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Particulate matter from Saudi Arabia induces genes involved in inflammation, metabolic syndrome and atherosclerosis

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Abstract

Airborne particulate matter (PM) exposure is a major environmental health concern and is linked to metabolic disorders, such as cardiovascular diseases (CVD) and diabetes, which are on the rise in the Kingdom of Saudi Arabia. This study investigated changes in mouse lung gene expression produced by administration of PM_{10} collected from Jeddah, Saudi Arabia. FVB/N mice were exposed to 100 μg PM_{10} or water by aspiration and euthanized 24 hr later. The bronchoalveolar lavage fluid (BALF) was collected and analyzed for neutrophil concentration and TNF-α and IL-6 levels. RNA was extracted from the lungs and whole transcript was analyzed using Affymetrix Mouse Gene 1.0 ST Array. Mice exposed to PM_{10} displayed an increase in neutrophil concentration and elevated TNF-α and IL-6 levels. Gene expression analysis revealed that mice exposed to PM_{10} displayed 202 genes that were significantly up-regulated and 40 genes that were significantly down-regulated. PM_{10} induced genes involved in inflammation, cholesterol and lipid metabolism, as well as atherosclerosis. This is the first study to demonstrate that Saudi Arabia PM_{10} increases *in vivo* expression of genes located in pathways associated with diseases involving metabolic syndrome and atherosclerosis.

Keywords

gene expression; metabolic diseases; lungs; cholesterol; particulate matter; mouse

Introduction

Airborne particulate matter (PM) exposures are a major environmental health concern. Epidemiological studies have linked air pollution with a number of detrimental outcomes such as, hospital admissions (Colucci et al. 2006; Vigotti et al. 2007), chronic obstructive pulmonary disease (Gan et al. 2013), lung cancer (Yanagi et al. 2012), asthma (Karakatsani et al. 2012) and cardiovascular disease (Beckerman et al. 2012; Brook et al. 2010; Chang et al. 2013; Chiu et al. 2013; Mazzoli-Rocha et al. 2010; Nishiwaki et al. 2012). PM has been

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found to be the major component of air pollution that has the most deleterious effects on human health (Colucci et al. 2006; Samet and Krewski 2007). PM exposure has been associated with premature mortality (Tagaris et al. 2010), decreased birth weight (Pedersen et al. 2013), and increased asthma morbidity (Delfino et al. 2014). PM may be composed of a number of constituents depending on the source, including organic matter, mineral oxides, liquid droplets, soil, dust, smoke, and gaseous chemicals (Araujo and Nel 2009; Simkhovich et al. 2008). PM may vary in size, chemical composition, and origin (Ghio et al. 2012). Coarse particulates (2.5– 10 μm) deposit in the nasopharyngeal region and the upper regions of the lung, while fine (≤ 2.5 μm) and ultrafine (≤1 μm) particulates may penetrate deep into the alveolar region (Ghio et al. 2012).

Several studies have reported on *in vivo* gene expression changes after particulate matter exposure. (Thomson et al. 2013) investigated the gene expression changes in kidney, heart, lungs, liver, and spleen in rats exposed to 5 or 50 mg/m^3 urban particulate matter and found that mRNA profiles were similar across organs for many genes including redox/ glucocorticoid-sensitive genes and inflammatory genes. An interesting study by (Motta et al. 2013) found that human subjects exposed to metal-rich PM displayed a unique expression of microRNAs (miRNAs) in their blood. The study found four differentially expressed PM-responsive miRNAs and eleven miRNA-mRNA pairs that regulate inflammatory gene expression.

Recently, (Khodeir et al. 2012) conducted a multi-week, multiple site sampling campaign to study the source apportionment and elemental composition of PM_{10} in Jeddah, the second largest city in Saudi Arabia. The major source factors for PM_{10} were soil re-suspensions, oil combustion, mixed industrial sources, traffic sources, and marine aerosols. Components of the PM_{10} from Jeddah have been characterized in (Khodeir et al. 2012).

There are many factors that indicate Jeddah, SA as a setting for high PM exposure. The city has stationary (power plant, airport, oil refinery, industries) and mobile (over 1.4 million cars) sources of air pollution. While an early study by (Nasralla 1983) concluded that concentrations of airborne particulates and other pollutants in Jeddah often exceeded air quality standards, a more recent study by (Elassouli et al. 2007) reported that PM_{10} in the city of Jeddah routinely exceeds the average hourly standard for PM_{10} established by the Presidency of Meteorology and Environment in Saudi Arabia, which is 80 μg/m^3.

In previous studies, a 24 hr exposure of PM_{10} collected from Jeddah induced genes involved in NRF2-mediated response to oxidative stress in human bronchial epithelial cells (BEAS-2b) (Sun et al. 2012). (Huang et al. 2011) found that airway epithelial cells treated with an acute exposure of fine or ultrafine PM also displayed altered mRNA profiles involving genes in the NRF2-mediated oxidative stress response pathway. A 4-day exposure in BEAS-2b revealed that genes related to cholesterol and lipid synthesis pathways were also increased (Sun et al. 2012), thus prompting further investigation to assess whether such a metabolic response would be observed *in vivo*.

Metabolic diseases, such as cardiovascular disease and diabetes, are often associated with obesity and have become major health concerns in Saudi Arabia. A study by (Al-Othaimeen

AI 2007) involving19,598 Saudi Arabian citizens found that 38% of males and 28% of females were overweight and a number of other studies highlighted the obesity problem in Saudi Arabia (Al-Malki et al. 2003; Madani et al. 2000). Given the previous *in vitro* findings that Saudi Arabia PM_{10} induces cholesterol and lipid metabolism genes, investigations were conducted to determine if PM_{10} would induce these genes *in vivo* in order to further evaluate the possibility that exposure to PM_{10} may contribute to the development of metabolic disorders.

Metabolic syndrome is a combination of risk factors, when occurring together, increase the risk of cardiovascular disease and diabetes. Many studies linked $PM_{2.5}$ exposure with metabolic disorders (Brook et al. 2013; Brook et al. 2010); however, it is still unclear how ambient particulates mediate adverse health effects. While metabolic disorders are commonly associated with changes in the liver, various studies demonstrated that after a toxic insult, lungs and liver display similar changes in mRNA levels (Crespo et al. 1999; Flohe et al. 1999; Ghoshal et al. 2001; Rosen et al. 2007; Zhang et al. 1998).

