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The priming effect of diesel exhaust on native pollen exposure at the air-liquid interface

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ABSTRACT

Pollen related allergic diseases have been increasing for decades. The reasons for this increase are unknown, but environmental pollution like diesel exhaust seem to play a role. While previous studies explored the effects of pollen extracts, we studied here for the first time priming effects of diesel exhaust on native pollen exposure using a novel experimental setup.

Methods: Human bronchial epithelial BEAS-2B cells were exposed to native birch pollen (real life intact pollen, not pollen extracts) at the air-liquid interface (pollen-ALI). BEAS-2B cells were also pre-exposed in a diesel-ALI to diesel CAST for 2 h (a model for diesel exhaust) and then to pollen in the pollen-ALI 24 h later. Effects were analysed by genome wide transcriptome analysis after 2 h 25 min, 6 h 50 min and 24 h. Selected genes were confirmed by qRT-PCR.

Results: Bronchial epithelial cells exposed to native pollen showed the highest transcriptomic changes after about 24 h. About 3157 genes were significantly up- or down-regulated for all time points combined. After pre-exposure to diesel exhaust the maximum reaction to pollen had shifted to about 2.5 h after exposure, plus the reaction to pollen was desensitised as only 560 genes were differentially regulated. Only 97 genes were affected synergistically. Of these, enrichment analysis showed that genes involved in immune and inflammatory response were involved.

Conclusion: Diesel exhaust seems to prime cells to react more rapidly to native pollen exposure, especially inflammation related genes, a factor known to facilitate the development of allergic sensitization. The marker genes here detected could guide studies in humans when investigating whether modern and outdoor diesel exhaust exposure is still detrimental for the development of allergic disease.

1. Introduction

Allergic diseases have been steadily rising since the middle of the last century, reaching epidemic levels (Platts-Mills, 2015) with about one billion people currently suffering from some type of allergic disease (Pawankar, 2014). This increase was explained by the western lifestyle hypothesis, but is being replaced by the exposome and "biodiversity hypothesis", which states that a changed exposure (for instance by a changed epithelial barrier) or a changed human microbiome, among other factors, are important for the allergy epidemic (Akdis, 2021; Haahtela, 2019; Joubert et al., 2020; Melén et al., 2021; Pawankar, 2014; Platts-Mills, 2015).

Many allergies are triggered by pollen and the allergic effect can be increased with the concomitant exposure to combustion aerosols and Particulate Matter (PM) derived from fossil fuels. Several epidemiological studies have related the adjuvant effect of anthropogenic combustion aerosols to the exacerbation of allergic diseases (Beck et al., 2013; Bettiol et al., 2021; Gauderman et al., 2005; Hauptman et al., 2020; Lipsett et al., 1997; McCreanor et al., 2007; Strand et al., 1998). Diesel exhaust is constantly changing in composition, and environmental

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pollution is (in most places) steadily declining (Hesterberg et al., 2011; Lee et al., 2019; Milando et al., 2016; Santos et al., 2019). However, modern air pollution, to which (diesel) car emissions contribute, still have substantial detrimental health effects even with pollution values below the legal limit (Wolf et al., 2021).

Birch pollen is the one of the main outdoor allergens in Central and Northern Europe (Burbach et al., 2009). Due to climate change, the magnitude and duration of the birch pollen season have been shown to increase (Anderegg et al., 2021; Biedermann et al., 2019; Rojo et al., 2021). Studies have shown that the combined exposure of diesel particles (or compounds from diesel exhaust) and allergen enhanced allergic sensitization in humans (Carlsten et al., 2016; Diaz-Sanchez et al., 1999; Hosseini et al., 2016; Schaumann et al., 2014). However, the composition of contemporary versus older diesel exhaust used in those studies is very different. European regulations, for example, have been stricter with modern diesel in order to reduce their pollutant emissions (Martin and Ray, 2016; Parliament and Union, 2009). If modern diesel exhaust still shows these effects, regulations to further reduce fossil fuel emissions could be beneficial for the reduction of asthma and allergic rhinitis symptoms, and even may decrease the incidence of allergic diseases.

Real life exposures are needed in order to study the association between combustion aerosols with the rise of allergic diseases. Therefore, in the present study, we hypothesize that the combined exposure to fresh diesel combustion aerosol and intact birch pollen enhances allergic inflammation compared to exposure to birch pollen alone.

Mouse models are routinely used to mimic human exposure to Diesel Exhaust Particles (DEP) and PM (Alessandrini et al., 2006; Brandt et al., 2020; Kim et al., 2016; Ouyang et al., 2018), because invasive human sampling is ethically problematic. A limited number of human studies that combine both pollen and DEP exposure have shown an enhanced effect of allergy-related gene expression and secreted proteins (Carlsten et al., 2016; Diaz-Sanchez et al., 1997; Mookherjee et al., 2018; Rider et al., 2016). Working with DEP in a laboratory setting often omits the gaseous phase of exhaust, which is also biologically active (Sapcariu et al., 2016). A real life exposure including both DEP and the gaseous phase of diesel exhaust in vitro, without the use of human subjects, would therefore expand the spectrum of analysis.

When investigating the effects of environmental exposure on biological systems, the current trend is to work at the air-liquid interface and the use of human material. Air-liquid interface exposure allows inclusion of (combustion) gases, which were shown to be equally biologically active as diesel particles alone (Oeder et al., 2015). The most prominent allergic sensitizations in northern Europe are against pollen, mainly birch and grass pollen (Burbach et al., 2009; Smith et al., 2014). Until today, an air-liquid co-exposure to intact pollen was not possible. This was because pollen is difficult to work with, due to their very large diameter (20–40 μm) and stickiness (Brown and Irving, 1973; Niklas, 1985; Pohl, 1937; Schäppi et al., 1997). We showed in a previous study, however, that such studies are possible (Candeias et al., 2021).

The use of pollen extracts is also not representative of a real-life exposure as pollen is (generally) inhaled intact. We therefore developed a Pollen Sedimentation Chamber (pollen-ALI), which allows exposure of cells at the air-liquid interface to an environmentally valid low dose whole pollen (Candeias et al., 2021). The co-exposure to fresh complete diesel exhaust gases at the air-liquid interface was achieved in this study using a VITROCELL® exposure station (Ihantola et al., 2020; Oeder et al., 2015; Sapcariu et al., 2016).

2. Material and methods

2.1. Cell culture

The culture of the immortalized human bronchial epithelial cell line BEAS-2B (ATCC® CRL-9609™) was performed as in Oeder et al. (2015). For all exposures, 0.4×10^6 cells were cultured on transferable 24 mm Transwells® inserts with a 0.4 μm pore polyester membrane (Corning, Tewksbury, USA). Cells attached in submerged conditions for 24 h were then kept at the air-liquid interface for another 24 h until the start of exposure. Only cells with 85–95% confluence, determined microscopically, were used.

2.2. Diesel- and pollen-ALI exposure

The diesel fuel used in this study was obtained from a local gas station, in Munich, Germany. A Combustion Aerosol Standard generator (diesel CAST, Jing mini-CAST 5201D, Switzerland) was used to generate the diesel model aerosol. This instrument allows the production of a stable aerosol, by the use of a diffusion flame, which mimics the physical and chemical properties of a real fuel emission (Moore et al., 2014). Diesel exhaust (gas and particle phase) pre-exposure was achieved as described in previous studies (Mason et al., 2020; Mueller et al., 2016) and was diluted (~1:200) with filtered air before exposing cells. The aerosol flow in the in vitro exposure system was 100 ml/min for each insert and the system was conditioned to 85% relative humidity and 37 °C. Control cells were exposed to clean air (CA named in the study). Cells were exposed to the diluted aerosol for 2 h as described by Oeder et al. (2015), and then placed in a cell culture incubator with fresh basal medium until pollen exposure. After 24 h, all cells were exposed to 4 mg of whole birch pollen grains. For the combined exposure, three different time points were analysed (2 h 25 min, 6 h 50 min and 24 h) (Fig. 1).

