ability to interact with ICAM-1 on activated endothelium, and is thus a key protein for neutrophil extravasation from the blood stream [36]. These data show that peripheral neutrophils of COPD patients switch to a more activated state after DE exposure than those of a never-smoker. Interestingly, there was no effect modification in ex-smokers, suggesting that this elevated risk was reserved to the COPD group in our acute exposure study. Although previous work postulates that ex-smokers have more pronounced lung inflammation even one year after quitting [37], it is possible that the degree of inflammatory signalling, or its persistent and relentless nature in COPD relative to ex-smokers, led COPD patients to a susceptibility threshold that was not met by ex-smokers in our study. In addition, COPD patients can experience greater pollutant deposition for the same level of exposure, increasing their effective exposure dose and thus susceptibility to inflammatory effects [38].

Band cells are a marker of bone marrow stimulation and release of neutrophils into the circulating pool from the bone marrow [39, 40]. Observational studies show that peripheral band cells are increased by prolonged and high ambient air pollution and wood smoke exposure [39, 40], which contrasts our results and suggests that bone marrow stimulation may only occur above a certain exposure threshold. The reduction in circulating band cells after DE shown herein is in agreement with previous work demonstrating a disappearance of band cells from the blood immediately following cigarette smoke exposure in an animal model [41], where band cell disappearance results from their limited deformability and sequestration in the lung microvasculature, contributing to lung tissue damage associated with smoke-induced emphysema [41, 42]. This lends a conceivable mechanism through which band cells could contribute to lung

tissue damage in a chronic TRAP exposure setting. However, without a tracer study we cannot confirm the reason for reduced band cell counts in the current study. The reduction in circulating neutrophils 24h after exposure, with no increase in band cells, suggests that neutrophil recruitment to lung spaces seen in multiple previous acute DE exposure studies [13-15, 43] may represent recruitment of existing neutrophil pools (and thus reduction in the percentage of peripheral neutrophils), rather than stimulation of neutrophil release from the bone marrow (in which case peripheral neutrophil levels would presumably remain normal or high, despite recruitment into lung spaces).

Acute DE exposure increased BAL NET formation in 11 out of the 13 participants who had complete data. Increased amounts of NETs in the lung have been described in the context of COPD [20, 21], cigarette smoking [44], and e-cigarette use [45], but this is the first study to describe the same in an acute human TRAP exposure model. Clinically, increased NET formation is associated with COPD severity [22] and correlates with airflow limitation [21] in COPD patients. In addition, proteolytic neutrophil proteins contained in NETs are capable of damaging endothelium and epithelium [46, 47], while exposed histones can promote inflammation by acting as damage-associated molecular patterns [48]. Therefore, NET formation in the lung is a mechanism through which TRAP exposure could cause lung tissue damage characteristic of emphysema, and promote chronic inflammation in a repeated exposure setting. Interestingly, four participants had particularly marked NET formation that was not explained by any particular patient phenotype as there was at least one participant from each phenotype in this ‘high-responding’ group, nor was it explained by smoking history, or sex. Additional insight into

which factors increase susceptibility to biological effects of TRAP exposure including NET formation, such as gene-environment interactions, may be useful.

Our *in vitro* findings were largely supportive of our *in vivo* results. Using flow cytometry and fluorescence microscopy, we showed that DEPs stimulate NET formation in peripheral neutrophils. In addition, the *in vitro* response to ionomycin was enhanced in the presence of DEPs, suggesting that ionomycin and DEPs may stimulate NETs through different pathways. Thus, DEPs deposited into the lung could be responsible for stimulating NET formation in neutrophils that travel to the site following exposure, with the caveat that blood neutrophils in this *in vitro* study may be phenotypically different from those in the lung [49]. Alternatively, some studies have demonstrated DEPs can translocate into the blood, where they would indeed interact with peripheral neutrophils, though likely at lower concentrations. In this case, the potential for NET formation in the vasculature has implications on known cardiovascular effects of TRAP exposure [1, 50, 51].

