

# Assessment of Changes in Genetic Transcriptome in Nasal Epithelial Cells Exposed to Black Carbon and Pollen Allergen by High-throughput Transcriptomics

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## Research

**Keywords:** Black carbon, Pollen allergen, Differential gene expression, Gene Ontology, High-throughput RNA sequencing, Human nasal epithelial cell cultures (hNECs), inflammation, KEGG pathway analysis, Transcriptome

**Posted Date:** December 4th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-118284/v1>

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# Abstract

**Background:** Evidence suggests that air pollution may be associated with an increase in airway responsiveness to allergens, an increase in bioavailability of airborne allergens and possibly exacerbation of allergic rhinitis (AR). Environmental black carbon (BC) is an important constituent of atmospheric particulate matter (PM), for which the mechanisms underlying its effects have not been fully elucidated in AR. The objective of the present study was to determine the BC and pollen-induced alterations in the transcriptome in human nasal epithelial cells (hNECs) in vitro.

**Methods:** hNECs were prepared from nasal epithelial mucosal samples of healthy individuals undergoing nasal surgery (turbinoplasty or septoplasty). The hNECs were established as air-liquid interface (ALI) cultures and exposed to BC alone or in combination with pollen allergen. The changes in the transcriptome were analyzed by high-throughput RNA sequencing (RNA-Seq). Some of the differentially expressed genes were verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Enrichment analysis based on Gene Ontology (GO) and KEGG database for each Gene was performed to determine their major biological functions and pathways.

**Results:** Exposure to  $\geq 50$   $\mu\text{g/ml}$  BC or  $25\mu\text{g/ml}$  BC +  $200\mu\text{g/ml}$  pollen significantly decreased cell viability in the hNECs compared to control ( $p < 0.05$ ) or  $25\mu\text{g/ml}$  BC alone ( $p < 0.05$ ). Expression of 114 genes (including 80 up-regulated and 34 down-regulated) and 293 genes (including 233 up-regulated and 60 down-regulated genes) was significantly altered following exposure to  $200\mu\text{g/ml}$  BC and  $25\mu\text{g/ml}$  BC +  $200\mu\text{g/ml}$  pollen respectively. For  $200\mu\text{g/ml}$  BC, up-regulated GO terms were mainly associated with hypoxia stress response, whereas for  $25\mu\text{g/ml}$  BC +  $200\mu\text{g/ml}$  pollen treatment the top enriched GO terms were associated with inflammatory response including regulation of neutrophil migration and chemotaxis, macrophage differentiation and chemotaxis, mast cell activation, and phagocytosis. KEGG pathway analysis indicated the top 10 upstream regulators to be IL1B, CSF1, CCL2, TLR2, LPL, IGF8, SPP1, CXCL8, FCER1G and IL1RN. The expressions of inflammation related gene IL1B, CSF1 and FCER1G were elevated as measured by RT-qPCR assay.

**Conclusion:** BC and pollen allergen may induce innate immune and allergic inflammation in hNECs, and therefore potentially exacerbate the symptoms of AR in affected individuals.

## Introduction

Allergic rhinitis (AR) is common inflammatory disease of the nasal mucosa, the prevalence of which has markedly increased over the past three decades and currently affects 10–40% of the population worldwide.<sup>1</sup> Epidemiologic studies have correlated the increase in AR in China with an increase in industrialization and air pollution over the last two decades.<sup>2</sup> Some studies have suggested that air pollution might induce an increase in airway responsiveness to allergens and the increase bioavailability of airborne allergens.<sup>3</sup> In this context, pollen is a typical outdoor aeroallergen that is an independent risk factor for the development of allergic respiratory diseases;<sup>4</sup> with about 30–58% of AR patients have been

shown to be sensitized to pollen. Studies investigating the effect of nitrogen dioxide (NO<sub>2</sub>) and sulphur dioxide (SO<sub>2</sub>), two major fossil fuel-derived air pollutants, have demonstrated that these increase the allergenicity of pollen,<sup>5</sup> thus possibly aggravating or inducing AR in susceptible individuals..

Black carbon (BC) produced by the incomplete combustion of fuel is an important constituent of atmospheric particulate matter (PM) and is the second-largest contributor to global warming.<sup>6</sup> When emitted into the atmosphere, BC undergoes an aging process during which its particle morphology, chemical features, and redox activity may change.<sup>7</sup> In the present study we have investigated the alterations in the transcriptome in human nasal epithelial cell (hNECs) exposed to BC and pollen allergen in vitro.

## Materials And Methods

### Isolation and cultivation of human nasal epithelial cells (hNECs)

Nasal epithelial tissues were obtained from seven patients undergoing nasal surgery (turbinoplasty or septoplasty). Nasal polyps and the nasal tissues from patients with allergies or other chronic epithelial diseases were excluded. None of the patients had other systemic diseases or had received glucocorticoids or antibiotics within 3 months before the study. The study protocol was approved by the Ethics Committee of Beijing Tongren Hospital, and all patients provided written informed consent prior to any samples being taken for investigation.

Freshly obtained nasal mucosal samples were washed in phosphate buffer saline (PBS) with 200 U/mL penicillin and 50 mg/mL streptomycin, and then incubated overnight at 4 °C in 0.1% pronase (Protease XIV; Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified eagle media (DMEM) culture medium to enzymatically digest the tissue. Following incubation, the separated epithelial cells were collected and washed by centrifugation at 100 g for 5 minutes and re-suspension in fresh DMEM. The washed cells were seeded at a concentration of  $1 \times 10^6$  cells on porous membrane inserts (Corning® Transwell polycarbonate membrane inserts, 0.4 µm; 6.5 mm diameter; Corning Inc., N.Y., USA) coated with 150 µl collagen I (66 ng/ml; Sigma-Aldrich, St. Louis, Mo., USA), and cultured at 37 °C in 5% CO<sub>2</sub> in air atmosphere. Once the cultures had reached 70–80% confluence by day 4, the culture medium was removed from the inserts, and BEGM: DMEM (1:1) medium was added to the basolateral side (insert wells) to differentiate cells. The cell cultures were assessed for transepithelial resistance (TER) using Millicell-ERS Volt-Ohm Meter (Millipore, Temecula, CA, USA). When the TER of individual cultures exceeded  $2000\Omega \times \text{cm}^2$ , the cultures were established as air-liquid interface (ALI) cultures, and subsequently used to assess the effects of exposure to BC and pollen.

### BC and pollen preparation and exposure

Ozone-oxidized black carbon (BC), which is consistent with black carbon in the real environment,<sup>8</sup> was obtained from State Key Joint Laboratory of Environmental Simulation and Pollution Control, College of Environmental Sciences and Engineering, Peking University. Prior to use, the BC was dissolved in deionized water at a concentration of 2 mg/ml and sonicated for 10 min using an ultrasonicator.

*Artemisia annua* pollen were collected from Beijing and aqueous protein extracts of the pollen were prepared by resuspending 2 g of pollen grains in 35 mL PBS buffer (0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>) and shaking for 12 h at 4 °C. The supernatants were collected for estimation of the protein concentration, using the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Carlsbad, CA, USA). The pollen protein was then dissolved in deionized water at a concentration of 10 mg/ml.

The cells on Transwell membranes of ALI cultures were washed twice with sterile PBS, and the supernatant was removed. The membranes were exposed to either doubling concentrations ranging 12.5 to 300 µg/ml or doubling concentrations of pollen protein ranging from 50 to 300 µg/ml for 24 h, and following exposure, the medium from the basolateral compartment was transferred to a 1.5 ml vial and stored at – 20 °C until further analysis. The cells were trypsinized off from the membrane, lysed in RLT buffer (RNeasy mini kit, Qiagen, Ven- lo, The Netherlands), and stored at – 80 °C until further analysis.