2.3. Pollen exposure to BEAS-2B at ALI

Whole natural birch pollen was collected in 2018 in Munich, and dosed as by (Candeias et al., 2021). In brief, 4 mg of birch pollen were resuspended with 0.5 bar pressured air into the Pollen Sedimentation Chamber (Pollen-ALI) with cell culture inserts placed at the bottom. The whole set-up was placed inside a standard cell culture incubator. Pollen was allowed to sediment on BEAS-2B cells for 10 min.

For pollen dose assessment, four cover glasses covered with Vaseline (Bombastus-Werke, Freital, Germany) were placed inside the pollen chamber. Pollen dose for each exposure was manually counted with a light microscope (Leica Microsystems GmbH, Germany), using 100× magnification (Candeias et al., 2021).

After pollen sedimentation, cells were taken out of the Pollen Sedimentation Chamber, the pollen stayed on the cells which were then incubated for different incubation times (2 h 25 min, 6 h 50 min and 24 h) in another cell culture incubator (Fig. 1). At all times, exposure occurred at normal cell culture conditions (37 °C, 5% CO_2 and about 95% humidity).

For each incubation time, cytotoxicity and viability of the cells were assessed, according to the manufacturer instructions using the LDH-Cytotoxicity Detection Kit assay (Roche Diagnostics GmbH, Mannheim, Germany) and Alamar Blue® (Thermo Scientific™, UK), respectively.

2.4. Physical composition of diesel CAST

For chemical and physical characterization of the combustion aerosol, online and offline measurements were used. The aerosol was additionally 10x more diluted compared to the in vitro cell exposures using an ejector diluter (PALAS® VKL 10 E, Germany). Regarding the online measurements, several instruments were monitoring the diesel-CAST model aerosol: an Aethalometer® (Magee Scientific, Model AE33-7, Slovenia), used to measure Black Carbon (BC) and total PM; and a Condensation Particle Counter (CPC, TSI, Model 3070, USA) in combination with an Electrostatic Classifier (TSI, Model 3082, USA) were used for determining size distribution.

For offline measurements, quartz fiber filters (QFFs) were used to collect elemental and organic carbon (EC and OC, respectively), which were analysed using a thermal-optical carbon analyser (Desert Research Institute Model, 2001A, Atmoslytic Inc., Calabasas, CA, USA), according

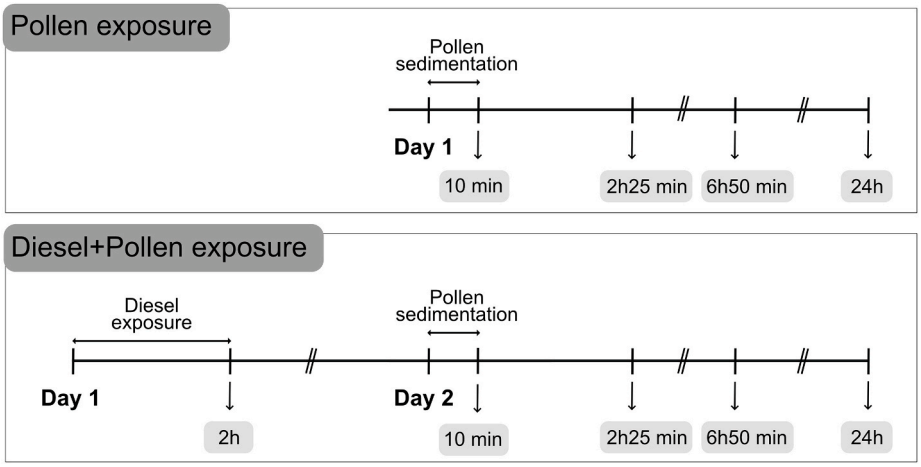

Fig. 1. Experiment Setup. When using only pollen, BEAS-2B cells at ALI were exposed to 4 mg birch pollen. Pollen was let to sediment for 10 min and stayed on the cells in ALI conditions for the indicated times. For the diesel + pollen exposure, cells were pre-exposed to fresh diesel-CAST for 2 h at ALI and then incubated for 24 h before pollen exposure. Controls were treated identical but were exposed to clean air (CA) instead of diesel CAST (N ≥ 3 independent experiments per time point).

to the IMPROVE_A Protocol (Chow et al., 2007).

2.5. Chemical composition of diesel CAST

2.5.1. Sample Preparation

Sampled QFFs were cut (diameter of 6 mm) and placed into deactivated glass inserts for thermal desorption (glass inserts were deactivated with trimethylchlorosilane, TMCS, Fisher scientific, Germany, prior to use). First, 1 μL of an internal standard (ISTD) and 1 μL of a derivatization standard (DSTD), then 10 μL of the silylation reagent N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, fisher scientific, Germany) were added to the QFFs by an autosampler (Shimadzu AOC-5000 Plus, Germany) (Table A1). In-situ Derivatization and Thermal Desorption (IDTD) was conducted (Orasche et al., 2011; Weggler et al., 2016).

2.5.2. Chemical analysis and data processing

Particle-bound semi-volatile organic compounds (SVOCs) were analysed on a GC × GC-ToFMS system (Agilent 6890 Gas Chromatograph, LECO Pegasus 4D ToF MS). An Optic 3 injection system (ATAS GL, Netherlands) was used for programmable temperature vaporization (PTV) desorption (further information in Table A2 and A3). SVOCs were first separated on a polar column (BPX50, SGE, Australia), then focused and released by a modulator onto a second non-polar column (BPX1, SGE, Australia). Finally, SVOCs were transferred into a mass spectrum, ionized with 70eV and analysed. The sample was measured in triplicates. See Table A4-A6 for more information on chromatographic and mass spectral parameters.

Samples were pre-processed with ChromaTOF (LECO, Version 4.50.8.0 optimized for Pegasus). Baseline correction, peak find, library search and area calculation were conducted. Afterwards, data was exported as. csv-file. Column bleed, contaminations and unknowns were manually excluded from the sample list. Additionally, only compounds found in at least 2 out of 3 technical replicates were included in further data inspection. See Table A7 for further information on data processing.

2.6. Whole-genome expression analysis

Transcriptomic analysis of BEAS-2B cells was performed for all incubation times. After the indicated exposure times, cells were lysed with Buffer RLT (QIAGEN, Hilden, Germany) and 1% β-Mercaptoethanol (Roth, Karlsruhe, Germany) was added to deactivate possible RNases. Total RNA was extracted with the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol. RNA was quantified with an UV–vis spectrophotometer (NanoPhotometer® N60, IMPLEN, Munich, Germany). Total RNA quality was analysed using the Agilent 2100 Bioanalyzer RNA Nano chip (Agilent Technologies, Waldbronn, Germany) and only RNA samples with a RIN higher than 8 were used for whole genome analysis (all samples). Samples were spiked (One-Color RNA Spike-in Kit, Agilent Technologies, Waldbronn, Germany) and Cy3-labelled (Low Input Quick Amp Labeling Kit, one-color, Agilent Technologies, Waldbronn, Germany) for further purification in RNeasy mini spin columns (QIAGEN) and hybridization on One-Color SurePrint G3 8 × 60 K Human gene expression arrays (Agilent Technologies, Inc, Waldbronn, Germany) according to the manufacturer's protocol. Extraction and analysis (Agilent Microarray Scanner, Agilent Technologies, Waldbronn, Germany) were performed as detailed in (Candeias et al., 2021). Microarray raw data can be accessed through the GEO Series accession number GSE185399, at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO).