One limitation of the current study was that BAL samples were used to assess neutrophil effects in the lung due to practical challenges of sample reliability (*e.g.* sputum) and low cell counts (*e.g.* bronchial wash) in other samples. Neutrophil recruitment following DE has been previously identified in bronchial wash and sputum [13-15, 43], and NETs have largely been studied in COPD sputum [20-22]. Nonetheless, a consistent NETs response was still seen in presumably the more protected lung compartment (lower airways) for pollutant-induced neutrophilic inflammation. Another limitation is sample size - this study was powered to detect a main effect of DE exposure, but we recognize the risk of type II error associated with considering clinical

phenotype as a modifying effect; still, even with low numbers, we detected clear effect modification therein. Overall, this crossover study adds to the existing knowledge of neutrophil recruitment following DE exposure, demonstrating that neutrophil activation is enhanced more in those with at-risk phenotypes. We postulate that acute DE exposure results in recruitment of existing neutrophil pool, and show that DE increases NET formation both *in vitro* and *in vivo*, highlighting novel mechanisms through which TRAP exposure may exert its harmful effects.

The Lancet Commission on Pollution and Health named research, including exploring emerging causal links between pollution, disease and subclinical impairment, as one of its six key recommendations to combat the massive global burden of pollution-related disease [1]. While a number of human exposure studies have begun to explain these mechanisms in healthy participants, much of the real-life burden occurs in vulnerable populations [1]. This study adds key mechanistic insight that can be used to support the policies pertaining to air pollution regulation that are paramount to public health protection, in addition to development or improvement of therapeutic approaches targeting neutrophilic inflammation in the context of air pollution exposure and respiratory disease.

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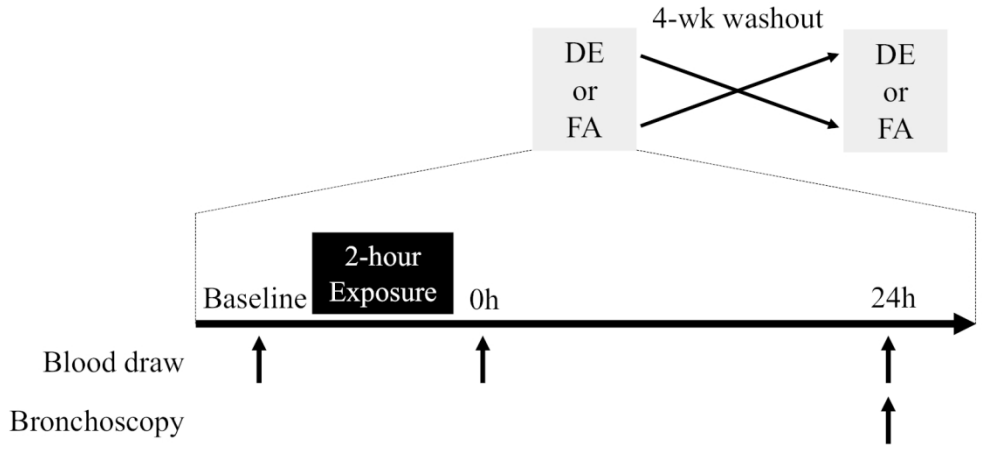

Figure 1. Study design. This was a randomized, controlled human exposure crossover study to diesel exhaust (DE, 300μg/m^3 $PM_{2.5}$) and filtered air (FA). 18 participants from three phenotypes (N=7 never-smokers, N=5 ex-smokers, and N=6 mild-moderate COPD) were exposed for 2 hours on separate occasions to DE and FA, in random order, separated by at least 4 weeks. Blood samples were obtained before and after exposures, and bronchoalveolar lavage was obtained during a bronchoscopy procedure 24h after exposure.