## Cell viability

Cytotoxicity effects of BC and pollen in hNECs were determined using the Cell Counting Kit-8 (CCK8 CK04-500T Dojindo, Japan), according to manufacturer's protocol. Cells ( $5 \times 10^4$ /well in 96-well plates) were exposed to BC and/or pollen for 24 h at 37 °C. 10 µL CCK8 solution was added to each well and the cells were incubated for a further 2 h at 37 °C. At the end of this incubation, the fluorescence of individual wells was determined at 450 nm using fluorescence microplate reader (Hitachi, Ltd., Tokyo, Japan).

## High-throughput RNA sequencing (RNA-Seq)

For the transcriptomic studies, ALI-hNECs were treated with 25 µg/ml BC ± 200 µg/ml pollen for 24 h at 37 °C in CO<sub>2</sub> incubator. At the end of incubation the hNECs were harvested and total RNA was extracted from the cells using RNeasy mini kit 147 ((Qiagen, Germany)). All RNA samples were stored at -80 °C until use. For each experiment, three different batches of cultured cells were used and assays were performed in triplicate.

Paired-end libraries were synthesized by using the TruSeq<sup>TM</sup> RNA Sample Preparation Kit (Illumina, USA) following TruSeq<sup>TM</sup> RNA Sample Preparation Guide. Briefly, the poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads.

Following purification, the mRNA was fragmented into small pieces by incubation with divalent cations at 94 °C for 8 min. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then underwent an end repair process; to add a single 'A' base and

ligation of the adapters; before being purified and enriched with PCR to create the final cDNA library. Purified libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies, USA) and validated by Agilent 2100 bioanalyzer (Agilent Technologies, USA) to confirm the insert size and calculate the mole concentration. Cluster was generated by cBot with the library diluted to 10pM and were then sequenced on the Illumina NovaSeq 6000 (Illumina, USA).

The library construction and sequencing was performed at Shanghai Sinomics Corporation.

## **Quantitative Real-time reverse transcription(qRT-PCR)**

ALI-hNECs were exposed to 25 µg/ml BC ± 200 µg/ml pollen in the absence or presence inhibitors (100 µM NAC, 10 µM MCC950, or 50 µM YVAD) for 24 h at 37 °C in CO<sub>2</sub> incubator, and at the end of incubation total RNA was extracted from the cells using the TaKaRa MiniBEST Universal-RNA Extraction Kit (TAKARA BIO INC, Kyoto, JPN). The RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA), and subjected to real-time PCR analysis using TB Green™ Premix Ex Taq™ II (TAKARA BIO INC, Kyoto, JPN) in a Bio-Rad real-time PCR detection system. GAPDH was used as an endogenous reference for mRNAs. All primers employed for PCR analysis were obtained from TSINGKE (TSINGKE Biological Technology, Beijing, CHN), as follows: GAPDH: sense 5'-ACCACCATGGAGAAGGC-3', and antisense 5'-GGCATGGA CTGTGGT CATGA - 3'; IL-1β: sense 5'-GTGGTGGTTCGGAGATTCGTAG-3', and antisense 5'-GAAATGATGGCTTATTACAGTGGC-3'; CSF1: sense, 5'-AGTATTGCCAA GGAGGTGTCAG-3', and antisense 5'-ATCTGGCATGAAGTCTCCATTT-3'; FCER1G: sense, 5'-GAGAGCCTCAGCTCTGCTAT-3', and antisense 5'-TGGTT ATAGCTGCCTTTCGCA-3. Relative expression was calculated using the comparative cycle threshold method.

## **Statistical analysis**

Data from at least three independent experiments were expressed as mean ± standard deviation (SD), and analyzed for significance using Spearman correlation coefficients, two-tailed indirect Student's t-test or one-way analysis of variance (ANOVA), followed by the LSD post hoc test for multiple comparisons. All statistical analyses were performed using SPSS 25.0 statistical software and a P value < 0.05 was considered statistically significant.

The parameter Q value was used for statistical screening of differential genes. The difference screening criterion was q value < 0.05, and FC2, that is, the change of the expression value was up 2 times (FC ≥ 2) or down 2 times (FC ≤ 0.5). Enrichment analysis of different genes based on Gene Ontology (GO) and KEGG database for each Gene, was additionally performed to determine the major biological functions and pathways of the different genes.<sup>9,10</sup>

## **Results**

### **Effect of BC ± pollen on viability of hNECs**

hNECs were exposed to 12.5, 25, 50, 100, 200 and 300 µg/ml BC or 50, 100, 200 and 300 µg/ml pollen. Exposure to BC progressively decreased the viability of hNECs from concentrations above 50 µg/ml; with a concentration of 50 µg/ml significantly decreasing the viability to  $73.57 \pm 8.270\%$ , compared to control and a BC concentration of 25 µg/ml ( $P < 0.01$ ,  $P < 0.05$ , respectively) (Fig. 1A). However, addition of 200 µg/ml pollen to hNECs treated with BC 12.5 µg/ml or 25 µg/ml significantly decreased the viability of hNECs compared to BC 12.5 µg/ml or BC 25 µg/ml alone ( $P < 0.05$  and  $P < 0.01$ , respectively) (Fig. 1A). Furthermore, survival rate of cells treated with BC concentrations higher than 50 µg/ml, was negatively correlated with the concentration of BC ( $r = -0.850$ ,  $p = 0.00001$ ) (Fig. 1A). In contrast, exposure to pollen alone did not alter the viability of hNECs at any concentration of pollen investigated (Fig. 1B).

## Transcriptome alteration and functional enrichment analysis

A subset of 58,300 human genes was assayed in this study. Gene expression changes were analyzed by comparison between BC, pollen and BC + pollen treated groups and the control groups using statistical criteria of > 2.0-fold changes with a p-value of < 0.05. Expression of 40 genes was significantly altered (17 genes up-regulated and 23 genes down-regulated) following exposure to 200 µg/ml pollen (Fig. 2A), 21 genes significant altered (6 genes up-regulated and 15 genes down-regulated) following exposure to 25 µg/ml BC (Fig. 2B), and 114 genes significantly altered (34 genes up-regulated and 80 genes down-regulated) following exposure to 200 µg/ml BC (Fig. 2C). Moreover, exposure to a combination of 25 µg/ml BC + 200 µg/ml pollen resulted in significant alterations in even greater number of genes (233 genes up-regulated and 60 down-regulated) (Fig. 2D).

Gene ontology (GO) biological processes and KEGG pathway enrichment analysis of the altered genes further demonstrated that no GO terms were substantially enriched in hNECs exposed to 25 µg/ml BC or 200 µg/ml pollen alone (Fig. 3A and B). However, for hNECs exposed to 200 µg/ml BC, the up-regulated GO terms were mainly associated with hypoxia stress response (i.e., cellular response to oxygen species and response to hydrogen peroxide) (Fig. 3C); whereas the top enriched GO terms for hNECs exposed to a combination of BC (25 µg/ml) and pollen (200 µg/ml) were mostly associated with the inflammatory response (i.e., regulation of neutrophil migration and chemotaxis, macrophage differentiation and chemotaxis, mast cell activation, degranulation and mast cells mediated immunity, and phagocytosis) (Fig. 3D).