2.7. Validation of transcriptome results with qRT-PCR

A selection of genes from different enriched GO terms related to allergy (i.e. immune response (CCL20, IL9R), homeostasis (MUC5B, ALDH8A1), inflammation (TGFB2, BACE1) and cell junction (ITGA4, MADCAM1)) were differentially expressed by microarray analysis, at different time points and for either pollen alone or combined diesel exhaust and pollen exposure, and validated by quantitative real-time PCR. For this, total RNA samples were reverse transcribed with the High-Capacity cDNA kit (Applied Biosystems, USA), according the manufacturer's instructions. The quantitative RT-PCR was performed using the FastStart Universal SYBR(R) Green Mastermix (Roche, Switzerland) and the ViiA 7 Real-Time PCR System (Applied Biosystems, USA) using commercially available human primer Assays (QIAGEN, Hilden, Germany), including endogenous controls (β-actin and 18 S). The mRNA expression was normalized to the endogenous controls and relative quantification was calculated using the comparative Ct method ($2^{-\Delta\Delta CT}$) (Schmittgen and Livak, 2008). All amplifications were carried out in duplicate and fold-changes in mRNA expression of BEAS-2B cells exposed to pollen or the combined diesel and pollen were compared to their respective control cells.

2.8. Statistical methods

At least three independent experiments were performed for all exposures. For data with two groups, an independent sample T-test was performed. ANOVA was used for multiple groups, with the adequate Tukey's Post-Hoc test. P-values were corrected using the Benjamini-

Hochberg method for all tests (p-values <0.05 were considered significant). Standard deviation (s.d.) was used to describe the variability of the mean, unless otherwise stated.

For transcriptomic analysis, data were analysed with the statistical programming environment R (version 4.0.2) (R Core Team, 2020) and pre-processing of the microarrays was performed using the Limma package (Ritchie et al., 2015). Enrichment analysis was performed using the Metascape web-based portal (Zhou et al., 2019) as described in (Candeias et al., 2021). The complete enrichment list for the experiments is given in Appendix A.2-A.11.

3. Results

3.1. Birch pollen doses at the BEAS-2B

BEAS-2B at pollen-ALI were exposed to 4 mg whole natural birch pollen for all exposures. For birch pollen exposure alone, 1318 ± 322 pollen grains/cm^2 had sedimented on the cells, with no significant differences observed between the different incubation times (Table 1). For the combined diesel and pollen exposure 1076 ± 54 pollen/cm^2 was dosed onto the cells. Doses were not significantly different between the two exposures for any time point.

3.2. BEAS-2B exposure to pollen alone

No cytotoxicity >15% was observed and viability of cells was above 85% at all times (Fig. 2A and B).

BEAS-2B cells exposed to birch pollen alone showed a high differential regulation of genes versus control cells at 24 h after exposure, with 2622 genes up- or down-regulated (Fig. 3A). At 6 h 50 min, fewer genes (526) were differentially regulated, while for the 2 h 25 min after pollen exposure, just a few genes were differentially regulated: 7 up- and 2 down-regulated genes.

At 6 h 50 min, a high expression of genes that are relevant for the immune response was observed. Focusing on the enriched GO Terms, we observed an up-regulation of the following biological processes: Leucocyte migration, chemokine signalling pathway, cytokine-cytokine receptor interaction, T cell differentiation/activation, regulation of immune effector processes, regulation of cell adhesion, etc. (Appendix A.2). Genes including *IL6*, *IL23A*, *IER2*, *CCL8*, *MAP3K3*, *CXCL6*, *CCL18*, *CXCL9*, *IL1RL2*, *MUC3* and *FOXA3* were up-regulated (Fig. 3C). Regarding the 147 down-regulated genes, the enriched GO Terms were mostly associated with leucocyte and T cell migration, receptor-mediated endocytosis and regulation of complement activation (Appendix A.3), like *CCL26*, *CASP2* and *IGLV1-44* (in the heatmap as *ENST00000390297*).

We observed the highest differential regulation at 24 h after pollen exposure. The up-regulated genes, e.g. *CASP3*, *CXCL16*, *IL17*RB, *IL17B*, *ITGA2*, *ITGA4*, *IL15*RA, *IL16*, *IL19*, *MAP3K14*, *MAPK15*, *CLDN20*, *CCL20*, *TGFB2*, *METTL11B*, *METTL24*, SERPINA11, CDH24, SERPINA6, and SOD3 (Fig. 3D) were associated with the following enriched GO Terms: tight junction, cellular response to lipid, intracellular receptor signalling pathway and TGF-beta signalling pathway (Appendix A.4). The relevant down-regulated GO Terms are related to integrin and cell-cell junction organization, oxidation by cytochrome P450, cellular protein disassembly and lymphocyte activation (Appendix A.5), as genes such as *MUC5B*, *TLR1*, *IFNL2*, *IL7R*, *IL3R*, *IL2RB*, *IL17F*, *ITGAL*, *CLDN22*, *FCER2*, *TGFB3*, *CCL16*, *CYP3A5* and *CYP7A1* were down-regulated. Each incubation time gave different responses, and we did not observe common differential regulated genes between the different times.

Table 1
Dose of birch pollen to the cells at the different exposures and incubation times. No significant dose differences were observed between the exposures ($p > 0.05$, independent sample *t*-test). Mean and s.d. are given. N ≥ 3 for all time points.

Incubation time	Pollen exposure	Diesel + Pollen exposure
	Pollen doses (pollen/cm^2)	Pollen doses (pollen/cm^2)
2 h 25 min	1018 ± 182	1109 ± 218
6 h 50 min	1385 ± 215	1105 ± 139
24 h	1218 ± 81	1014 ± 132

3.3. Physical and chemical analysis of the diesel-CAST combustion aerosol

Diesel-CAST exhaust physical characterization is summarized in Table 2. Following 2 h pre-exposure to fresh diesel model aerosol, we observed a geometric mean between all exposures of 136 ± 8 nm, a concentration of particles of 240 ± 59 μg/m^3 and about the same concentration of black carbon (207 ± 50 μg/m^3). Almost no organic carbon was measured in the quartz fiber filters, however the chemical composition of the combustion aerosol shows compounds typical of a diesel exhaust aerosol.

Table 3 shows the most abundant compounds of the diesel aerosol for different compound classes. Oxygenated species, such as cyclopentanedione, butylated hydroxytoluene, hexadecanoic acid and phenol, were detected in our diesel exhaust. Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, acenaphthylene, pyrene, fluorine and fluoroanthene were emitted as well as compounds like propanamide, benzenesulfonamide and N-butyl- and 2,6-ditert-butyl-4-nitrophenol. See Fig. S.1 for detailed GC × GC-ToFMS chromatogram.

3.4. Combined exposure of BEAS-2B to diesel CAST and pollen

In the combined diesel + pollen exposure, cells did not show any cytotoxicity >15% and viability was >85% for all the conditions tested in the combined exposure (Fig. 4 A and B). For unknown reasons, the lysis with 2% Triton-X in the viability assay did not result in complete lysis of the cells. Nevertheless, viability of the treated cells was compared to unlysed cells, which were set to 100%.

After pre-exposure to diesel, pollen exposure resulted in fewer differentially regulated genes compared to clean air pre-exposed controls (all cells here treated with pollen). Regarding transcriptome analysis, we noticed the highest differential gene expression at 2 h 25 min after pollen exposure (214 genes up- and 262 down-regulated), compared with the other time points (Fig. 5). Genes related to the GO Terms cell adhesion, response to extracellular stimulus, detoxification and organic hydroxy compound metabolism were up-regulated (Appendix A.6), with genes like *ITGA4*, *CD34*, *SERPINH1*, *HGB1* and *GNGT2* being expressed. Regarding the down-regulated genes, e.g. like *IL9R*, *CYP11A1*, *MAPK11*, *GALNT13*, *CCK* and *CCNA2*, the enriched GO Terms show a regulation of protein glycosylation, amine transport, negative regulation of G protein-coupled receptor signalling pathway and cellular response to organic cyclic and nitrogen compounds (Appendix A.7). At 6 h 50 min, 14 genes were up-regulated and 12 down-regulated, mostly with unknown function. Two of the up-regulated genes, *MED1* and *FBP1*, are known to be associated with asthma (Appendix A.8) (Hu et al., 2021; Schoettler et al., 2019; Valette et al., 2021). At 24 h post combined exposure, only 7 genes were up-regulated, where *ALDH8A1*, an aldehyde dehydrogenase gene is included; and 49 genes were down-regulated, e.g. *CCNA2*, *CCK* and *PPEF1*.