Materials and Methods

Animals

Male FVB/N mice (11 weeks old, body weight 22–30 g) were obtained from Taconic Farms (German-town, NY) and housed in our AAALAC accredited housing facility in Tuxedo, NY. FVB/N mice respond well to perturbations of the airways and have been used in PM studies (Keith et al. 2004). Approval was given by NYU Institutional Animal Care and Use Committee (IACUC) for the use of animals in experimental studies. After a 16 day acclimation period, treatment mice (n=6) were exposed via aspiration to 100 μg PM_{10} (3.92 mg/kg) collected from Jeddah, Saudi Arabia and control mice (n=3) were exposed to an equivalent volume of distilled water. The $PM_{2.5}/PM_{10}$ ratio of the PM was 0.33. The dose of $PM_{2.5}$ received by each mouse was 1.29 mg/kg. All procedures were conducted in compliance with New York University's guidelines for ethical animal research. All procedures were conducted in compliance with New York University's guidelines for ethical animal research. Details regarding the particle collection and extraction techniques, as well as, the components of PM_{10} have been previously described (Khodeir et al. 2012; Sun et al. 2012). PM_{10} was analyzed by XRF for the concentration of 27 elements. Re-suspended soil and oil combustion contributed 82% of the mass and mixed industrial sources, traffic sources, and marine aerosols were also present. The PM was heavily concentrated with silicon, calcium, sulfur, aluminum and iron. Some of the other metals present include nickel, vanadium, arsenic, lead, cadmium, manganese, titanium and magnesium.

Animals were anesthetized in a chamber containing 3 ml isoflurane. After 5 min, the animal was removed from the chamber and attached to a suspension slide with its tongue held down by a wire and its upper mouth held open by a rubber band. The appropriate volume of PM_{10} or distilled water was injected in the back of the animal's mouth between breathes. Prior to exposure, PM_{10} was re-suspended by sonication for 10 min.

At 24 hr post-exposure, animals were euthanized with sodium pentobarbital (150–200 mg/kg) via an intraperitoneal injection. Animals were injected with ketamine (0.1 mg/ 10 g)

5–6 minutes prior to euthanasia. Blood was taken from the heart immediately following euthanasia. The trachea was cannulated and the lungs lavaged twice with 1.2 ml phosphate buffered saline without calcium and magnesium (PBS, Invitrogen, Carlsbad, CA). Bronchoaveolar lavage fluid (BALF) was stored on ice until analysis. Both lungs were extracted from the chest cavity and placed in a cryogenic tube and frozen in liquid nitrogen for future analysis of mRNA expression.

BALF Assays

For cell differentials, an aliquot of lavage fluid was prepared using a cytospin (Shandon, Southern Products, UK). An aliquot from each lavage sample (100 μl) was placed into the cytospin for 7 min. Slides were subsequently stained with Hemacolor® staining (EM Science, Gibbstown, NJ). Neutrophil and macrophage populations were enumerated by counting 100 total cells. The remaining lavage fluid was centrifuged at 400 g for 10 min and the collected supernatant was frozen at −20° C until further analysis. BALF supernatants were analyzed for tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by Enzyme-Linked Immunoassays (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN). ELISA assays were run in duplicates. OD values were transferred to Microsoft Excel and a two- tailed t- test was performed. The coefficients of variation within each assay were 0.049 for TNF-α and 0.291 for IL-6. The amount of each cytokine was derived from standard curves specific to each cytokine.

RNA extraction and microarray hybridization

Total RNA was extracted from lungs of control and PM-exposed mice using Trizol (Invitrogen) and further purified using RNeasy Plus Micro Kit (Qiagen). One hundred ng of total RNA was used to synthesize double-stranded cDNA (dsDNA). cRNA was synthesized from dsDNA template, and subsequently used to produce sense single-stranded cDNA (ssDNA) with incorporated deoxyuridine triphosphate. The ssDNAs were fragmented, end-labeled, and hybridized to Affymetrix Mouse Gene 1.0 ST Array (Affymetrix). Hybridization and scanning of the arrays were performed using a standard procedure.

Microarray data analysis

Gene expression analyses were performed using R. Gene expression data were imported and normalized in batches using the Affymetrix package version 1.36.1. in R 2.15.1 GUI 1.42 Leopard build 64-bit and robust multichip average (RMA) (Bolstad et al. 2003; Gautier et al. 2004). Significance of gene expression changes between controls and PM_{10}- treated mice were assessed using a gene-wise linear model approach with LIMMA 3.14.3, which utilizes an empirical Bayes approach to generate moderated t-statistics by taking into account the standard errors and estimated log-fold changes (Smythe et al. 2005). P-values were subjected to FDR correction for multiple hypothesis testing and adjusted p-values with $p<0.05$ were considered significant (Benjamini Y 1995). Gene network and pathway analyses were performed using Ingenuity Pathway Analysis software (http://www.ingenuity.com).

Real time PCR

Total RNA extracted from control and treated lung tissue was converted to single stranded cDNA using Superscrip® III (Invitrogen). Quantitative real-time PCR analysis was performed using SYBR green PCR system (Applied Biosystems) on ABI prism 7900HT system (Applied Biosystems). Relative gene expression levels were normalized to ACTB expression. All PCR reactions were performed in duplicate.

Results

Neutrophil, TNF- α and IL-6 Concentrations in BALF

During the acute phase of inflammation, neutrophils are one of the first responders that migrate to the inflammatory site and increased neutrophil concentration is a reliable indication that an inflammatory response has been elicited (Scapini et al. 2000). In order to measure the neutrophil concentration in bronchoalveolar lavage fluid (BALF) after PM_{10} exposure, FVB/N mice were treated via aspiration with either 100 μg PM_{10} or distilled water and BALF was collected 24 hr later and analyzed for neutrophil concentration (Table 1). The dose was generated from a previous experiment (Table 2). Our aim was to select a dose that would generate immune cell counts of 40–50 % neutrophils in the BALF. Mice treated with PM_{10} or water contained an average of 59.6 and 0.67% neutrophils/total immune cells in BALF, respectively. Other immune cells identified included macrophages, while eosinophils and basophils were not present. Mice treated with PM_{10} or water contained an average of 3.79×10^5 and 2.45×10^5 total immune cells/ ml, respectively.

Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are produced by lung epithelial cells, alveolar macrophages and other immune cells after an insult (Kaplanski et al. 2003; Rosenblum and Donato 1989). In order to determine the effects of PM_{10} on TNF-α and IL-6 levels, an ELISA was performed using BALF supernatant of PM_{10}-exposed and control mice. TNF-α levels were higher in PM_{10}-treated mice compared to control (Figure 1). The average TNF-α levels were 26.2 ± 7.0 pg/ml in PM_{10} and 4.01±1.9 pg/ml in control BALF, respectively. The TNF-α levels in the PM_{10} BALF were significantly increased compared to control. IL-6 levels were also significantly higher in PM_{10} BALF compared to control (Figure 1). Average IL-6 levels were 29.9 ± 9.9 pg/ml in PM_{10} and 4.06 ± 2.7 pg/ml in control BALF, respectively.

Gene expression profiles of PM_{10}-exposed mice

PM is known to affect the expression of many genes involved in various molecular pathways that contribute to the inflammatory microenvironment as well as cholesterol and lipid metabolism (Huang et al. 2011; Kooter et al. 2005; Sun et al. 2012). Whole lung gene expression profiles of 4 mice exposed to PM_{10} and 3 control mice exposed to distilled water were examined. Whole transcript analysis using Affymetrix Mouse Gene 1.0 ST Array was performed and data analyzed using R and Ingenuity Pathway Analysis (IPA) software.

To explore the global impact of PM_{10} on gene expression, principal component analysis (PCA) was first performed to visualize the profile of all genes without any filtering. The

PCA in Figure 2a demonstrates that gene expression in mice treated with PM_{10} or distilled water exhibits distinct differences in clustering based on exposure.

Similar effects were seen in hierarchical clustering analysis of genes significantly changed by PM_{10} (Figure 2b). Gene expression changes between controls and PM_{10}-treated mice were assessed using a gene-wise linear model approach with LIMMA 3.14.3 (Smythe et al. 2005). 202 genes were identified that were significantly up-regulated and 40 genes that were significantly down-regulated in PM_{10}- treated mice. Table 3 displays the top 10 up-regulated and down-regulated genes for the treatment group versus the control.

To assess the biological relevance of the differentially expressed genes in PM_{10}- treated versus control the Ingenuity Pathway Analysis (IPA) tool was used. IPA identified many significantly up-regulated genes that participated in lung inflammation: serum amyloid-A3 (*SAA3*) (42.4-fold), tissue inhibitor of metalloproteinase 1 (*TIMP1*) (8.46-fold), chemokine (C-X-C motif) ligand 2 (*CXCL2*) (7.84-fold), tumor necrosis factor receptor superfamily, member 9 (*TNFRSF9*) (2.59-fold) and chemokine (C-C motif) ligand 2 (*CCL2*) (3.00-fold). The top molecular and physiological function categories (ranked by p-value) were “cell-to-cell signaling and interaction” and “immune cell trafficking” suggesting that one of the early events in PM_{10} –exposed mice is the migration of immune cells to the lungs due to a cascade of signaling and recruiting events. Genes associated with top molecular and physiological function categories included: *CCL2, CXCL2* and *TNFRSF9*. Interestingly, the top pathway identified in each toxicity category – such as cardiotoxicity, hepatotoxicity, nephrotoxicity, was inflammation.

Along with many inflammatory genes, PM_{10} induced genes involved in lipid and cholesterol metabolism, as well as, others associated with metabolic disorders. *SAA3* (42.4-fold), *CH25H* (cholesterol 25 hydroxylase) (2.29-fold), *STEAP4* (six transmembrane epithelial antigen of prostate 4) (3.76-fold), *LBP* (1.46-fold) (lipopolysaccharide binding protein) and *PLA2G2D* (phospholipase A2, group IID) (−1.51- fold) were differentially expressed between the control and treated mice. RT-PCR revealed another up-regulated gene: *OLR1* (oxidized low density lipoprotein (lectin-like) receptor 1) (6.50-fold).

Some of the top IPA canonical pathways were involved in CVD, diabetes and obesity. LXR/RXR activation pathway ($p= 7.65 \times 10^{-10}$) was largely influenced by PM_{10} exposure. The liver X receptor (LXR) and retinoid X receptor form heterodimers and regulate cholesterol, lipid, and glucose metabolism (Wente et al. 2007). Atherosclerosis signaling ($p= 5 \times 10^{-6}$) was another pathway affected by the exposure. IPA identified TNF- α, Il-6 and *PLA2G2D* to be involved in this pathway. Other pathways that were induced that are related to liver function include, hepatic cholestasis pathway ($p= 2.26 \times 10^{-5}$) and hepatic fibrosis ($p= 3.81 \times 10^{-5}$).

Induced genes and their upstream regulators

TNF-α and IL-6 were significantly elevated in the BALF of PM_{10}-treated mice. The gene expression profiles of PM_{10}-treated mice contained numerous genes that were downstream of TNF-α and IL-6. The differentially expressed genes, therefore, may have been attributed to the interaction of upstream regulators.

IPA's upstream regulator analysis represented a novel approach to predict the activation or inhibition of upstream regulators based on the expression pattern of genes downstream to those regulators. In the PM_{10}- treated mice, TNF-α was one of the top upstream regulators that was activated ($p= 5.81 \times 10^{-35}$). TNF-α played a role in the up-regulation of several genes including *SAA3, CCL2, TIMP1, STEAP4, CH25H*, and *LPB*. IL-6 was also found to be an upstream regulator that played a role in inducing gene expression ($p= 2.76 \times 10^{-26}$). IL-6 activation in PM_{10}- treated mice contributed to the up-regulation of many of the same genes that were also up-regulated by TNF-α, such as, *CCL2, SAA3, CXCL2*, and *LPB*.

Interestingly, APOE ($p= 1.08 \times 10^{-12}$) was identified by IPA to be activated upstream of many of the differentially expressed genes (Figure 3). Apolipoprotein E (APOE) is produced by the liver and macrophages and is involved in cholesterol metabolism (Frikke-Schmidt 2000). According to IPA, induction of *TIMP1, CXCL3*, and *CCL3* were attributed to upstream activation of APOE.

Another interesting upstream regulator that may have contributed to the differentially expressed genes was NFκB ($p= 6.46 \times 10^{-31}$). According to IPA, NFκB promotes expression of many of the same genes that are activated by both TNF-α and IL-6 such as *TIMP-1, CCL13* and *CH25H*. NFκB was also involved in the down-regulation of *PLA2G2D*. Our analysis suggested that following PM_{10} exposure, TNF-α, IL-6, APOE, and NFκB were upstream regulators that worked together to activate genes involved in inflammation, cholesterol, and lipid metabolism.