Further investigation of the main altered genes associated with innate immune and inflammatory response in hNECs exposed to the combination of BC (25 µg/ml) and pollen (200 µg/ml) are shown in Table 1. In particular, differentially expressed genes associated with regulation of neutrophil migration and chemotaxis included IL1B, IL1R1, IL1A, ITGAM, CSF3R, S100A9, RAC2, CCL20, CXCL5, CXCL3, and CXCL8; Genes associated with macrophage differentiation and chemotaxis included CSF1, CSF1R, TLR2, HCLS1, MMP9, LPL, and CD36; genes associated with phagocytosis included IL1B, SLC11A1, ITGSAM, DOCK2, RAC2, CD14, CD36, PTPRC, and MERTK; and genes associated with regulation of mast cell activation, degranulation and mast cells mediated immunity included FCER1G, FGR, LAT2, SYK, FES, RAC2, and VAV1.

Table 1

The differentially expressed genes associated with inflammatory response; including; regulation of neutrophil migration and chemotaxis, macrophage differentiation and chemotaxis, mast cell activation, degranulation and mast cells mediated immunity, phagocytosis, and cell response for lipoteichoic acid in hNECs exposed to 25 µg/ml BC + 200 µg/ml pollen vs control hNECs.

Biological function	Related Gene	Log2FC	q-value	Biological function	Related Gene	Log2FC	q-value
Regulation of neutrophil degranulation, migration and chemotaxis	CCL20	8.81	5.01E-37	Regulation of phagocytosis	TLR2	1.27	3.71E-21
	ITGAM	7.72	8.65E-22		ITGAM	7.72	8.65E-22
	CXCL8	7.59	3.29E-68		DOCK2	6.53	5.20E-17
	IL1B	6.22	2.87E-56		IL1B	6.22	2.87E-56
	S100A9	5.99	7.24E-06		CD14	5.86	3.80E-14
	CXCL5	4.76	2.44E-06		PTPRC	5.55	9.34E-07
	IL1R1	3.63	7.47E-09		CD36	3.72	1.71E-10
	CSF3R	3.15	1.04E-05		SLC11A1	3.12	2.54E-14
	RAC2	3.05	2.40E-16		RAC2	3.05	2.40E-16
	CXCL3	2.01	1.51E-09		MERTK	2.82	1.21E-07
IL1A	1.14	1.01E-07	Regulation of mast cells degranulation, activation and mast cells mediated immunity	VAV1	4.76	2.44E-06	
Macrophage differentiation, chemotaxis	HCLS1	7.29		7.19E-19	FGR	4.43	1.89E-10
	MMP9	6.06		1.24E-48	FES	3.40	0.000367
	LPL	5.59		5.24E-07	RAC2	3.05	2.40E-16
	CSF1	4.23		4.08E-15	MILR1	1.87	0.01
	CD36	3.72	1.71E-10	LAT2	1.26	2.48E-07	

Biological function	Related Gene	Log2FC	q-value	Biological function	Related Gene	Log2FC	q-value
	CSFR1	3.37	7.25E-18		FCER1G	1.21	8.12E-17

## KEGG pathway analysis

Assessment of the upstream regulators associated with inflammatory response in hNECs exposed to the combination of BC and pollen compared with control indicated that the top 10 upstream regulators were IL1B, CSF1, CCL2, TLR2, LPL, IGF8, SPP1, CXCL8, FCER1G and IL1RN (Table 2).

Table 2  
upstream regulators associated with inflammatory response in hNECs exposed to 25 µg/ml BC + 200 µg/ml pollen vs control hNECs.

Upstream regulator	Expr Log Ratio	Activation z-score	p-value of overlap
IL1B	6.00	4.41	1.13E-14
CSF1	4.01	3.68	1.87E-12
CCL2	3.93	1.37	4.51E-10
TLR2	6.75	3.39	6.33E-08
LPL	3.88	0.71	1.67E-07
IGF8	6.47	2.01	6.48E-07
SPP1	10.13	2.74	1.101E-06
CXCL8	5.57	2.29	9.69E-08
FCER1G	3.21	2.11	0.0000018
IL1RN	7.89	-2.61	3.07E-08

## Gene expressions validation by RT-qPCR analysis

In order to confirm the results of microarray, RT-qPCR was applied to further examine the expressions of target genes. Pro-inflammatory factor IL1B was up-regulated in hNECs exposed to 25, and 200 µg/ml BC and 25 µg/ml BC + 200 µg/ml pollen (Fig. 4A). For inflammation related gene expressions, the primary regulator of mononuclear phagocytes, CSF-1 was over-expressed in hNECs exposed to 25 µg/ml BC and 25 µg/ml + 200 µg/ml pollen; whereas for mast cell mediated immunity related gene expressions, FCER1G was over-expressed in hNECs exposed to 200 µg/ml pollen and 25 µg/ml BC + 200 µg/ml pollen. Although these results were consistent with the microarray data, there was a small difference in terms of

the degree of over-expression between microarray method and RT-qPCR assay, which may be due to differential sample preparation and sensitivity of the measuring method.

## Discussion

This study determined the effect of BC on pollen-sensitized human nasal epithelial cells (hNECs) and showed that exposure to more than 50 µg/ml of BC or 25 µg/ml BC + 200 µg/ml pollen significantly decreased the viability of epithelial cells. Whilst exposure to 200 µg/ml BC, mainly up-regulated GO terms associated with hypoxia stress response, exposure to a combination of 25 µg/ml BC + 200 µg/ml pollen upregulated GO terms related to innate immune and inflammatory responses, including regulation of neutrophil migration and chemotaxis, macrophage differentiation and chemotaxis, mast cell activation, degranulation and mast cells mediated immunity, and phagocytosis. Furthermore, increased expression of genes IL1B, CSF1 and FCER1G were found to be the main upstream regulators involved in the inflammatory process.

IL1B is a potent proinflammatory cytokine, belonging to the IL-1 family cluster that includes the IL-1a, and IL1RN genes. IL-1β can be activated by caspase 1 and induces neutrophil influx and activation, T-cell activation and cytokine production, B-cell activation and antibody production, and fibroblast proliferation and collagen production. IL1R1 (interleukin-1 receptor 1) as a member of IL-1 family cluster may regulate the process of inflammation in organisms.<sup>11</sup> One recent study has reported that exposure to BC induced differential expression of IL-1R1 in A549 cells,<sup>12</sup> which is consistent with the findings from the present study. Similarly, our findings for differential expression of other genes influencing neutrophils are consistent with other studies. For example, TGAM (integrin alpha M chain), a leukocyte-specific integrin, has been shown to be important in the regulation of neutrophil migration and phagocytosis-induced apoptosis in extravagated neutrophils.<sup>13</sup> Similarly, Rac2, a member of the Rho family GTPases, which are critical in regulating neutrophil activation, is involved in the control of the neutrophil actin cytoskeleton, cell migration, and the NADPH oxidase.<sup>14</sup> CSF3R (granulocyte colony-stimulating factor receptor) plays a crucial role in the proliferation, differentiation and survival of cells along the neutrophilic lineage. One of the most important functions of neutrophils is the production of oxidative metabolites for killing invading micro-organisms. Studies have suggested that S100A9 inhibits chemotaxis toward pro-inflammatory molecules and increased oxidative metabolism by neutrophils<sup>15</sup>, which is consistent with our finding of the anti-oxidative and anti-inflammatory effect of S100A9 on neutrophils. An in vitro study has confirmed that CCL20 has a direct chemotactic effect in neutrophil recruitment.<sup>16</sup> In accordance with these studies, the present study has indicated that epithelium-derived neutrophil-activating peptide 78 (CXCL5), and neutrophil chemoattractants IL-8 (CXCL8) and CXCL3 are likely to be involved in the inflammatory response induced by BC and pollen in hNECs.