3.5. Pre-exposure to diesel exhaust changes the reaction of epithelial cells to pollen

To find the genes that were synergistically affected by both treatments, the two sets of regulated genes were superimposed. A total of 97 differentially regulated genes were found (Fig. 6A). An enrichment analysis was performed with the genes common to both treatments (Appendix A.11). Fig. 6B shows a heatmap of all the common differentially regulated genes and Fig. 6C a subset of genes of interest i.e. known

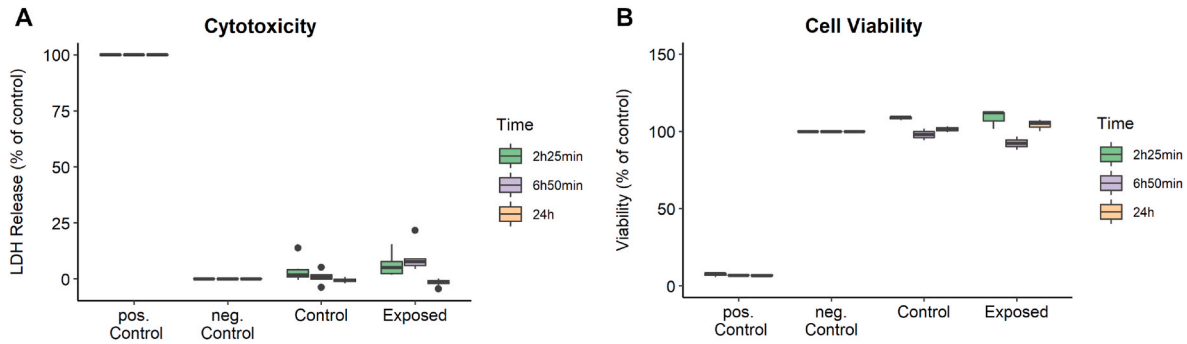

Fig. 2. Toxicity and viability of BEAS-2B cells at ALI after pollen exposure, without pre-exposure to fresh diesel model aerosol. (A) cytotoxicity (LDH assay) and (B) viability (Alamar Blue assay) of BEAS-2B after exposure to 4 mg birch pollen for the different incubation times. "pos. control" and "neg. controls" were cells that stayed all the time in a cell culture incubator, but "pos. controls" were lysed with 2% Triton-X 100. "neg. controls" served as reference for 0% toxicity or 100% viability, respectively. "Control" cells were treated as exposed cells inside the pollen-ALI chamber but were not exposed to pollen. For all exposures, negative and positive controls were performed (N ≥ 3 independent experiments per time point). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

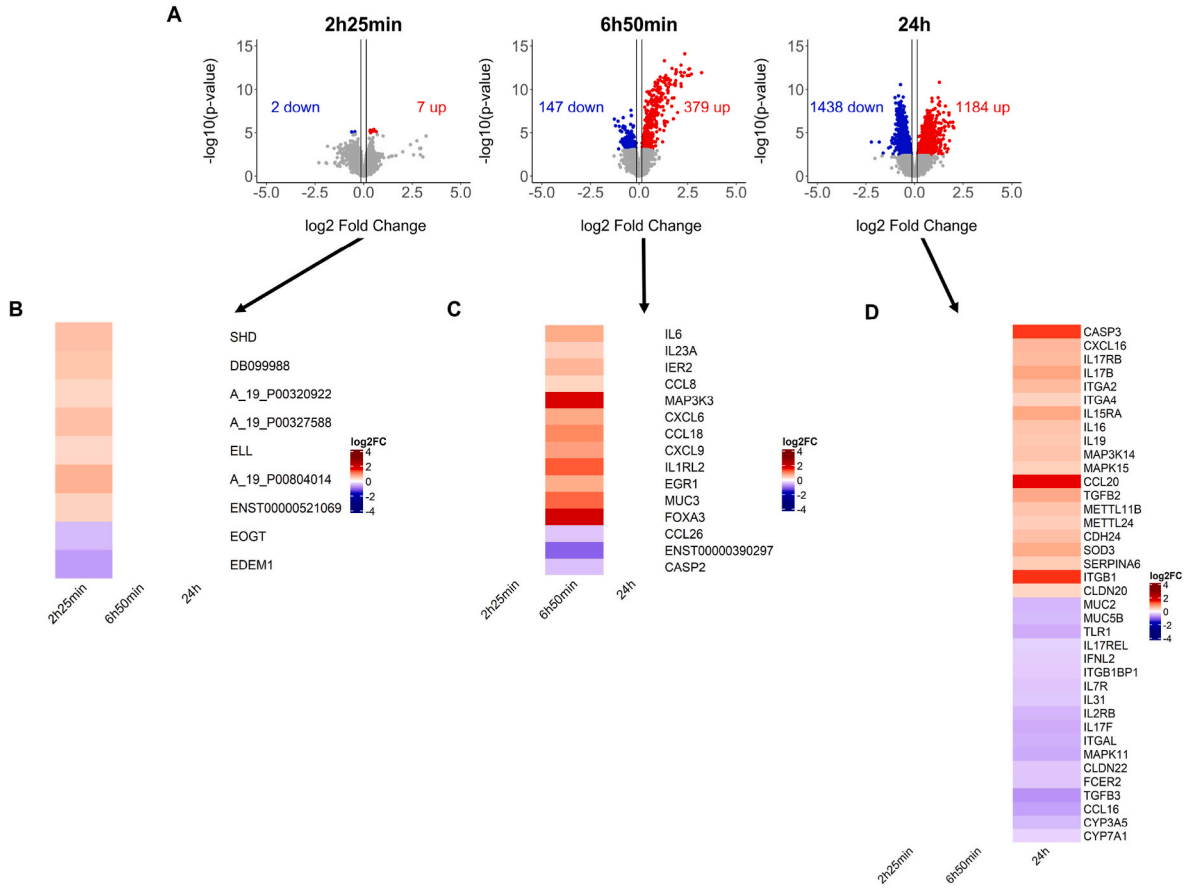

Fig. 3. Transcriptomic analysis of BEAS-2B exposed to only whole birch pollen (4 mg) at the air-liquid interface. (A) Volcano plots for the different incubation times. (B) heatmap of all the differential regulated genes at 2 h 25 min; heatmaps of significant and selected relevant genes, related to immune and allergic responses, for (C) 6 h 50 min and (D) 24 h after pollen exposure. Significant regulated genes are marked in color. N = 3 independent experiments per time point. Identical treated but not pollen exposed cells served as reference for fold-change analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to be relevant in immune response. We observed an earlier up- or down-regulation of genes when BEAS-2B cells were pre-exposed to diesel compared to the pollen exposure alone.

Focusing on the relevant enriched GO-terms previously described, the following were differentially regulated: genes correlated with Class B/2 and adenylate cyclase-activating G protein-coupled receptor signalling pathway (*CRH*, *GIPR*, *GLP1R*, *GNGT2*, *PTH1R*, *ADGRB3*); detection of external stimulus (*GNGT2*, *BACE1*, *GRK7*), response to toxic substance/detoxification (*BMP7*, *HBG1*, *SLC15A2*, *EHMT1*), cell adhesion related genes such as *ITGA4* and *MADCAM1*; and cell response to nitrogen compounds (*CRH*, *GLP1R*, *HRH1*, *ITGA4*, *RYR1*, *BACE1*, *LYPD1*) (Fig. 6C). Most of these genes were up-regulated by both exposures, and diesel pre-exposure enhanced their expression, while a few changed the direction of regulation (e.g. *MADCAM1* and *PTH1R*).

Table 2
Diesel exhaust (CAST) exposure characteristics. Mean and s.d. are given of concentrations encountered by the BEAS-2B cells in the in vitro exposure system. N ≥ 3 for all time points.