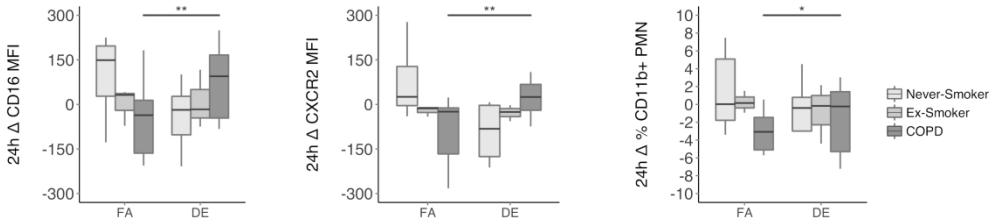

Figure 2. Patient phenotype modifies peripheral neutrophil activation 24h after diesel exhaust. The effect of acute diesel exhaust (DE) exposure on peripheral neutrophil CD16 and CXCR2 expression, and the percentage of polymorphonuclear cells (PMN) expressing the activated form of CD11b (N=17) was assessed using a linear mixed effect model with patient phenotype as an interaction term and participant ID as random effect. Neutrophil activation was significantly modified by patient phenotype such that those with COPD demonstrated increased expression in CD16, CXCR2, and CD11b expression relative to the change in never-smokers. For CD16 and CXCR2 panels, Y-axis denotes the change in expression from previous-day baseline sample (Δ = post-exposure minus pre-exposure), measured as median fluorescence intensity, and normalized to an unstained sample by subtraction. *=$p<0.05$, **=$p<0.01$, *FA = filtered air, DE = diesel exhaust, MFI = median fluorescence intensity, COPD = chronic obstructive pulmonary disease.*

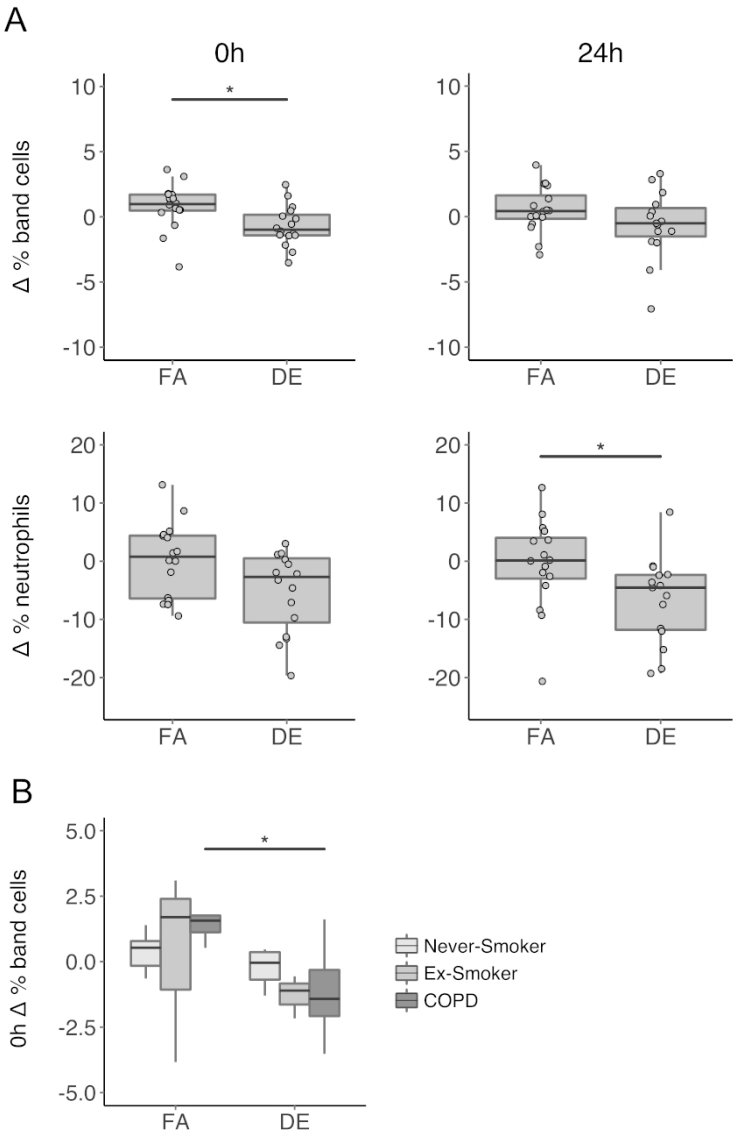

Figure 3. Diesel exhaust reduces percentage of peripheral blood band cells and neutrophils. (A) Differential neutrophil and band cell counts from blood smears (N=17) taken before exposure, at 0h (immediately after exposure) and 24h for all participants. Acute human exposure to diesel exhaust (DE) reduced the percentage of circulating band cells at 0h, and decreased the percentage of circulating total neutrophils (band + mature) 24h after exposure, compared to filtered air (FA). (B)The effect of exposure at 0h was modified by patient phenotype such that the decrease in band cells was greater in those with COPD than never-smokers. Effects were analyzed using a linear mixed effects model. Values are expressed as the change from same-exposure baseline (Δ = post-exposure minus pre-exposure), circles represent individuals.* = *p<0.05, FA = filtered air, DE = diesel exhaust.*