Gene expression validation

To validate the gene expression changes observed in the microarray analysis, RNA from lungs of mice was used to perform quantitative real-time PCR (qRT-PCR) of selected genes and the fold changes with qRT-PCR were compared to those from microarrays (Table 4).

Discussion

The objective of this study was to characterize the inflammatory responses and gene expression changes in the lungs of mice exposed to PM_{10} collected from Jeddah, Saudi Arabia. This study demonstrated that acute exposure to PM_{10} in mice stimulated neutrophil influx into the respiratory tract, elevated TNF-α and IL-6 levels in BALF, and altered the gene expression profiles of lung cells. Many of the induced genes function to promote the inflammatory microenvironment by participating in pathways, such as, immune cell trafficking and cell-to-cell signaling. Along with up-regulated inflammatory genes, genes involved in cholesterol and lipid metabolism were found to have increased expression levels in PM_{10}-treated mice. This is the first study to demonstrate that PM_{10} collected from Saudi Arabia raises *in vivo* expression of genes involved in pathways associated with metabolic diseases.

The PM collected from Jeddah, SA is different than other PM sources in a number of ways. A large fraction of the Saudi Arabia PM was coarse PM. The PM's average ratio of $PM_{2.5}/PM_{10}$ was 0.33, which was significantly lower than in most other urban locations (Marcazzan et al. 2003; Rodriguez et al. 2004; Yatkin and Bayram 2008). The large portion

of coarse PM in the Jeddah PM sample is likely due to the high level of wind-blown dust and sand occurring in that region. Also, nickel (Ni) and vanadium (V) are higher in the PM sample than some of the other metals probably due to the large amount of fossil fuel combustion in the area. Ni and V are common components in many particulate matter samples and studies investigating the effects of PM containing Ni and V have observed CVD endpoints (Afridi et al. 2011; Zhang et al. 2009). (Lippmann et al. 2006) exposed $ApoE^{-/-}$ mice to PM containing high concentrations of Ni. Electrocardiograms of the mice revealed that Ni was significantly associated with acute changes in heart rate and their variability. A study by (Campen et al. 2001) demonstrated that Ni and V combined produced increased toxic effects than just Ni alone.

Metabolic disorders have become major health concerns in Saudi Arabia. Metabolic syndrome is a combination of risk factors, when occurring together, increase the risk of cardiovascular disease and diabetes. Some of the risk factors include: obesity, high blood pressure, high glucose, low high-density cholesterol levels, and high serum triglycerides (Schivo M 2013). Several studies have demonstrated the association of PM exposure with risk factors of metabolic syndrome. A recent study by (Liu et al. 2013) found that PM mediates insulin resistance by regulating visceral adipose tissue inflammation, hepatic lipid metabolism, and glucose utilization in skeletal muscle. An epidemiological study by (Nascimento and Francisco 2013) found that in a city in Brazil as PM exposure increased so did hospitalization due to hypertension. A $10\mu g/m^3$ increase in concentration of particulate matter was associated with a 13% increase in risk of hospitalization. In 2002, 35% of deaths in Saudi Arabia were due to cardiovascular disease (CVD) (Taha Abdullah Kumosani 2011). Diabetes mellitus is another chronic disease that occurs at high rates among Saudi Arabians (Alqurashi et al. 2011). Studies have shown that individuals with diabetes are at a higher risk for the toxic effects of particulate matter (Kappos AD 2004). Obesity increases an individual's chances of developing a chronic disease such as CVD and diabetes. Several studies were conducted documenting high rates of obesity among Saudi Arabian citizens (Al-Malki et al. 2003; Madani et al. 2000). One shocking study revealed that the prevalence of obesity in Saudi Arabian adults was 83% (Madani et al. 2000).

Exposure to air pollution may influence the current problems of obesity and obesity-related diseases in Saudi Arabia. Previously, our lab reported that BEAS-2b cells (human bronchial epithelial cells) exposed to PM_{10} collected from Saudi Arabia for 4 days contained increased gene expression profiles of genes involved in cholesterol and lipid synthesis pathways (Sun et al. 2012). Interestingly, our current study reports similar findings *in vivo* and supports the conclusion that PM from Saudi Arabia may be involved in the etiology of metabolic syndromes.

Other studies exposing mice to PM demonstrated that PM affects parameters associated with metabolic syndrome – insulin resistance (Xu et al. 2011) and adipocyte inflammation (Sun et al. 2009). One investigation exposed mice to $PM_{2.5}$ by inhalation 5 days/week for 10 months and found insulin resistance and a decrease tolerance to glucose. Mice exposed to PM contained a decreased amount of circulating leptin and adiponectin and reduced mitochondrial number and size (Xu et al. 2011). Another study found that mice exposed to a high- fat diet and PM displayed increased systemic inflammation, insulin resistance, and

elevation in visceral fat with higher levels of adipose tissue macrophages (Sun et al. 2009). A strain of rabbits that naturally develop atherosclerosis was exposed to 1.5 mg/kg PM_{10} by intratracheal instillation and the results demonstrated that PM promotes the recruitment of circulating monocytes into atherosclerotic plaques (Yatera et al. 2008).

While metabolic disorders are commonly associated with changes in liver, various studies demonstrated that after exposure to chemical or biological stress, lungs and liver display similar changes in mRNA levels (Crespo et al. 1999; Flohe et al. 1999; Ghoshal et al. 2001; Rosen et al. 2007; Zhang et al. 1998). A study reported that after *in utero* exposure to perfluorooctanoic acid (PFOA), full term mouse fetuses displayed similar changes in gene expression in lungs and liver, with the majority of changes occurring in genes involved in lipid homeostasis (Rosen et al. 2007).