Macrophages are indispensable as members of the innate immune system, as they regulate normal physiology as first responders by communicating with the host's adaptive immune system. Macrophage phenotypes include classically activated macrophages (M1) and alternatively activated macrophages

(AAMφ or M2), of which M2 play a role in resolving inflammation. Canonical induction of M2 polarization is mediated by PPARs (peroxisome proliferator-activated receptors; PPARδ, PPARγ) and PGC1β (PPARγ coactivator-1 beta) activation, induction of cluster of differentiation 36 (CD36) and lipoprotein lipase (LPL). Several studies indicate that LPL is a primary regulator of macrophage lipid uptake and a modulator of macrophage polarity.<sup>17,18</sup> Thus, the finding for increased expression of LPL in the present study would suggest that exposure to BC and pollen may induce macrophage polarization to M2. This is consistent with the finding for also increased expression of Colony-stimulating factor-1 (CSF-1, also known as macrophage-CSF); the primary regulator of the survival, proliferation, differentiation and function of mononuclear phagocytes; and CSF-1 receptor (CSF1R) following exposure to BC and pollen. Indeed, it has been demonstrated that CSF-1 enhances cytotoxicity, superoxide production, phagocytosis, chemotaxis and cytokine production in monocytes or macrophages when CSF-1 receptor (CSF1R) expressed on these cells is activated.<sup>19</sup> Similarly, CSF-1 can also prime some innate immune responses while suppressing others by modulation of Toll-like receptors (TLRs), such as TLR2, another gene found to be differentially expressed following exposure to BC and pollen. Moreover, our findings for differential expression of MMP-9 and HCLS1 are also in accordance with studies that have demonstrated that MMP-9 activation is important for macrophage migration, and HCLS1 enhances the function of monocytes/macrophages.<sup>20</sup>

Phagocytosis is primarily carried out by specialized cells termed professional phagocytes, which include cells of the immune system such as macrophages, neutrophils and dendritic cells. Of the differentially expressed genes associated with regulation of phagocytosis in the present study, pro-inflammatory cytokines IL1B (IL-1β) and TNF-α, have been shown to upregulate Fc receptor-mediated phagocytosis.<sup>21</sup> SLC11A1 (Solute Carrier Family 11, Member 1) was formerly known as natural resistance associated macrophage protein 1 (NRAMP),<sup>22</sup> and NRAMP1 is a member of the metal transporter protein family, which transfers iron (Fe) ions across the phagosome membrane.<sup>22</sup> Integrin ITGSAM is important in the phagocytosis of complement coated particles, and may regulate phagocytosis-induced apoptosis in extravagated neutrophils.<sup>23</sup> DOCK2 is a new member of the CDM family proteins, which plays an important role in phagocytosis and NADPH oxidation by functioning upstream of RAC2.<sup>24</sup> Protein Tyrosine Phosphatase Receptor Type C (PTPRC, CD45) has been shown to be involved in phagocytosis as a positive regulator of Src family kinases (SFKs).<sup>25</sup> CD36 and CD14 have been shown to interact with TLRs to induce phagocytosis and inflammation involving monocytes and macrophages.<sup>26,27</sup> MERTK, a MER/AXL/TYRO3 receptor kinase family, plays a role in various processes such as macrophage clearance of apoptotic cells, platelet aggregation, cytoskeleton reorganization and engulfment.<sup>28</sup>

The present study has demonstrated that exposure to BC and pollen also lead to differential expression of genes associated with regulation of mast cells. Mast cell mediator release plays a vital role in the initiation of inflammatory reactions associated with allergic disorders. This involves a chain of reactions following antigen-mediated aggregation of immunoglobulin E (IgE)-occupied high-affinity receptors for IgE (FceRI) on the mast cell surface; involving activation of the Src family tyrosine kinase (Syk, include Lyn and Fgr), phosphorylation of the transmembrane adapter molecules linker for activated T cells 1

(LAT1) and LAT2, activation of protein kinase C (PKC) and liberation of intracellular calcium. These signals lead to mast cell degranulation and contribute to activation of transcription factors required for cytokine and chemokine production. The role of LAT2 in mast cell activation is still enigmatic; however, it has been proposed to both upregulate and downregulate antigen-mediated responses, and to enhance FcεRI-dependent degranulation.<sup>29</sup> In this pathway, tyrosine phosphorylation activates Vav1, a regulator of PLCγ-activated calcium signals to induce migration and activation of mast cells.<sup>30</sup> FGR positively regulates mast cell degranulation, production of eicosanoids and cytokines,<sup>31</sup> and FES protein-tyrosine kinase, a downstream effector of KIT signalling in mast cells, is required for migration of mast cells.

## Conclusion

In summary, exposure to 25 g /ml BC and 200 g /ml pollen significantly decreases the viability of epithelial cells, and induces innate immune and allergic inflammation in hNECs. IL1B, CSF1, and FCER1 are main the upstream regulators in the inflammatory response induced by BC and pollen.

## Abbreviations

ALI cultures – air-liquid interface cultures

AR – Allergic rhinitis

BC – Black carbon

BCA – bicinchoninic acid

CSF- 1(R) – Colony-stimulating factor-1(Receptor)

DMEM – Dulbecco's modified eagle media

FcεRI – high-affinity receptor for immunoglobulin E

GO – Gene ontology

hNECs – Human nasal epithelial cells

IgE – immunoglobulin E

IL – interleukin

IL1R1 – interleukin-1 receptor 1

LAT 1/2 linker for activated T cells 1/2

LPL – lipoprotein lipase

M1/M2 – classically activated macrophages/alternatively activated macrophages

NO<sub>2</sub> – nitrogen dioxide

NRAMP – natural resistance associated macrophage protein 1

PBS – phosphate buffer saline

PM – Particulate matter

RNA-Seq – High-throughput RNA sequencing

PKC – protein kinase C

PPAR – peroxisome proliferator-activated receptor

PTPRC – Protein Tyrosine Phosphatase Receptor Type C

RT-qPCR – reverse transcription-quantitative polymerase chain reaction

SFKs – Src family kinases

SLC11A1 – Solute Carrier Family 11, Member 1

SO<sub>2</sub> – sulphur dioxide

TER – transepithelial resistance

TLRs – Toll-like receptors

SLC11A1 – Solute Carrier Family 11, Member 1

## **Declarations**

### **Ethics approval and consent to participate:**

Not applicable

### **Consent for publication:**

Not applicable

### **Availability of data and materials:**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Competing interests:

The authors declare that they have no competing interests.

## Fundings:

This work was supported by grants from Beijing science and technology commission science and technology planning project (Z191100009119013) and the Beijing Health Bureau Program for High Level Talents (2014-3-017)

## Authors' contributions:

Yuhui Ouyang analyzed and interpreted the results of high-throughput RNA-Seq, and was a major contributor in writing the manuscript. Ying li and Zhaojun Xu performed the cell culture and RT-qPCR of the hNECs. All of authors read and approved the final manuscript.

## Acknowledgements:

Not applicable.