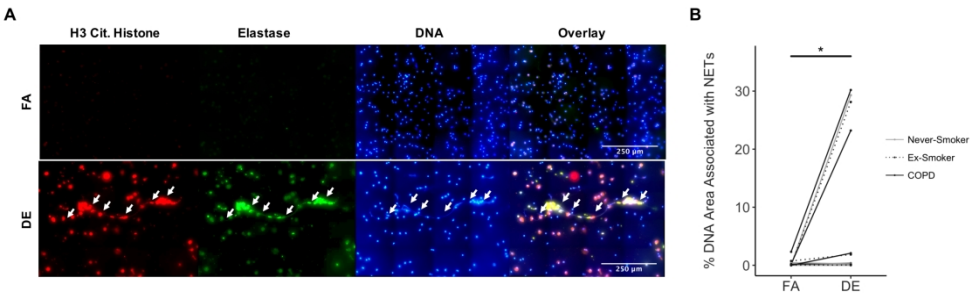

Figure 4. Representative NETs images in bronchoalveolar lavage. (A) Representative images and quantification of neutrophil extracellular traps (NETs) found in bronchoalveolar lavage (BAL) following acute filtered air (FA) and diesel exhaust (DE) exposure (N=15). Each pane shows a representative 4x4 stitched image of BAL after pelleting, resuspending and seeding onto glass slides, then staining for the following markers of NETs used to quantify BAL NETs: citrullinated histones (H3 Cit. Histone, red), neutrophil elastase (green) and DNA (blue, DAPI). NETs can be identified as fibrous strands of elastase- and histone-coated DNA, visible 24 hours following acute diesel exhaust (DE) exposure and denoted by white arrows. Normal intact cells following a filtered air (FA) exposure are shown for comparison. (B) Images were quantified to assess the effect of DE exposure on NET formation. The Y-axis denotes slide area (μm^2) covered by NETs markers divided by slide area (μm^2) covered by DNA x 100%. Each line is one participant. * = *$p<0.05$. FA = filtered air, DE = diesel exhaust.*