Many of the PM_{10}-induced genes are involved in cholesterol and lipid metabolism and have been implicated in metabolic disorders- *SAA3*, *CH25H*, *STEAP4*, *OLR1*, and *PLA2G2D*. *SAA3* (serum amyloid A- 3) was the most up-regulated gene and is an apolipoprotein associated with high density lipoprotein (HDL) in plasma (Uhlar and Whitehead 1999). This gene is primarily expressed in liver and involved in transport of cholesterol to the liver for secretion into bile (de Beer et al. 1994; Uhlar and Whitehead 1999) and both TNF-α and IL-6 were noted to activate *SAA3* (Bombini et al. 2004; Fasshauer et al. 2004). SAA protein plays a critical role in inflammatory responses and mediates neutrophil infiltration into the lungs after exposure. SAA was shown to activate the NLRP inflammasome (Ather et al. 2011) and its serum concentrations are increased during inflammatory disorders (Menschikowski et al. 2013). The role of SAA protein in inflammation may be contributing to metabolic syndrome and atherosclerosis since both of these conditions are influenced by inflammation. *SAA* has been identified as an active player in the development of atherosclerosis. (Dong et al. 2011) reported that $ApoE^{-/-}$ mice overexpressing *SAA* contained a larger aortic atherosclerotic lesion than that found in control mice. *SAA* also induced the expression of *VCAM-1* (vascular adhesion molecule- 1) (Dong et al. 2011), which is also involved in the development of atherosclerosis (Cybulsky et al. 2001; Nakashima et al. 1998). It should be noted here that SAA3 is an acute response protein that is synthesized during the acute phase of inflammation. Whether or not it was directly activated due to PM-induction of pathways involved in metabolic syndrome is unknown. It may have just been a bystander observation and long-term studies are needed to confirm its continued expression throughout PM exposure and its ability to promote the development of metabolic syndrome.

CH25H (cholesterol 25-hydroxylase) was significantly up-regulated in treated mice and its protein product functions to inhibit the synthesis of cholesterol by indirectly blocking the function of SREBP (sterol regulatory element binding proteins). SREBP are transcription factors that mediate a network of genes involved in cholesterol synthesis. Sterols in turn inhibit SREBP and thus SREBP regulation of sterols is controlled by a negative feedback mechanism (Eberle et al. 2004). *SREBF1* and *2* genes were found to be up-regulated by Saudi Arabia PM_{10} *in vitro* after 4 days of exposure (Sun et al., 2012). Ultimately, *CH25H* lowers cholesterol synthesis by negatively regulating SREBP and inducing degradation of HMG-CoA reductase (Gil et al. 1985), which is the enzyme targeted by the group of

cholesterol –lowering drugs known as statins. Since Saudi Arabia PM_{10} affects the levels of *SREBF* and *CH25H*, it would be of interest to conduct intermediate and long- term animal investigations in order to determine if the levels of *CH25H* decrease and *SREBF* increase.

STEAP4 (six transmembrane epithelial antigen of prostate 4) was also found to be significantly up-regulated in mice treated with Saudi Arabia PM and the gene may be involved in development of metabolic disease. An investigation found that a genetic variation of *STEAP4* in Chinese Uygur patients was associated with metabolic syndrome (Nanfang et al. 2010). *STEAP4* may be involved in obesity via its role in adipocyte development and metabolism. The protein encoded by *STEAP4* is termed TIARP (tumor necrosis factor-alpha-induced adipose-related protein) and acts as a transporter or channel on fat cells which mediate TNF- α effects on adipocyte development (Moldes et al. 2001). Future chronic studies will investigate whether or not PM-induced *STEAP4* is correlated with phenotypic changes of fat in exposed mice.

Other interesting genes altered by PM include- *OLR1* and PLA2G2D. These genes are associated with cholesterol metabolism (*OLR1*) (Khaidakov et al. 2011) and lipid metabolism (*PLA2G2D*) (Degousee et al. 2002). *OLR1* (oxidized low density lipoprotein (lectin-like) receptor 1) encodes a protein that binds, internalizes and degrades oxidized low-density lipoprotein. A polymorphism in this gene has been associated with metabolic syndrome (Palmieri et al. 2013). A study found that deletion of the *OLR1* gene in LDL receptor-deficient mice fed a high cholesterol diet decreases the outcome of atherogenesis in these mice. The fall in atherogenesis was marked by a reduction in inflammation: a decrease in the proinflammatory signal MAPK P38 and NFκβ, and an elevation in anti-inflammatory levels. A PM- induced up-regulation of *OLR1* may promote atherogenesis by increasing inflammatory responses (Mehta et al. 2007). Moreover, *OLR1* is up-regulated in the atherosclerotic arteries of humans and animals and has been implicated in diabetes in animals (Mehta et al. 2006). Based on the aforementioned findings, PM-induced *OLR1* may play a role in the development of metabolic disease. *PLA2G2D* (phospholipase A2, group IID) encodes a phospholipase that creates free fatty acids and lysophospholipids by hydrolyzing glycerophospholipids (Degousee et al. 2002). An increase in glycerophospholipids leads to the enrichment of lipid mediators which may promote insulin resistance and impair glucose tolerance in metabolic syndrome (Farooqui 2013). Thus, down-regulation of *PLA2G2D* in PM-treated mice may lead to metabolic syndrome by increasing glycerophospholipids. However, it should be noted here that another reason for *PLAG2D* down-regulation may be a cellular response to combat damage to the plasma membrane. Glycerophospholipids are structural components of the plasma membrane and increased synthesis of these lipids may be involved in other pathways besides promotion of metabolic syndrome. PM- induced alterations of lipid metabolism genes may be occurring to reinforce structural integrity to the damaged cell membrane of lung epithelial cells.

Many studies have linked PM exposure with metabolic disorders; however it is still unclear how ambient particulates mediate adverse health effects. Some of the gene expression changes that occur after an acute exposure to PM_{10} that may influence the development of metabolic diseases were characterized. Our studies to-date characterizing the gene expression changes induced by PM_{10} collected from Jeddah, Saudi Arabia have generated *in*

vitro and *in vivo* findings that suggest that PM_{10} may be mediating or exacerbating metabolic diseases- a health concern that is increasing rapidly in Saudi Arabia. While this study provides data that acute exposure to PM_{10} affects pathways involved in cholesterol and lipid metabolism, long- term studies are needed to confirm an association of PM_{10} exposure and the occurrence of metabolic syndrome risk factors.