Parameters	Mean (± s.d.)
Geometric mean (nm)	136 ± 8
Particle number (#/cm^3)	46,301 ± 5424
Total PM (μg/m^3)	240 ± 59
BC (μg/m^3)	207.5 ± 49.9
Total Carbon (μg C/m^3)	130.5 ± 5.9
OC/EC ratio	0.01 ± 0.01

Table 3
Diesel (CAST) chemical composition. For different compound classes, the most abundant compounds found in the diesel-CAST combustion aerosol (diesel exhaust) are listed. For PAHs, selected compounds, known to be relevant in diesel exhaust but with a lower abundance, were added. Mean of the area and unique mass is shown (* blank corrected). NIST: National Institute of Standards and Technology; DTIC: Deconvoluted Total Ion Chromatogram; PAHs: Polycyclic aromatic hydrocarbons.

Compound Class	NIST Hit/Name	Mean Area (DTIC)	Unique mass (m/z)
Oxygenated Species	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,5-dimethyl-3-(1-methylethylidene)-, (4ar-cis)-	6648540	161
	1,2-Cyclopentanedione	4130742	98
	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	3024325	219
	9-Octadecenoic acid, methyl ester, (Z)-	2365545	55
	Butylated Hydroxytoluene	1879953	205
	Hexadecanoic acid, methyl ester	1426061	74
	Heptadecanoic acid (derivatized with trimethylsilyl ester)	1259976	117
	Phenol, 2,6-bis(1,1-dimethylethyl)-	1207964	191
	Phenol	1153591	94
	9,19-Cyclolanostan-3-ol, acetate, (3á)-	1037367	135
PAHs	Naphthalene	774744*	128
	1H-Indene, 1-ethylidene-	288982	142
	Biphenyl	247602	154
	Acenaphthylene	153093	152
	Pyrene	127386	202
	Naphthalene, 2,6-dimethyl-	116680	156
	Fluorene	94139	166
	1,1'-Biphenyl, 4-methyl-	91557	168
	9H-Fluorene, 2-methyl-	76086	165
	Fluoranthene	63204	202
	Naphthalene, 1,4,6-trimethyl-	50824	155
	Naphthalene, 2-ethyl-	40294	141
Diverse	Propanamide, 3-[3,5-di (tert-butyl)-4-hydroxyphenyl]-N-(2-hydroxyethyl)-	3101757	147
	Benzenesulfonamide, N-butyl-	3067819	141
	2,6-ditert-butyl-4-nitrophenol	848617	236

3.6. Validation of selected genes by RT-qPCR for both exposures

A selection of genes related with allergy and immune responses was validated by RT-qPCR.

For the birch pollen exposure alone, three genes were validated (*CCL20*, *TGFB2* and *MUC5B*), where *CCL20*, *TGFB2* were up-regulated with about 2-fold expression and *MUC5B* was down-regulated, compared to control cells (Fig. 7), confirming the transcriptomic data.

Regarding the combined fresh model diesel exhaust and pollen exposure, *ALDH8A1* and *IL9R* were validated by qRT-PCR, which confirmed the microarray data, where *ALDH8A1* was over-expressed by more than 2-fold over-expressed, compared to control cells. *IL9R* was confirmed down-regulated by 2-fold, as in transcriptome analysis (Fig. 7).

A validation of selected genes that were in the birch pollen alone and in the combined exposure differentially expressed in the microarray data was performed. *ITGA4*, *MADCAM1* and *BACE1* genes were validated and the transcriptome data was confirmed for the three genes selected. *MADCAM1* was expressed higher in cells pre-exposed to diesel, compared to the cells only exposed to pollen. For *ITGA4* and *BACE1*, the up-regulation for both exposures was validated, with similar fold expression in both exposures.

4. Discussion

Human studies on the effect of combustion aerosols and allergic diseases have been performed (Carlsten et al., 2016; Diaz-Sanchez et al., 1997; Mookherjee et al., 2018; Rider et al., 2016). Here we demonstrate the effect of pre-exposure to diesel exhaust on the pollen exposure, both at the air-liquid interface. This mimics real-life exposure to seasonal pollen where combustion pollution is omnipresent, with a seasonal pollen exposure on top. We thus analysed the effects of exposure to pollen, then pre-exposure of diesel and pollen, and overlaid both results to find the synergistically affected genes.

The effects of diesel exhaust alone at the air-liquid interface was not carried out in this study, as it is not the focus of this work and is amply described elsewhere (Klein et al., 2017; Oeder et al., 2015; Sapcariu et al., 2016). By subtracting the effects of diesel exposure alone (our control condition), the synergistic effects by diesel priming on pollen exposure becomes better visible. We also used the bronchial epithelial derived cell line BEAS-2B as a model for reaction of the airway epithelium to pollen (Abbas et al., 2019). Although bronchial and nasal epithelium are not the same, within the concept of respiratory allergy as a systemic disease (or in its "limited" version of united airways disease) the concept of a united airway seems to be valid and shows a correlating reaction between both tissues (Zissler et al., 2018).

4.1. Pollen exposure alone

Exposure of immortalized human BEAS-2B bronchial epithelial cells in the pollen-ALI was described previously (Candeias et al., 2021). Dosing 4 mg of pollen resulted in 1000–1300 pollen/cm^2 (about 6 μg/cm^2, as 1 birch pollen grain weighs about 7 ng (Brown and Irving, 1973; Pohl, 1937; Schäppi et al., 1997)). The human nasal cavity where pollen is deposited is about 160 cm^2 (Gizurarson, 2012). Our dose is higher than a peak exposure during the pollen season in central Europe (up to 3000 birch pollen/m^3, see pollenscience. eu), but lower than in the heartland of birch trees in Russia where peaks concentrations of 10, 000–20,000 birch pollen/m^3 are common (Buters et al., 2018). Our dose is low compared to submerged cell exposures, where 10 mg pollen/ml (about 1,500,000 pollen/ml) and higher was common (Abós-Gracia et al., 2013; Blume et al., 2013, 2015; Gilles et al., 2020). We believe a lower dose is more representative of real life exposure.

BEAS-2B at Pollen-ALI, exposed to 1000 pollen/cm^2 intact birch pollen, showed a disturbance in the epithelial barrier and inflammation response from the earlier to the later incubation times. The up-regulation of *IL6*, *IL23A*, *CXCL9* and *CXCL6* shows that BEAS-2B cells are able to (try to) recruit effector cells in order to decrease inflammation, at 6 h 50 min after pollen exposure. A higher immune response, with expression of cytokines, was observed at later times (6 h 50 min and 24 h). *IL17B* is a low affinity ligand of *IL17*RB, playing an anti-inflammatory role by blocking the expression of *IL25*, and subsequent Th2 response (Reynolds et al., 2015; Tworek et al., 2016). These two genes, up-regulated in our exposure at 24 h, could indicate that at lower exposure times, the induction of *IL25*, an important regulator of the allergic reaction, might have happened and pro-inflammatory genes are replaced towards an anti-inflammatory reaction one day after. At the same time, we observe an up-regulation of *IL19* and *CCL20*, involved in allergic asthma and the amplification of a Th2 response (Liao et al., 2004; Pichavant et al., 2005; Vroling et al., 2008; Weng et al., 2019).