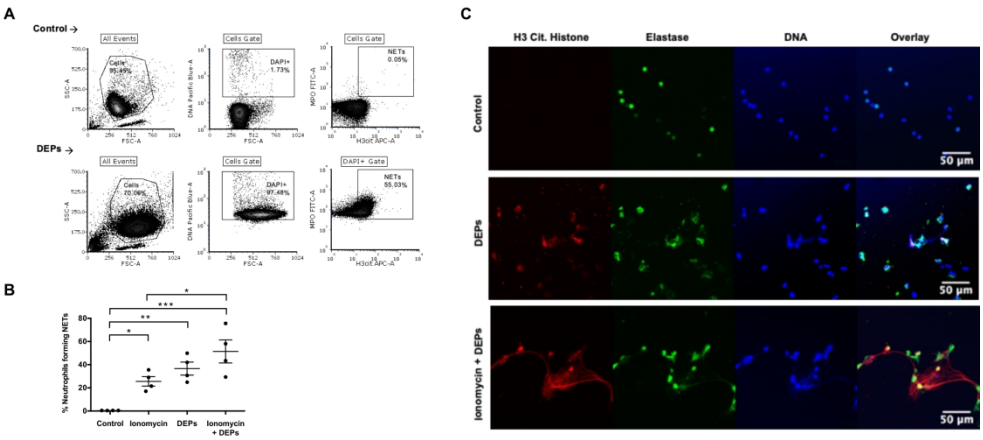

Figure 5. Diesel exhaust particles induce neutrophil extracellular trap (NET) formation in isolated peripheral blood neutrophils *in vitro*. (A) Representative flow cytometry gating is shown, where peripheral blood neutrophils (N=4) were isolated then stimulated *in vitro* for 2h with media alone (negative control), 4µM ionomycin (positive control), 100µg/mL diesel exhaust particles (DEPs) obtained from the outlet of our in-house diesel engine, or both ionomycin and DEPs. Samples were stained without a permeabilization step for hallmark markers of NETs (myeloperoxidase, MPO (flow cytometry); neutrophil elastase (microscopy), citrullinated histones, H3cit; DNA). For flow cytometric quantification, cells were gated on based on forward scatter area (FSC-A) and side scatter area (SSC-A) to exclude debris and contaminating lymphocytes. Then DAPI+ cells were gated on, followed by those cells expressing H3cit and MPO. NETs are defined as cells staining triple-positive for the aforementioned markers. (B) Ionomycin, DEPs, and the combination significantly increased the percentage of neutrophils forming NETs, and adding DEPs to ionomycin significantly enhanced NET formation, when measured by flow cytometry. Bars represent mean ± SEM, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. (C) Representative immunocytochemistry images demonstrate formation of NETs after DEPs and ionomycin + DEPs, but not control.

Supplemental Materials

DETAILED METHODS

Exclusion Criteria

Exclusion criteria were as follows: pregnant/breastfeeding, currently smoking or quit smoking <6 months prior, or smoking history 0-10 years, diagnosed with asthma, inhaled corticosteroid use, regular bronchodilator use, or significant comorbidities considered by the principal investigator to increase cardiovascular risk. In addition, an exercise test was performed to determine VO_2-max, used to set exercise intensity during exposures described below.

Data completeness

One participant did not consent to bronchoscopy and we were not able to obtain blood samples from one participant. Adequate cell counts were required for BAL assays, resulting in the following numbers with complete data: N=17 with blood draws, N=17 blood flow cytometry, N=17 blood smears (N=1 only one exposure), N=17 performed bronchoscopy, N=15 with complete BAL flow cytometry, N=15 with BAL NETs assay (N=1 only one exposure).

Peripheral blood neutrophil flow cytometry

50µl of blood was stained for neutrophil lineage and activation markers (APC-Cy7 CD45, PE-Cy7 CD16, BV421 Activated Mac-1, APC CD66b, BV510 CXCR2, ThermoFisher LIVE/DEAD Red Stain©). Live neutrophils (CD45+/CD16+/CD66b+/LIVE/DEAD-) were identified after excluding debris based on forward scatter, using CD45 APC-Cy7 BD Biosciences Cat #55783, and the following from ThermoFisher: CD16 PE-Cy7, Cat #25-0168-41, CD66b APC Cat #17-0666-41, LIVE/DEAD Red Stain Cat #L23102. Neutrophils were assessed for their expression of CD16, CD66b, Activated Mac-1 (CD11b

Activated BV412, BD Biosciences Cat #566313) and CXCR2 (CD182 BV510 BD Biosciences Cat #744196), quantified as the change from baseline median fluorescence intensity (MFI) across the exposure. An unstained sample was included for every timepoint and used to normalize MFI by subtraction, accounting for slight day-to-day variability. Gates were set using fluorescence minus one controls.

Peripheral blood neutrophil and band cell differential

Blood smears were prepared with 5µl of blood, fixed in ethanol and stained with eosin and methylene blue (Millipore Harleco Hemacolor Stain Set). 500 leukocytes per time point were identified by a skilled observer as mature polymorphonuclear cells (PMN), band cells, eosinophils, or others. For quality control, the percentage of neutrophils counted from blood smears was cross referenced with the complete blood cell differentials (CBCs) obtained from these same samples from Vancouver General Hospital. A coefficient of variance was determined, and referenced against the confidence interval values provide by the Laboratory Quality Assurance Program from the College of Physicians and Surgeons of Saskatchewan (1). Wherever there was disagreement greater than the referenced threshold, a second skilled observer re-counted the blood smears, and the observer count which more closely approximated the hospital CBCs was used for analysis. Our method of band cell counting was chosen as we deemed this more reliable than hospital-ordered counts, the latter of which consists of 200 manually counted cells by a single observer.