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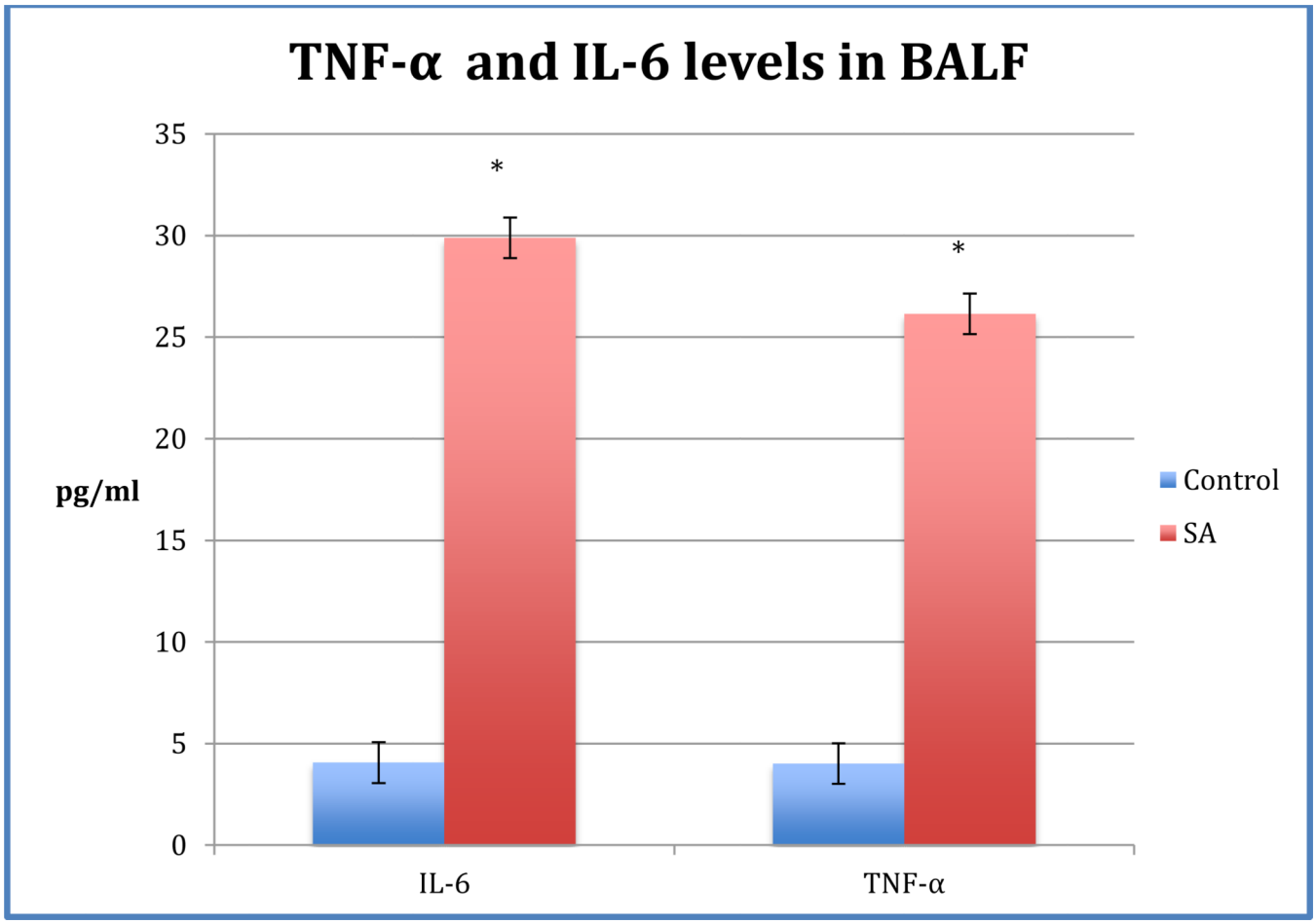
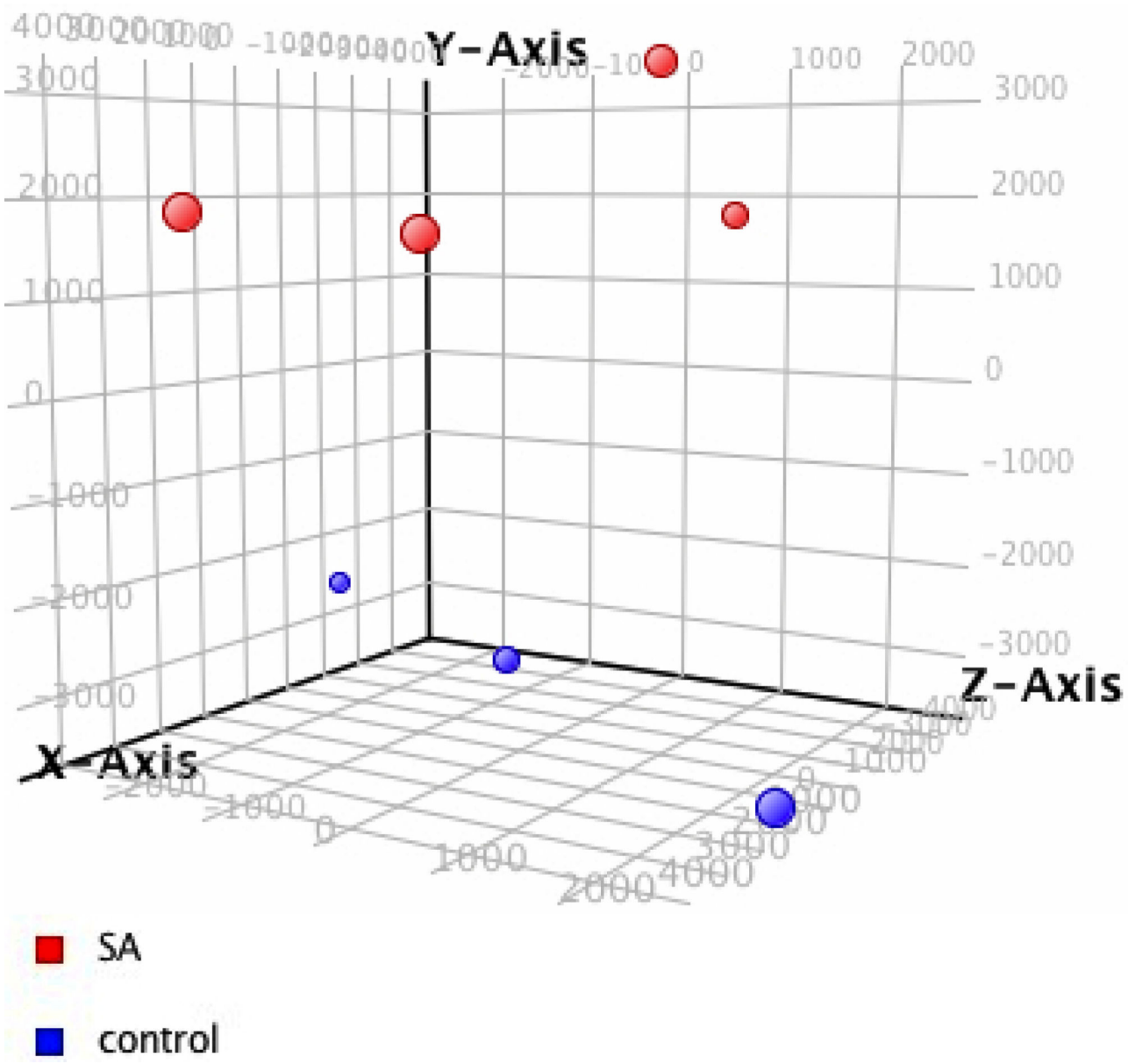