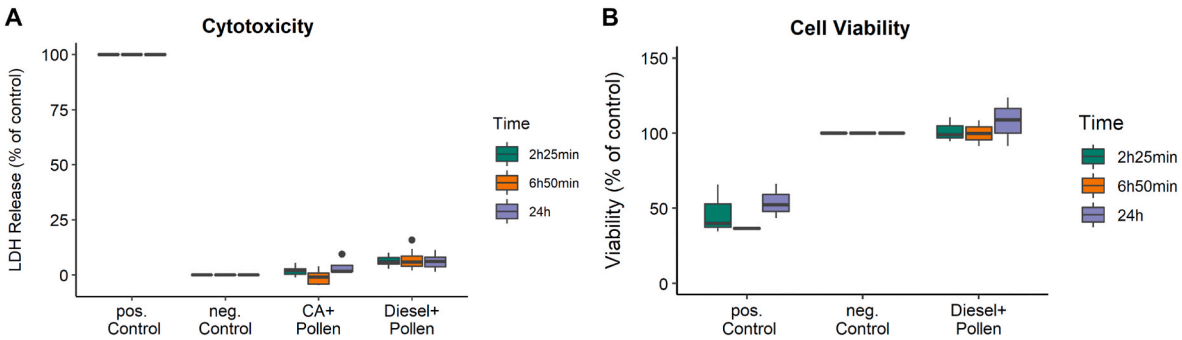

Fig. 4. Toxicity and viability of BEAS-2B cells at ALI after pollen exposure, with pre-exposure to fresh diesel model aerosol. (A) cytotoxicity and (B) viability of BEAS-2B after being exposed to fresh diesel-CAST combustion aerosol and to 4 mg birch pollen. "CA + Pollen" were cells exposed to filtered clean air (CA) and later to pollen. For "CA + Pollen" only cytotoxicity was measured. For all exposures, negative and positive controls were performed (N ≥ 3 independent experiments per time point).

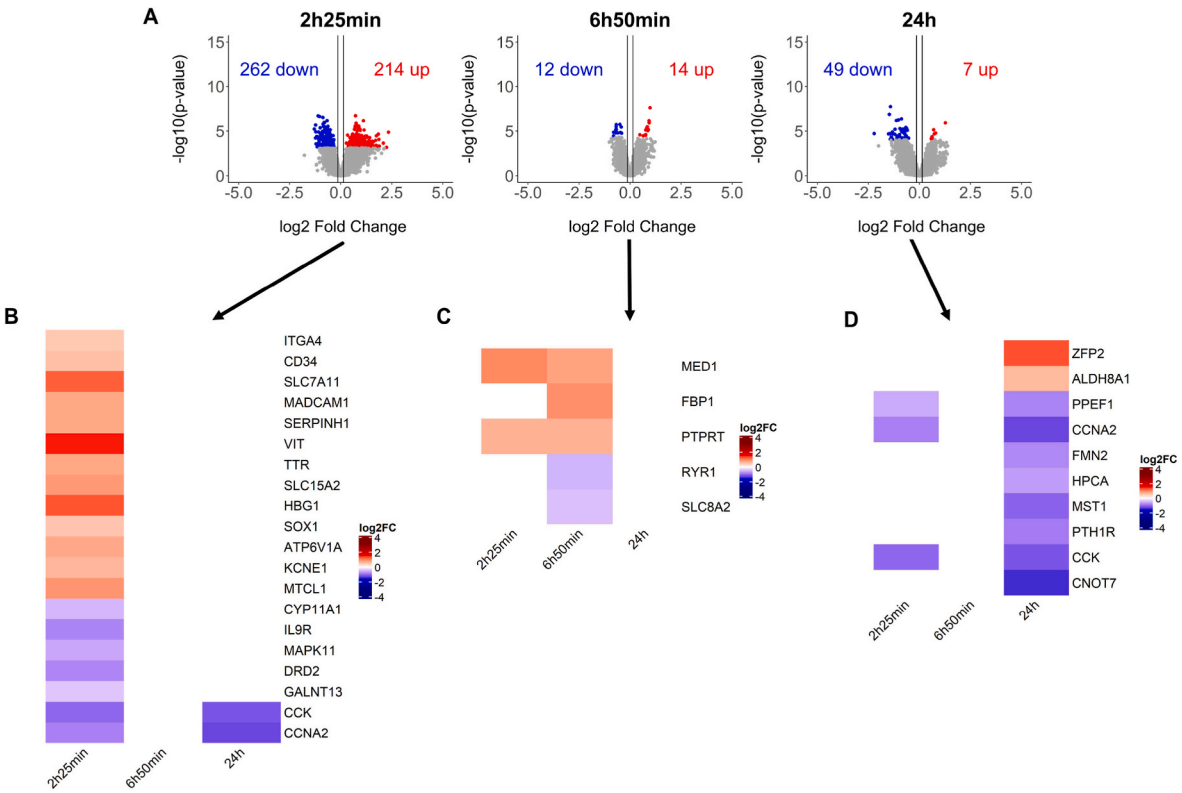

Fig. 5. Transcriptomic analysis of BEAS-2B cells exposed to first diesel exhaust and then 4 mg whole birch pollen at the air-liquid interface. (A) Volcano plots for the different incubation times. Heatmaps of selected significant regulated genes, related to allergic and immune responses for (B) 2 h 25 min, (C) 6 h 50 min and (D) 24 h after the combined exposure. Significant regulated genes are marked in color. N ≥ 3 independent experiments per time point. Identical treated cells with pollen but without diesel ("CA + pollen" cells) served as reference for fold-change analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Genes from the integrin family (*ITGA2*, *ITGA4*) are also up-regulated and, together with *CLDN20* and *TGFB2*, where *TGFB2* regulates allergen induced airway inflammation in asthma patients (Bottoms et al., 2010; Lopez-Guisa et al., 2012). This finding suggests that pollen could initiate bronchial epithelium remodeling processes already 24 h after exposure to birch pollen. In fact, genes involved in epithelial barrier regulation such as *MUC5B and CLDN20*, were also triggered (Cremades-Jimeno et al., 2021; Lachowicz-Scroggins et al., 2016; Ordoñez et al., 2001). Genes related with Th1 and Th2 response and immune effector cells recruitment were down-regulated (e.g., *IFNL2/IL28A*, *IL7R*, *IL2RB*, *FCER2*, *TGFB3*, *CCL16*, *IL31*), putting forward the idea of a decreased acute inflammatory response and increased homeostasis related expression of genes 24 h after exposure to whole birch pollen.

4.2. Diesel + pollen exposure

BEAS-2B cells pre-exposed to diesel exhaust reacted faster to birch pollen, than after exposure to pollen without the combustion pre-exposure. Cells exposed to diesel model aerosol and then pollen showed the highest differential regulation of genes at 2 h 25min, compared to the 24 h when pollen is dosed alone. Induced genes are related to cell adhesion, response to extracellular stimulus, detoxification and organic hydroxy compound metabolism. In contrast, inhibited genes were significantly related to processes of cellular response to organic cyclic and nitrogen compounds. Most of the genes are known to be up-regulated in allergic asthma and/or severe asthma. For examples, *ITGA4* binds to *MADCAM1* and recruits lymphocytes to the mucosa