BAL neutrophil extracellular traps

150,000 BAL cells were seeded onto uncoated round coverglass for 1h at 5% CO_2 and 37°C. Cells were fixed with 4% paraformaldehyde, blocked (ThermoFisher BlockAid Solution) and stained for hallmark NETs markers, using primary antibodies (anti-H3Cit Rabbit IgG, Abcam Cat #5103; anti-neutrophil

elastase mouse IgG, Dako Cat #M0752), secondary antibodies (Alexa555 goat anti-rabbit IgG, Invitrogen Cat#A21428; Alexa 488 mouse anti-goat IgG, Invitrogen Cat #A228175), and Hoechst 33342 DNA stain (BD Biosciences, Cat #561908). A 4x4 grid of photos for each slide was acquired at 60X magnification and stitched together into one large image (EVOS FL Auto Imaging System). The clearest image for each visit was used to quantify NETs using ImageJ. NETs were defined as structures meeting these three criteria: 1) clear extracellular fibrous citrullinated histone strands, 2) positive DNA stain, and 3) positive NE stain. NET-covered area was quantified by tracing an outline in the overlay image. Then total nuclear area (um^2) was assessed automatically by converting the DAPI channel image to binary with the color threshold tool. NETs were quantified as NET-covered area normalized to total nuclear area and expressed as a % of total nuclear area.

Peripheral blood neutrophil extracellular traps by flow cytometry and immunocytochemistry

$1x10^6$ isolated neutrophils were stimulated for 2h *in vitro* with 100μg/mL DEPs, 4μM ionomycin (positive control) or media alone (negative control). Using a previously described flow cytometry assay, NETs were quantified as the percentage of cells staining triple-positive, without a permeabilization step, for anti-myeloperoxidase (MPO-FITC, Mouse IgG, Abcam Cat #11729), anti-H3Cit (Rabbit IgG, Abcam Cat #5103), APC goat anti-Rabbit IgG (Jackson ImmunoResearch Cat #111-136-144) and DAPI DNA stain (BD Biosciences, Cat #564405). For the immunocytochemistry assay, anti-neutrophil elastase (Mouse IgG, Dako Cat #M0752) was used as a primary antibody, with Alexa488 Goat Anti-Mouse IgG (Invitrogen, Cat #A28175) as the secondary antibody.

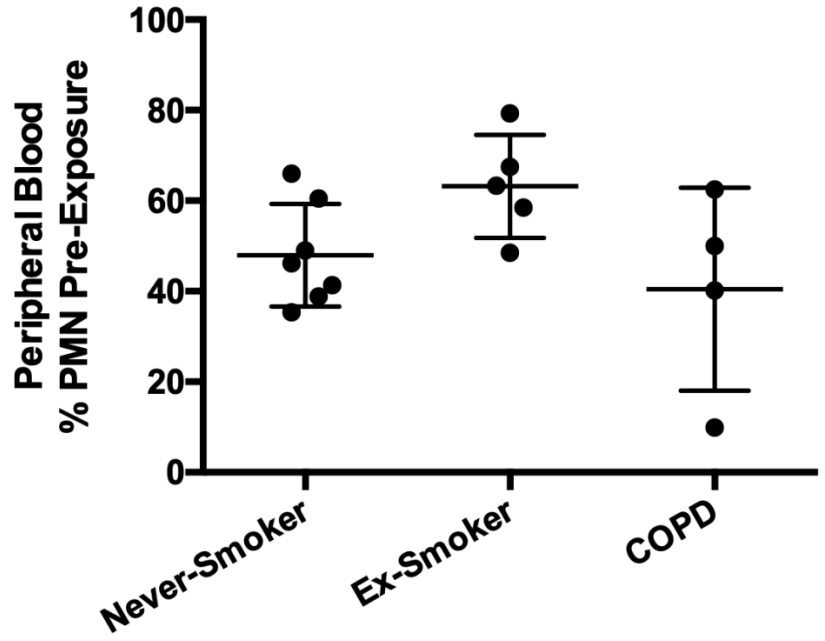

Figure S1. No difference in percentage of baseline polymorphonuclear cells (PMN). Percentage of mature neutrophils from pre-exposure, obtained from blood smear differentials, was compared across groups using repeated measures one-way ANOVA as an indicator of baseline inflammatory state.

REFERENCES

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