Fig. 1. TNF-α and IL-6 levels in BALF of mice treated with PM_{10}

Mice were exposed to 100 μg PM_{10} or distilled water.**TNF-α**. The average TNF-α levels were 26.2 ± 7.0 and 4.01±1.9 pg/ml in PM_{10} BALF and control BALF, respectively. TNF- α levels in PM_{10}-treated mice were significantly higher than control mice ($p < 0.0012$).**IL-6**. Average IL-6 levels ($p < 0.05$) were 29.9 ± 9.9 and 4.06 ± 2.7 pg/ml in PM_{10} BALF and control BALF. IL-6 levels in PM_{10}- treated mice were significantly higher than control mice ($p < 0.05$).

A)



B)

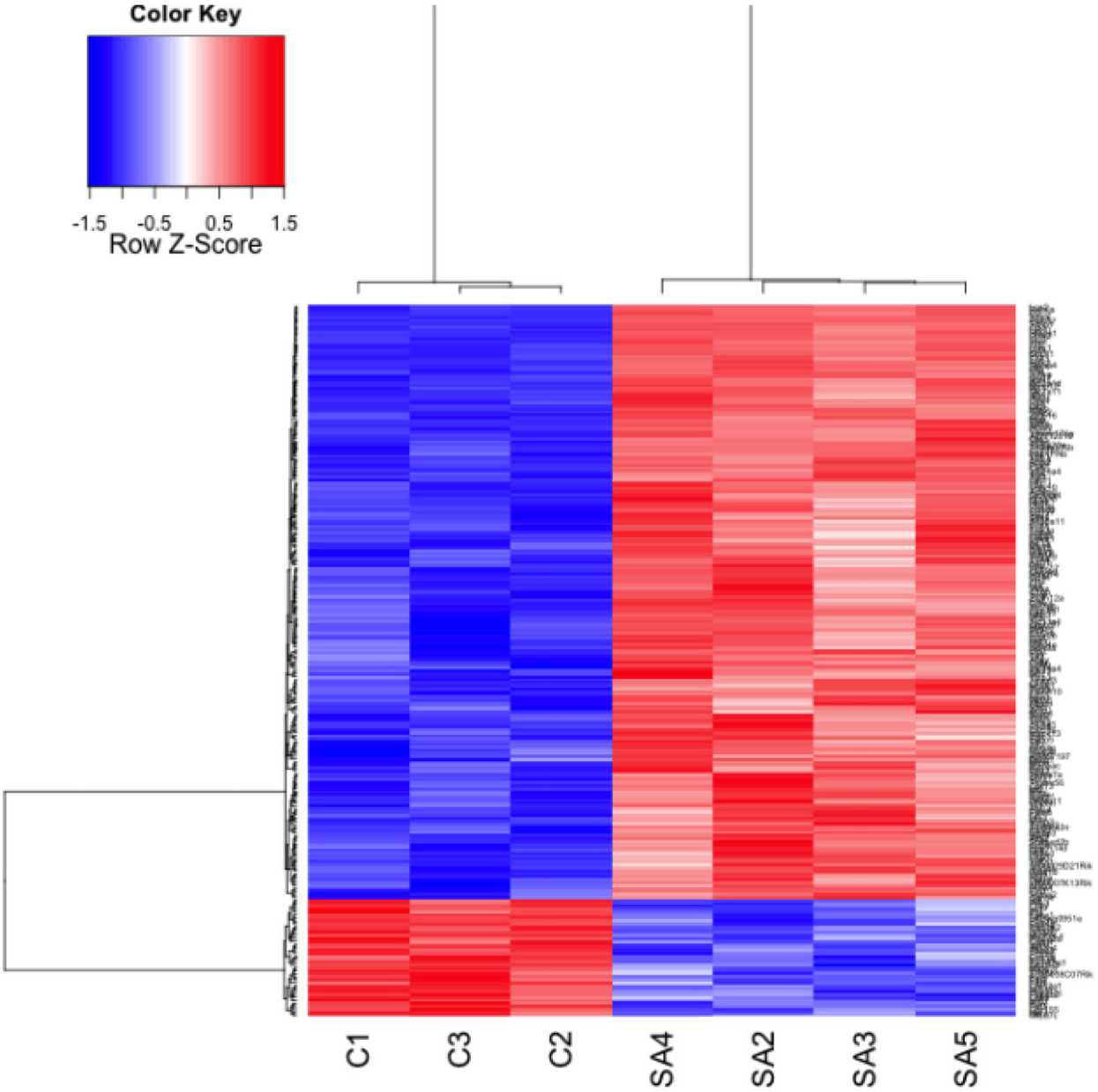


Fig. 2. Gene expression profiles of PM_{10}-exposed mice

(a) Principal component analysis (PCA) PCA revealed distinct separation between control mice vs. treatment mice Red: PM_{10}; Blue: control **(b) Heat Map**. Hierarchical cluster analysis of significantly differentially expressed genes in a PM_{10}- treated group compared to an untreated control group. The bar relates the color code to the expression value after quantile normalization and baseline transformation to the median levels of control samples.

