# Influence of fine particulate matter (PM$_{2.5}$) on the function of human immune cells

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## Abstract

Fine airborne particulate matter (PM$_{2.5}$) evidently contributes to morbidity and mortality of exposed populations, particularly in densely populated industrial areas with heavy traffic. Research on the toxic effects of PM$_{2.5}$ has indicated that numerous adverse health consequences of exposure to PM$_{2.5}$ result from their immunomodulatory and immunotoxic effects. Therefore, immunological assays are supposed to be a suitable screening tool for evaluating the harmful potential of PM. Using selected *in vitro* immunological assay, we have evaluated changes in both humoral a cell-mediated components of the innate and acquired immune response. Generation of reactive oxygen species was assessed using a chemiluminescence assay. Stimulation effects of the PM sample on the key component of cell specific immunity was monitored using a lymphocyte proliferation assay. Allergenic potential of the PM sample was investigated by a basophil degranulation assay. Changes in levels of selected cytokine were monitored using a multiplex technology ALBIA. Even if certain immunomodulatory effects of short-term incubation of the PM$_{2.5}$ sample in whole blood cultures were observed using *in vitro* immunological assays, changes in evaluated immune response parameters were not significant to prove impact on the immune cells' functions of healthy persons. However, health risks of long-term exposure, especially for individuals with genetic predisposition to certain diseases or already existing diseases, cannot be excluded.
*Keywords: PM$_{2.5}$, immunological assays, oxidative stress, inflammation, allergy, lymphocyte proliferation, cytokines.*

## 1    Introduction

Results of both the epidemiological and the toxicological studies have proved that inhalation of the air containing high concentrations of airborne particles influences various functions of the immune system [1]. Disruptions of the immune system functions may lead to the induction of immunodeficiency resulting from the suppression of various immune systems' components, or contrary to autoimmune diseases and allergies resulting from the excessive stimulation of the immune systems' components. These immune disorders usually arise from a genetic predisposition developed under prolonged activation of the immune system. Such activation can be induced by chronic systemic inflammation or long-term local irritation by undegradable substance provoking development of granulomatous inflammation.

After inhalation and deposition, airborne particles react with various cell types under various conditions. For these reasons, most likely there is no unified mechanism of all their immunomodulatory effects. Nevertheless, it is possible to find certain common features of action of different particle types after their deposition in different areas of the respiratory system, as well as after the particle translocation into the bloodstream and the other parts of the body. The common attributes of particle pathogenic effects are primarily the ability of particles to induce oxidative stress and inflammation in the body [2].

With regard to the crucial role of the immune system in development of various adverse health effects related to ambient PM exposure, we suggest that immunological assays may represent suitable screening tool for evaluating the harmful potential of PM. Using selected *in vitro* immunological assay, we have evaluated changes in both humoral a cell-mediated components of the innate and acquired immune response.

## 2    Materials and methods

### 2.1  PM sample preparation

PM was sampled at an industrial measuring station located in Ostrava-Radvanice, the district of the Ostrava City in the north-east of the Czech Republic. Air pollution measurements have revealed that Ostrava-Radvanice belongs to areas with the most polluted air in Europe. The average $PM_{2.5}$ concentration during the sampling period was 175 $\mu g/m^3$.

$PM_{2.5}$ was collected on mixed cellulose ester (MCE) membrane filters (Millipore Co., Ltd., USA, 47 mm diameter, 0.8 $\mu m$ pore size). The sampling flow rate was 16.7 l/min. PM sampling was conducted on November 13–14, 2011 (with a total sampling period 19.11 hours; total air volume 19.185 $m^3$). The filter removed from the sampler head was kept in Petri dish covered with paper box to restrain light exposition. After stabilizing at constant temperature (22–24°C) and humidity (40–50%), the filter was weighed on an electronic balance (Sartorius BP211D).

To obtain PM suspension, the filter was sonicated in a beaker filled with 250 ml of distilled water using Ultrasonic compact cleaner 4L PS04000A. Obtained suspension was stored in the refrigerator. Just before the use, the suspension was resonicated and diluted. A part of the suspension was chemically analysed by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) using model THERMO X-Series2. Chemical analysis was aimed at metal detection.

## 2.2 Biological material preparation

The immunological assays were performed on the whole blood cultures. Blood samples were obtained from three healthy volunteers (two men, one woman, aged from 25 to 55 years) into Vacutainer Tubes containing heparin. Immunological assays were performed within three hours after blood taking. Whole blood analysis (total red blood cell count, total and differential white blood cell counts) was performed using Sysmex XS-800i analyser. Blood cell counts of all three donors correspond to reference values [3]. Thus, results of immunological assays were not influenced by abnormalities of haematological parameters of the donors.