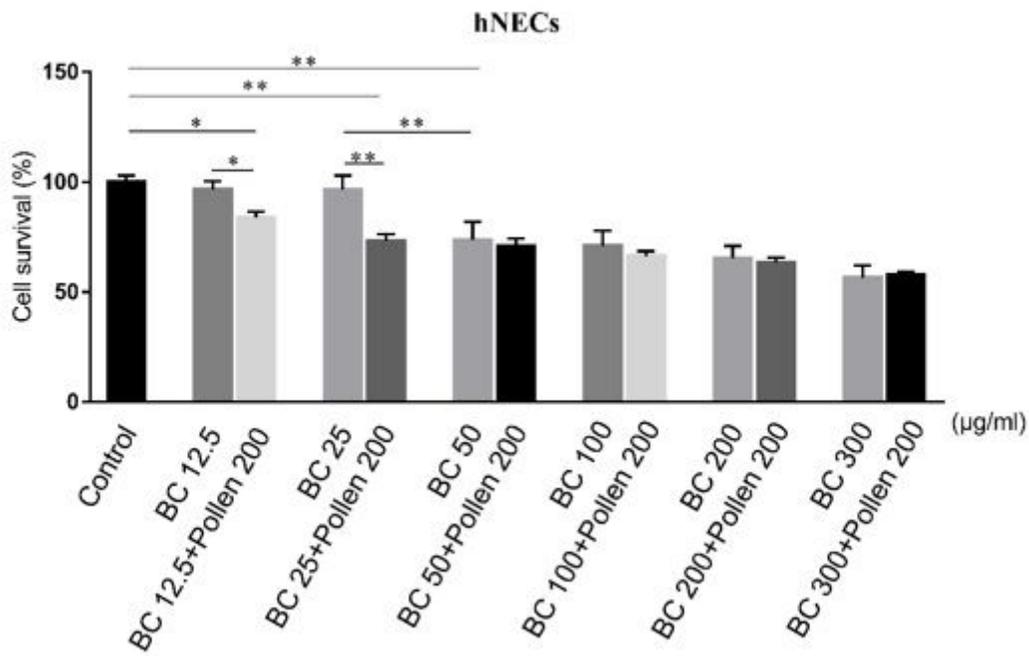
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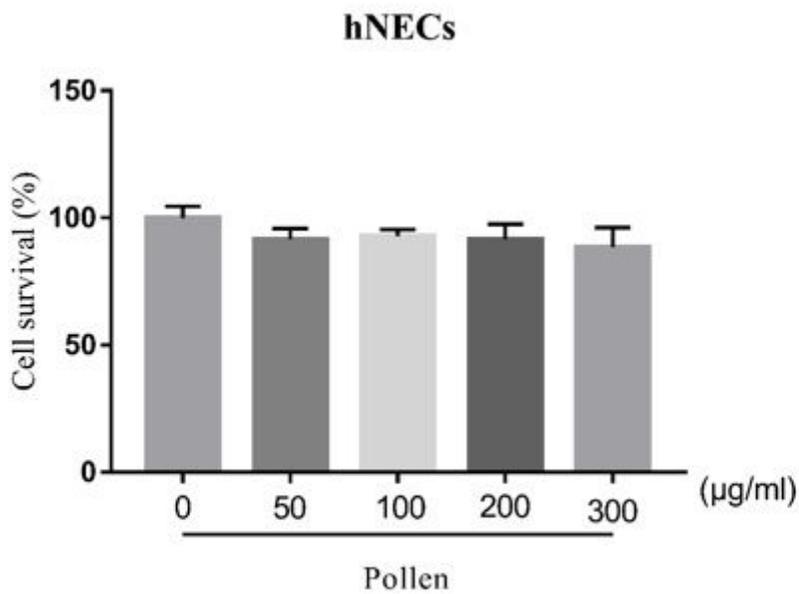
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## Figures



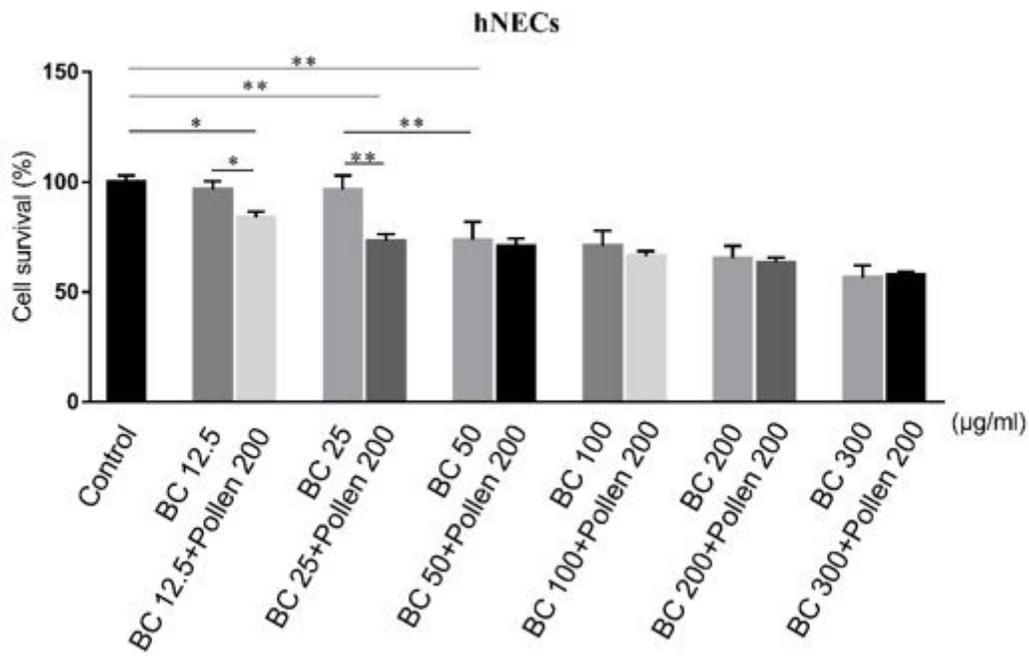
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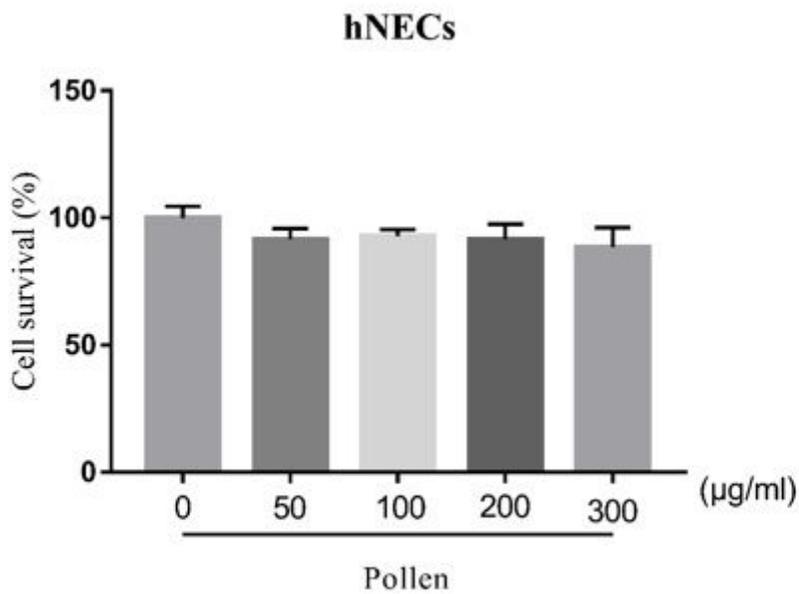
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**Figure 1**

Effect of BC and pollen on the viability of hNECs. (A) Cell viability assay showing decreased viability in hNECs exposed to 50 µg/ml BC and 25 µg/ml BC + 200µg/ml pollen. (B) Cell viability assay showing no changed in hNECs viability exposed to different concentrations of pollen.