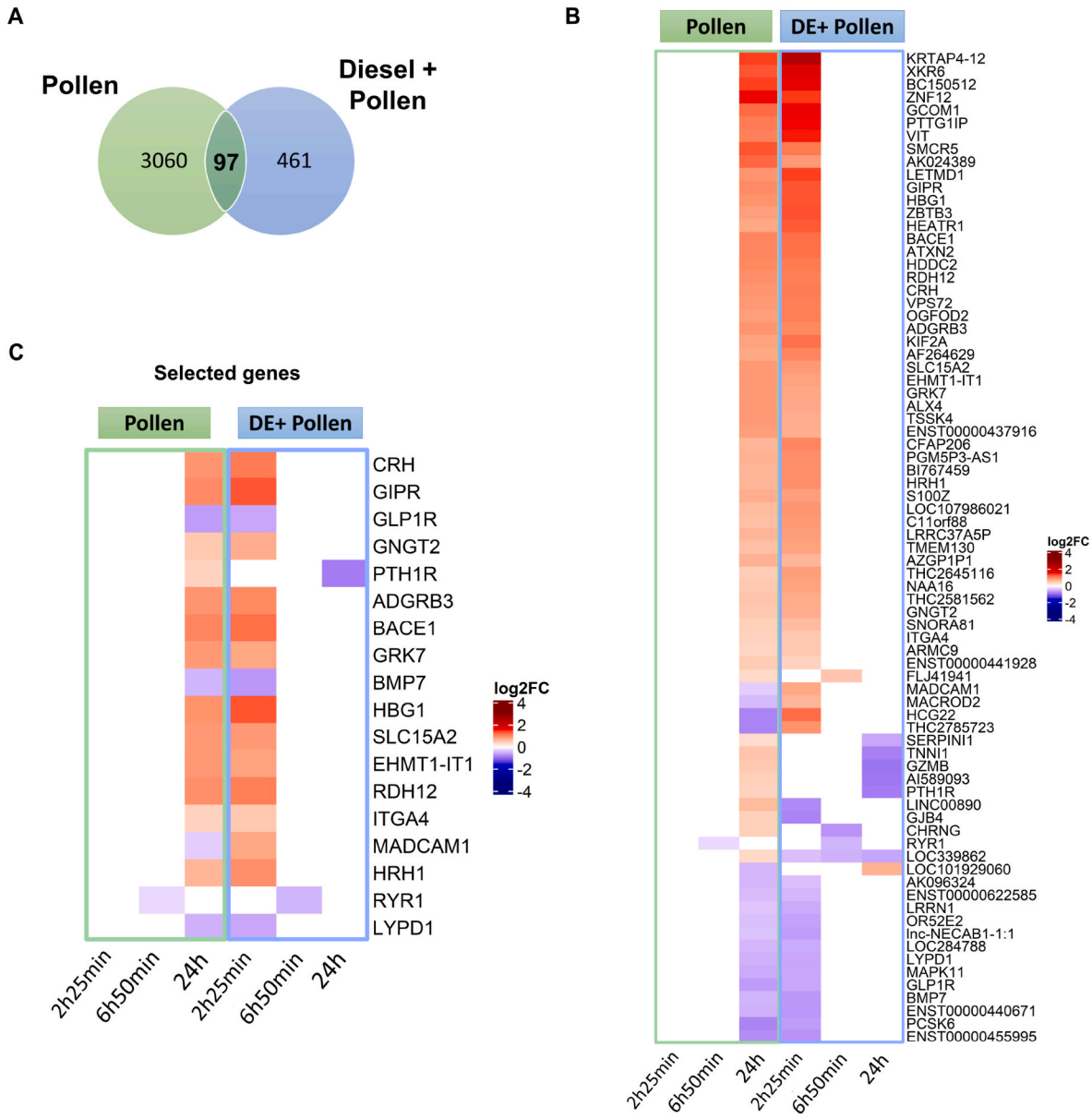

Fig. 6. Comparison between differential expressed genes of BEAS-2B cells exposed to pollen alone or with preceding diesel exhaust exposure. (A) Venn diagram summarizing the total number of differentially expressed genes for both pollen exposure alone and combined diesel + pollen exposure; (B) Heatmap of all the significant expressed common genes between both exposures (n = 97); (C) Heatmap of selected genes in both exposures, related to immune and allergic responses. (N ≥ 3 independent experiments per time point).

(Pacheco et al., 1998), and both genes have been implicated in asthma and chronic inflammation (Briskin et al., 1997; Wang et al., 2018). *BACE1*, as well as *FBP1* and *GNGT2* were shown to be correlated with asthma in other studies (Dong et al., 2020; Hu et al., 2021; Madore et al., 2016; Madore and Laprise, 2010). Regarding the down-regulated genes, we observed a decrease of genes related to the regulation of immune response and Th2 differentiation, such as *IL9R* – a pathway that is of particular importance for asthma (Musiol et al., 2021). The same reaction of BEAS-2B, towards a regulation of immune response, was seen for pollen exposure alone but then at 24 h incubation time, which confirms the adjuvant effect of diesel exposure particles to the allergic response.

4.3. Comparison pollen alone with diesel + pollen exposure

BEAS-2B cells pre-exposed to diesel exhaust displayed diminished effects in a subsequent pollen exposure. The diesel-mediated epithelial numbness was seen in other studies (Li et al., 2012; Rider et al., 2016).

Regarding the synergistically regulated genes, we observed that the enrichment of pathways related to inflammation were expressed at earlier times when BEAS-2B were pre-exposed with diesel particles. The majority of those genes were shown before to be associated with allergic asthma and pro-inflammation, such as ITGA4, SLC15A2, HRH1, GNGT2 and BACE1 (Dong et al., 2020; Laulajainen-Hongisto et al., 2020; Lilly et al., 2012; Madore et al., 2016; Pacheco et al., 1998; Song et al., 2020; Wang et al., 2018; Xie and He, 2005), The up-regulation of those genes in both exposures may reflect the damage of the cells after exposure to both conditions. Besides that, BMP7 for example, known to antagonize TGFB effect and reduce inflammation, is down-regulated in both exposures, which strengthens the idea of a dysregulation on the BEAS-2B.

The fresh diesel exhaust created with the CAST is comparable to other human chamber exposures (Birger et al., 2011; Carlsten et al., 2016) regarding particle concentration and size distribution. Diesel exhaust consists mainly of elemental carbon, organics, sulfates, nitrates and metals. The concentration of each substance depends on several

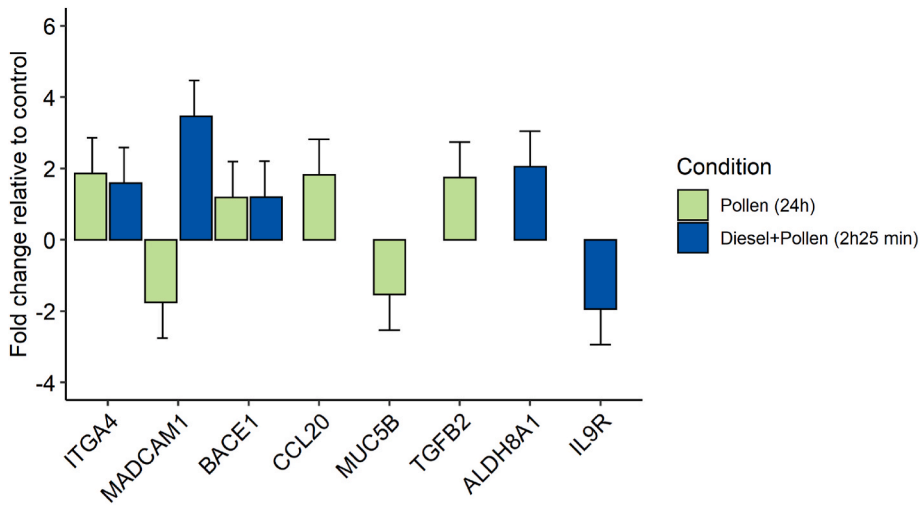

Fig. 7. mRNA expression of selected genes by qRT-PCR that were differentially expressed in transcriptome data, for pollen alone and/or combined exposure. N ≥ 2 for all experiments.

factors like engine, driving conditions or fuel characteristics (Kittelson, 1998). Monocyclic aromatic hydrocarbons (MAHs) and PAHs known to be produced by diesel exhaust (Corrêa and Arbilla, 2006) such as acenaphthylene, fluoranthene, fluorene and pyrene were identified in our study. In our chemical analysis, we observed high amounts of 1,2-Cyclopentanedione, 7-Hexadecenoic acid, methyl ester, which are among the most abundant exhaust compounds. In addition, 2,6-ditert-butyl-4-nitrophenol (DBNP, also known as Bayer 28,589), an alkylphenolic antioxidant usually added to fuels to prevent degradation (Jensen et al., 2014), is identified in the diesel model aerosol produced.

Our study also has limitations. Although bronchial epithelial cells responded similar as nasal epithelial cells (Zissler et al., 2018), they are not the same which might limit the results obtained. Due to technical space limitations of the pollen-ALI, we could not perform all necessary exposures simultaneously. The diesel exhaust used in this study is only a model aerosol, it is generated using a diffusion flame and not by combustion, therefore naturally lacking the nitrogen oxides and nitrated PAHs.

We addressed the adjuvant effect of fresh diesel exhaust particles to the reaction of the epithelium to whole birch pollen. The pre-exposure to diesel particles shifted the reaction to birch pollen to earlier times, which shows the adjuvant effect of pollutants in bronchial epithelial cells.