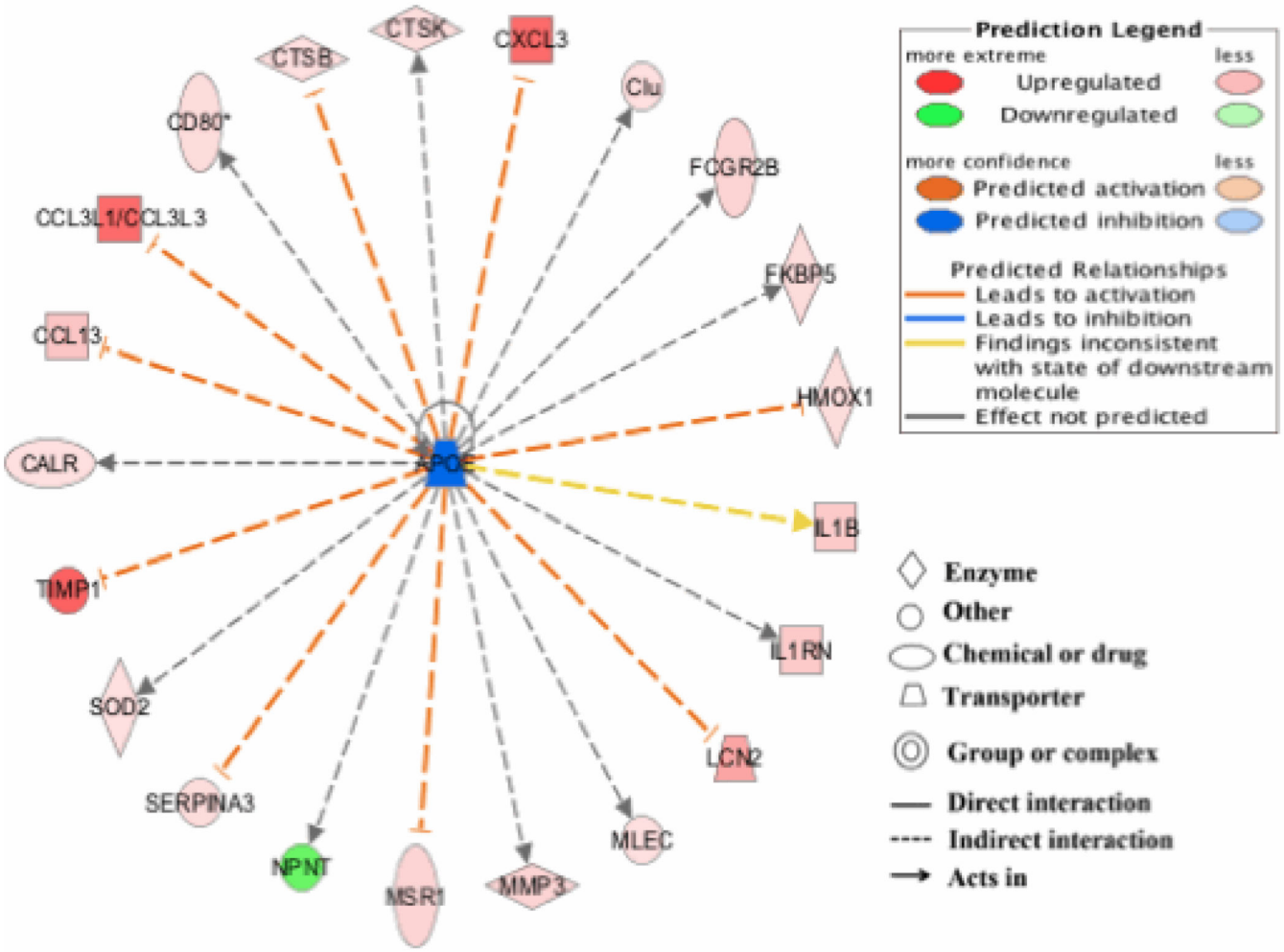


Fig. 3. APOE is a top upstream regulator influencing altered gene expression in PM_{10}-treated mice

APOE was identified as an upstream regulator of many of the genes induced by PM_{10}. Most of the genes affected by this regulator are up-regulated and only a few are down-regulated.

Table 1

Percent neutrophils and total immune cells present in [a]BALF.

SAMPLE	% Neutrophils	Total Immune Cells
Control	0.67%	2.45×10^5cells/ml
[b]PM_{10}	59.6%	3.79×10^5 cells/ml

[a]Bronchoalveolar lavage fluid

[b]PM_{10}- particulate matter collected from Jeddah, Saudi Arabia

Table 2

[a]BALF neutrophil concentrations to different doses of [b]PM_{10}.

Dose	% Neutrophils	Total Immune Cells
25 μg	8%	2.71×10^5cells/ ml
50 μg	14%	2.65×10^5cells/ ml
[c]**100 μg**	43%	3.39×10^5cells/ ml
250 μg	100%	5.05×10^5cells/ml
500 μg	99%	5.6×10^5cells/ml

[a]Bronchoalveolar lavage fluid

[b]PM_{10}- particulate matter collected from Jeddah, Saudi Arabia

[c]Dose chosen for acute exposure

Table 3

Top ten up- and down-regulated genes ($p < 0.05$) in the lungs of PM_{10}-treated mice versus control mice.

Affymetrix ID	Gene Symbol	Gene Name	Fold Change
10563597	Saa3	serum amyloid A 3	42.4
10429560	Ly6i	lymphocyte antigen 6 complex, locus I	11.0
10505451	Orm2	orosomucoid 2	10.6
10436095	Retnla	resistin like alpha	8.72
10598976	Timp1	tissue inhibitor of metalloproteinase 1	8.46
10545569	Reg3g	regenerating islet-derived 3 gamma	8.11
10389231	Ccl3	chemokine (C-C motif) ligand 3	7.85
10523156	Cxcl2	chemokine (C-X-C motif) ligand 2	7.84
10505438	Orm1	orosomucoid 1	6.72
10416837	Irg1	immunoresponsive gene 1	6.31
10529937	Kcnip4	Kv channel interacting protein 4	−1.47
10542929	Calcr	calcitonin receptor	−1.48
10509577	Pla2g2d	phospholipase A2, group IID	−1.51
10493449	Thbs3	Thrombospondin3	−1.54
10538802	A930038C07Rik	RIKEN cDNA A930038C07 gene	−1.55
10595211	Col12a1	collagen, type XII, alpha 1	−1.55
10384223	Igfbp3	insulin-like growth factor binding protein 3	−1.58
10580663	Ces1f	carboxylesterase 1F	−1.65
10580678	Ces1g	carboxylesterase 1G	−1.74
10523483	Prdm8	PR domain containing 8	−1.79

Table 4

Real-time validation of microarray results.

	qRT-PCR[a]	Microarray
TNFRSF9	92.5	2.59
SAA3	299.5	42.4
CXCL3	16.9	2.09
CES1G	−45.8	−1.74
CH25H	45.1	2.29
CA13	18.2	1.55
STEAP4	18.1	3.76
OLR1	6.5	-

[a]The RT-PCR data represent the means of duplicates and are presented as fold change to the level expressed in control mice lungs.