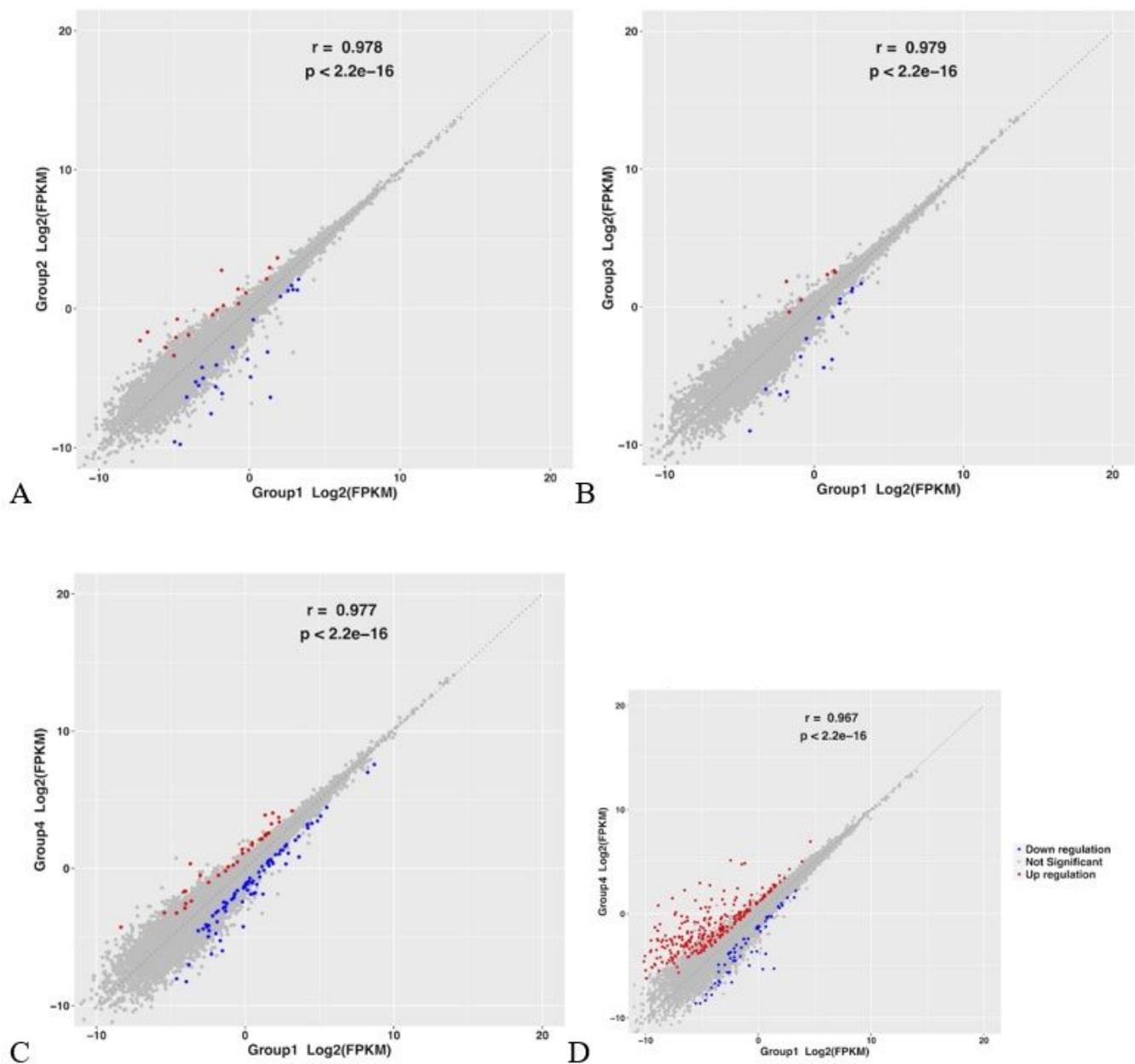
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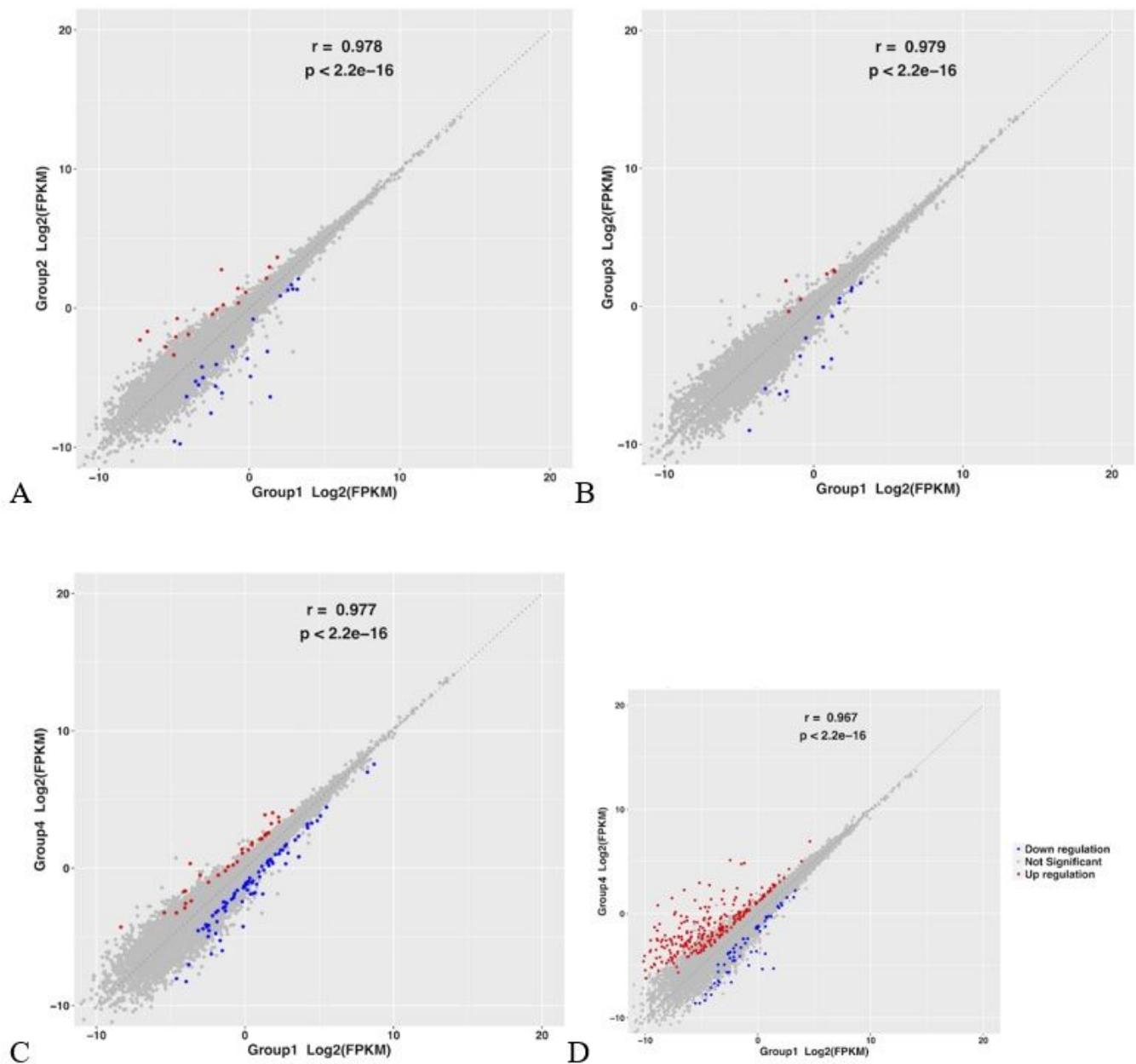