5. Conclusion

Genome wide transcriptome analysis of the pre-exposed immortalized human BEAS-2B epithelial cells with diesel exhaust shows a shift to a faster inflammatory response when pollen is encountered. Our results show that concomitant diesel exhaust exposure may not only result in stronger allergic reactions with the enhancement of inflammation related pathways, but also suggest that diesel disables the epithelial barrier.

Our results provide a way to faster generate evidence that could also guide human studies in which biomarkers are used to monitor the adjuvant effect of pollution particles. Here *BACE1, ITGA4, GNGT2, and MADCAM1* might be worthwhile candidates.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.112968.

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Supplemental Information

Table A1 – Internal standard (ISTD) and derivatization standard (DSTD) used for the analysis of Diesel-CAST samples.

ISTD	DSTD
Acenaphthylene-D8	Fumaric Acid-D2
Acenaphthene-D10	Adipic Acid-D10
Biphenyl-D10	P-Nitrophenol-D4
Fluorene-D10	Glycerol-D8
Anthracene-D10	Levoglucosane 13C6
Phenanthrene-D10	Vanillin 13C6
Fluoranthene-D10	Palmitic Acid-D31
Pyrene-D10	Dodecanol-D25
Benz[a]anthracene-D12	Cholesterol-D6
Chrysene-D12	
Benz[b]fluoranthene-D12	
Benz[k]fluoranthene-D12	
Benz[e]pyrene-D12	
Benz[a]pyrene-D12	
Perylene-D12	
Indeno[1,2,3-cd]pyrene-D12	
Dibenz[a,h]anthracene-D14	
Benz[ghi]perylene-D12	
Coronene-D12	
n-Hexadecane-D34	
n-Docosane-D46	
n-Tetracosane-D50	
n-Triacontane-D62	
Benz[a]anthracene-7,10-dione-D10	
9,10-Anthraquinone 13C6	

Table A2 – Flow parameters. Carrier gas used: Helium.

Column flow [mL min^{-1}]	Split flow [mL min^{-1}]	t [s]
0.1	10	1200
1	100	90
2.6	0	600
1	100	2510

Table A3 – Injection temperature profile.

T rate [°C s^{-1}]	T [°C]	t [s]
-	70	40
2	300	1350
-	250	3010

Table A4 – Column setup.

Column setup	Type	Length [m]
Pre-column	BPX5 (0.25 mm i.d. x 0.25 µm df)	2
1st Dimension	BPX50 (0.25 mm i.d. x 0.25 µm df)	58
2nd Dimension	BPX1 (0.1 mm i.d. x 0.1 µm df)	1.4

Table A5 – GC oven temperature profile. The secondary oven had a temperature offset of 5 °C relative to the GC oven. The modulator temperature offset was 15 °C relative to the secondary oven. The modulation time was 3 s.

T rate [°C min^{-1}]	T [°C]	Holding time [min]
-	40	5
5	300	-
-	300	15

Table A6 – Mass spectral parameters.

MS Parameters	Settings
Transferline temperature	280 °C
Acquisition rate	100 Hz
Acquisition range	29 – 750 m/z
Optimized Voltage Offset	100
Electron ionization	70 eV
Ion source temperature	250 °C

Table A7 – Processing parameters of Leco ChromaTOF. Version 4.50.8.0 optimized for Pegasus.

Baseline Correction		
Baseline Offset	1	auto no. of data points for smoothing
S/N	50	
Peak Find	**1st dimension**	**2nd dimension**
Peak Width [s]	12	0.1
Match required to combine Peaks	-	800
Subpeak	min. S/N	5
Library Search Settings		
Identity/Search Mode	normal/forward	
No. of Hits	10	
Molecular Weight Min./Max.	29/800	
Mass Threshold	50	
Similarity Match Min.	650	
Library	NIST	
Mass for Area Calculation	DTIC	
Export	.csv-files	

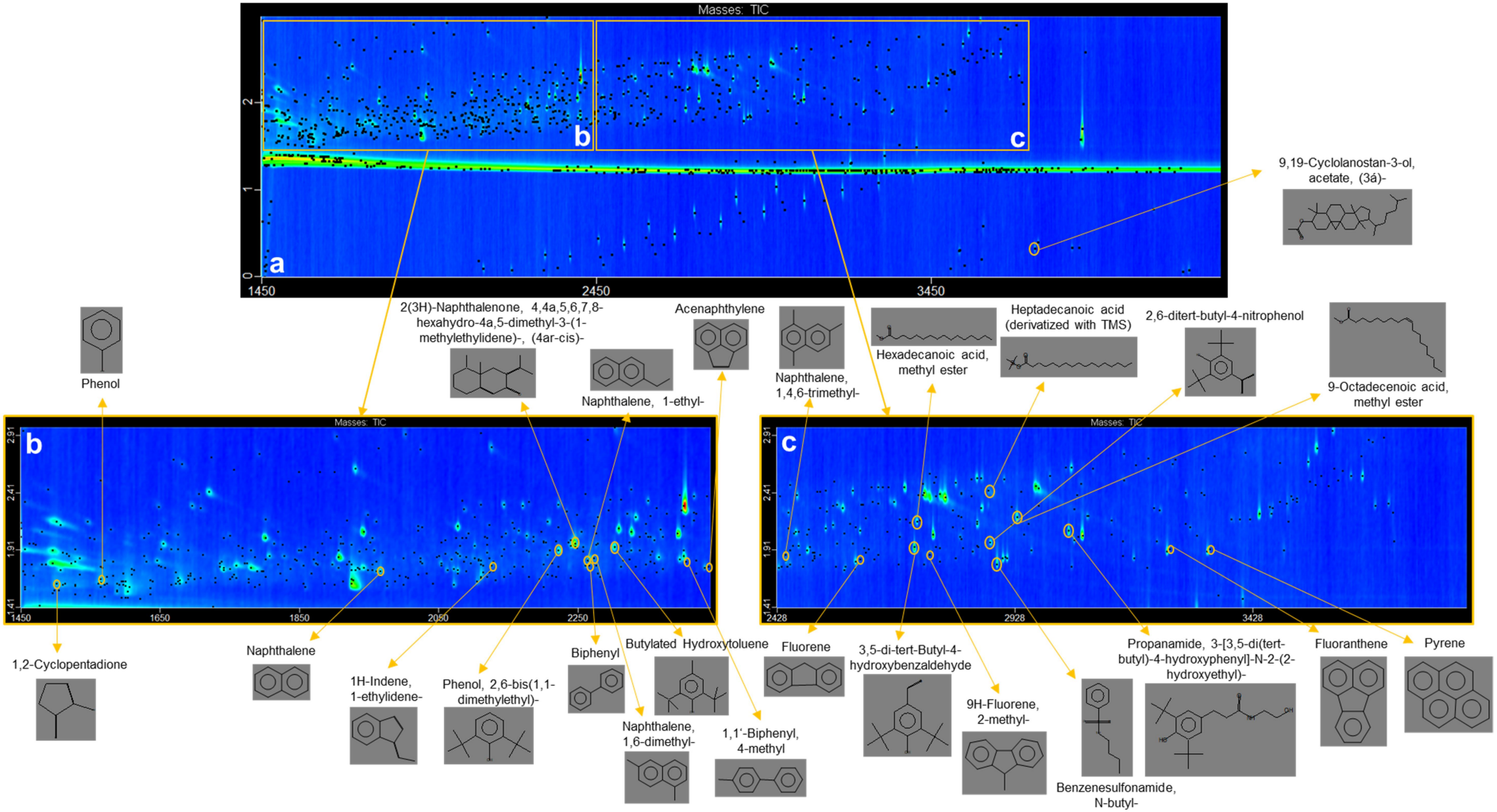

Fig. S1 – Chemical composition of diesel exhaust (CAST) by GC×GC-ToFMS. (a) Full GC×GC-ToFMS chromatogram. (b) and (c) Selection of specific areas of the chromatogram with the most abundant compounds highlighted.