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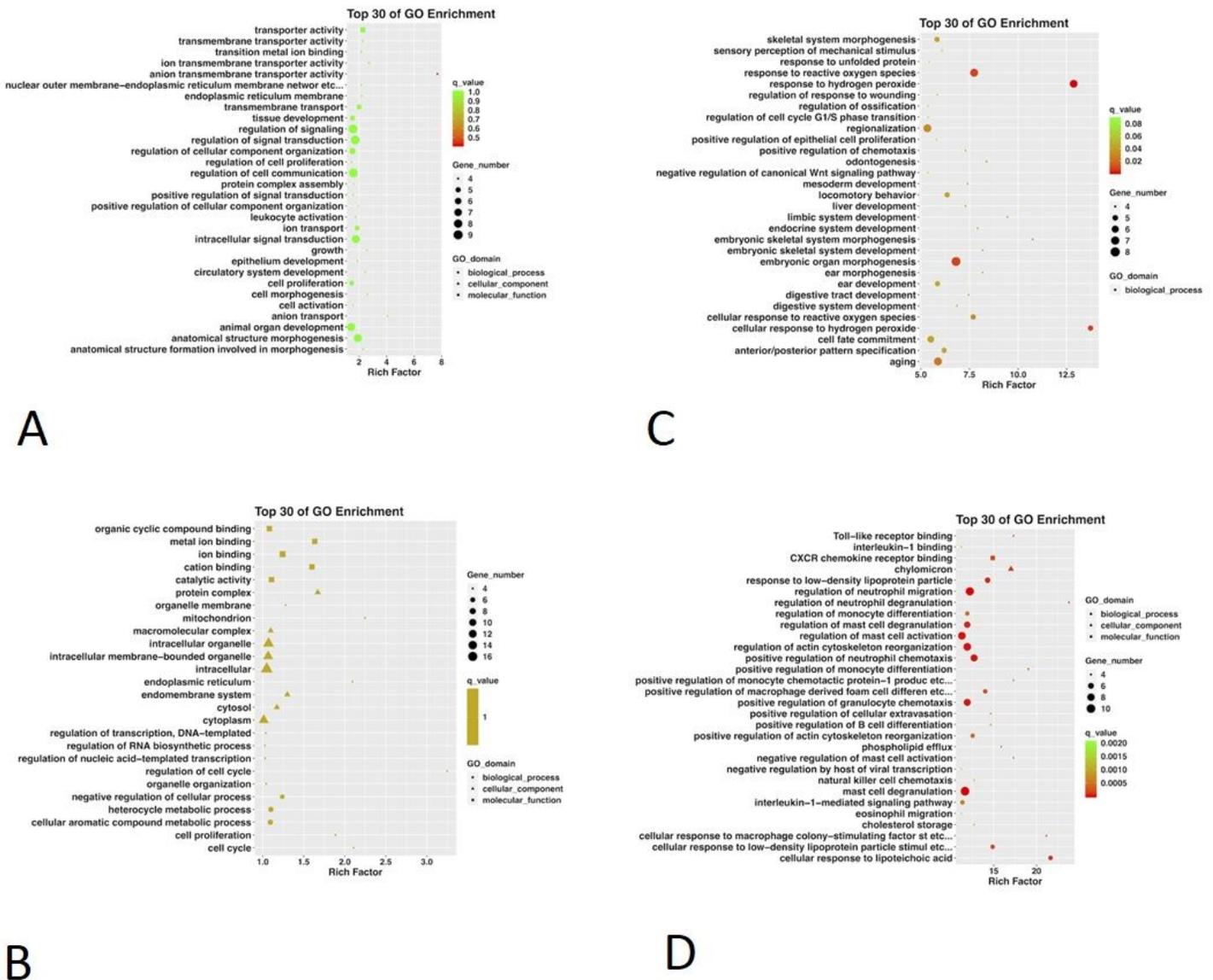
**Figure 2**

Transcriptomic alterations in hNECs treated by BC alone or in combination with pollen. Scatter diagrams of up-regulated (denoted in red) and down-regulated (denoted in blue) genes in hNECs treated by BC alone or in combination with pollen compared with control. (A) 200 µg/ml pollen treatment, (B) 25 µg/ml BC treatment, (C) 200µg/ml BC treatment, (D) 25 µg/ml BC + 200 µg/ml pollen treatment. q value <0.05 and FC2.



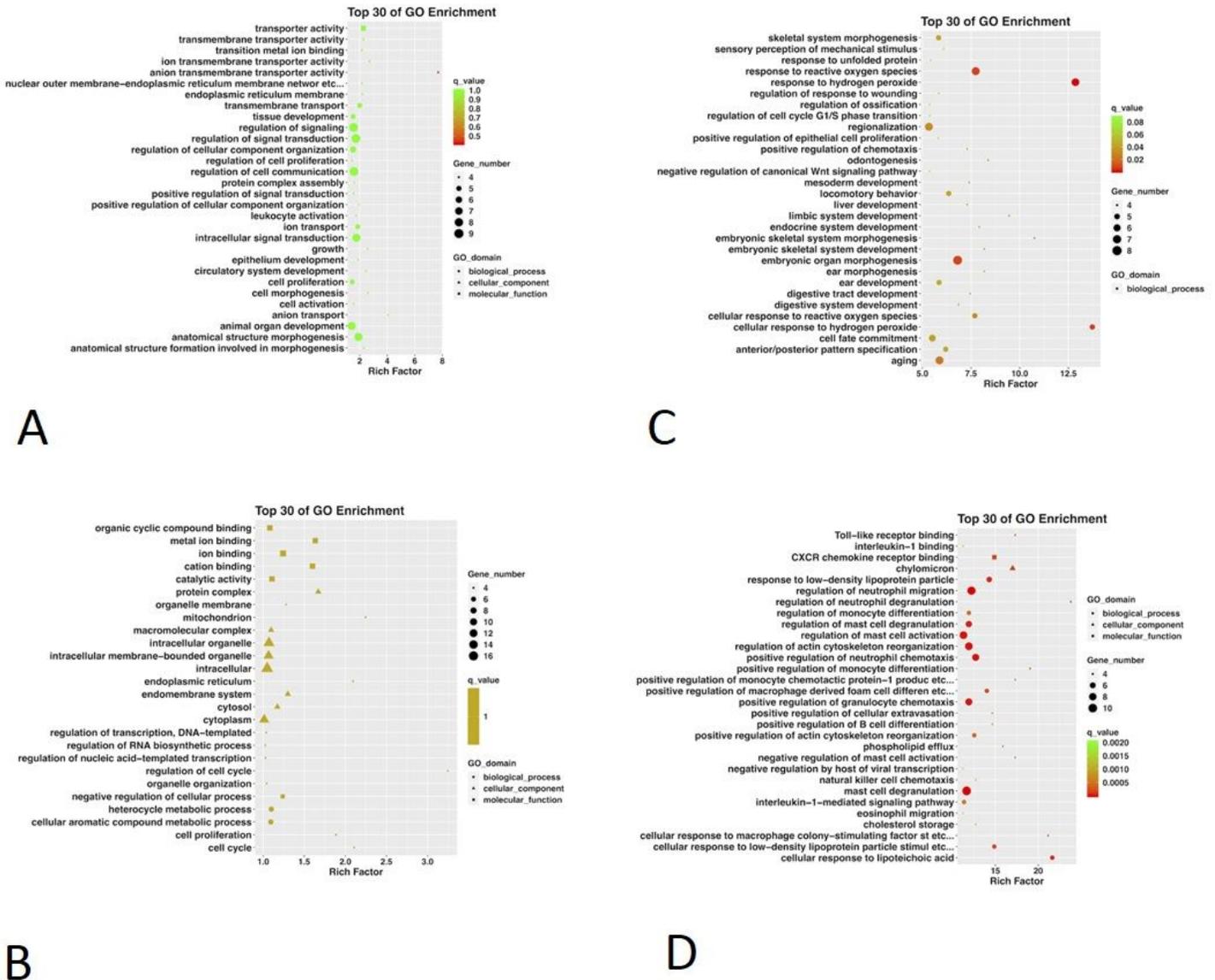
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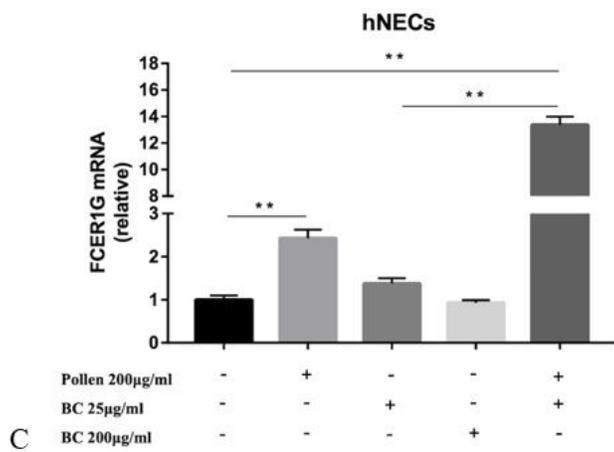
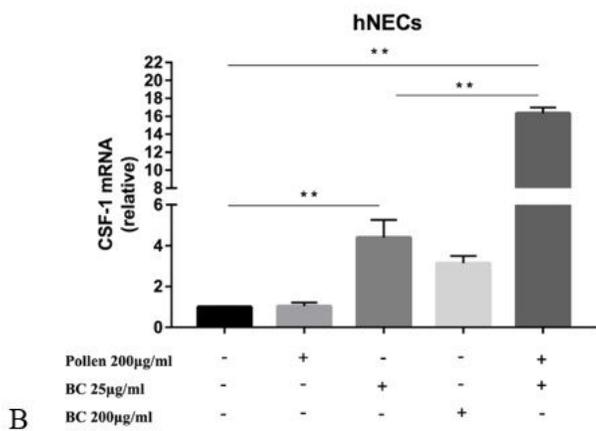
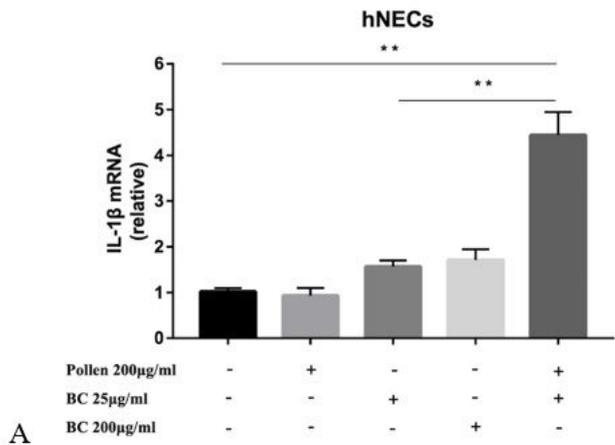
**Figure 3**

Gene ontology (GO) analysis by High-throughput RNA sequencing. Top 30 GO terms enriched by up-regulated and down-templated genes in BC (25 and 300  $\mu\text{g}/\text{ml}$ ) or pollen treated hNECs. In each plot, the GO terms were aligned from left to right according to their Rich Factor from low to high. (A) 200  $\mu\text{g}/\text{ml}$  pollen treatment, (B) 25  $\mu\text{g}/\text{ml}$  BC treatment, (C) 300  $\mu\text{g}/\text{ml}$  BC treatment, (D) 25  $\mu\text{g}/\text{ml}$  BC + 200  $\mu\text{g}/\text{ml}$  pollen treatment.  $q$  value  $< 0.05$  and FC2.



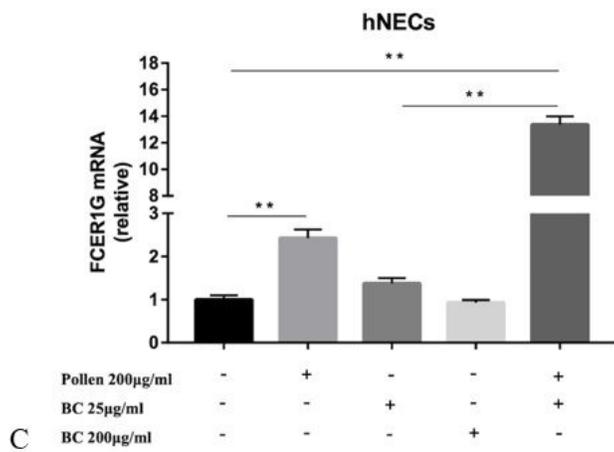
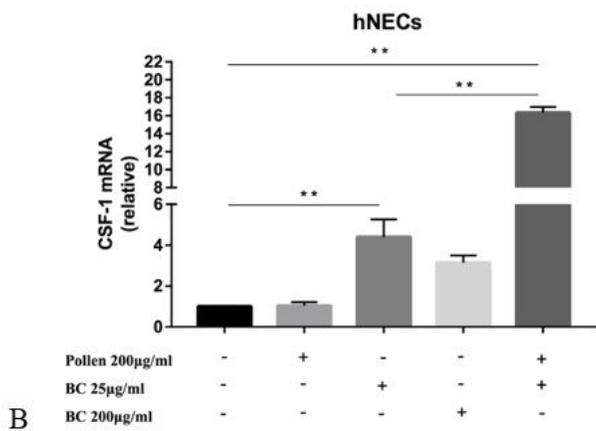
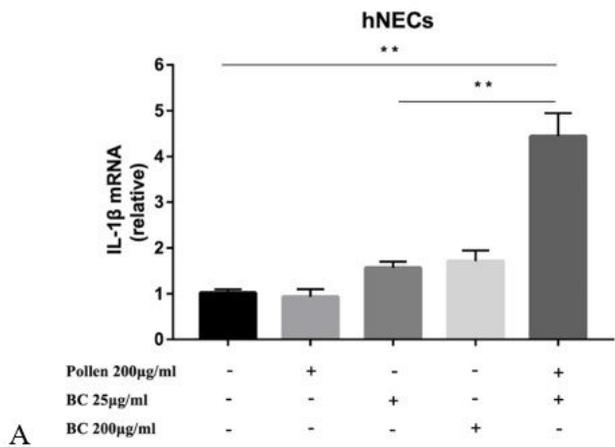
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**Figure 4**

The expressions of inflammation related gene by RT-qPCR assay. IL1B, CSF1 and FCER1G were elevated in hNECs exposed to 25  $\mu$ g/ml BC + 200  $\mu$ g/ml pollen as measured by RT-qPCR assay. n=7, \*p<0.05  
\*p<0.01



**Figure 4**

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