## 2.3 Chemiluminescence assay

The assay was performed according to SOP 25.47 CKL of Institute of Public Health Ostrava [4]. The PM suspension was diluted in Hank's Buffered Salt Solution (HBSS) to obtain required concentrations (30 pg/ml, 300 pg/ml, 3,000 pg/ml, 134 µg/ml) of original dust. 2 ml of HBSS and 10 µl of luminol were pipetted into all scintillation viols. A viol without any stimulant served as negative control. 50 µl of opsonized zymosan were added into the viol representing positive control. The tested particle sample in different concentrations was added into the other viols. 20 µl of whole blood culture were pipetted into the viols at 40-second intervals. After vortexing, all viols were placed into Tri-Carb Scintillation Counter and temperate for 15 minutes at 37°C. Results expressed in the terms of cpm (counts per minute) were recorded at 30, 60 and 90 minutes after addition of the blood. The obtained results were statistically analysed using pared Student's t-test and one-way ANOVA.

## 2.4 Lymphocyte proliferation assay

The assay was performed according to SOP 25.46 CKL of Institute of Public Health Ostrava [5]. The PM suspension was diluted in Minimum Essential Medium (MEM) to obtain required concentrations (30 pg/ml, 300 pg/ml, 3,000 pg/ml, 134 µg/ml) of original dust. The assay was performed in microplate wells in triplicates. Into three wells representing negative controls, only 200 µl of MEM were added. Into three positive control wells, 200 µl of cultivated media with standard mitogen phytohemagglutinine (PHA) were added. 200 µl of the tested sample in different concentrations were pipetted into the other wells. Then, 10 µl of whole blood were added, vortexed and incubated in atmosphere with 5% $CO_2$ for 72 hours. After incubation, 10 µl of [³H]thymidine were added

and the microplate was returned into the $CO_2$ incubator for 24 hours. Finally, the content of each well was recovered with a cell harvestor. A stripe of filter paper was put onto the sturdy paper and left to dry. Drought rounds of the filter paper were placed into particular flasks with 1.5 ml of scintillation liquid. The flasks were transported into Tri-Carb scintillation counter to measure the incorporated radioactivity. The assay results were counted as an average of triplicate values and expressed in the terms of cpm (counts per minute). The obtained results were statistically analysed using pared Student's t-test and one-way ANOVA.

## 2.5 Basophil degranulation assay

The assay was performed according to the user manual of Commerce kit BasoFlowEx® [6]. PM suspension was diluted in Physiological Buffered Solution (PBS) to obtain required concentrations (30 pg/ml, 300 pg/ml, 3,000 pg/ml, 134 µg/ml) of original dust. One tube left without allergens served as a negative control. Into positive control tube, 10 µl of stimulation control were added. PM sample in different concentrations was added into the others tubes. Into all tubes, 100 µl of whole blood were added and stimulated by addition of 100 µl of stimulation buffer for 15 minutes at 37°C. Afterwards, 300 µl of Staining Reagent were added and incubated for 20 minutes at 6°C. Thereafter, 300 µl of Lyzing Solution were added, mixed and incubated for 5 minutes at laboratory temperature. To red blood cell lysis, 3 ml of demineralized water were added into each tube and incubated for 5 minutes at laboratory temperature. Finally, the tubes were centrifuged for 5 minutes at 300 g, the supernatant was removed and pellet was resuspended in 0.2 ml of PBS. Cell surface expression of CD63 was measured by flow cytometry within 2 hours after staining. Obtained data were visualized on the side-scatter (SSC) versus fluorescence intensity in PE channel (FL2) dot-plot. The gate for basophil population (CD203c positive, SSC low) was set and the gated basophils were brought to histogram.

## 2.6 Cytokine release evaluation

The assay was performed according to the user manual of Commerce kit Human Cytokine 25-Plex Panel [7].The PM suspension was diluted in Roswell Park Memorial Institute Medium (RPMI) to obtain concentration 3,000 pg/ml of original dust. The filter plate was pre-wetted with 200 µl of working Washing Solution. Washing Solution was aspirated using a vacuum manifold. 25 µl of the beads with defined spectral properties conjugated to protein-specific capture antibodies were pipetted into each well and incubated for 2 hours. Afterwards, protein-specific biotinylated detector antibodies were added and incubated for one hour. The excess antibodies were washed and the wells were drought again. Streptavidin conjugated to the fluorescent protein R-Phycoerythrin was added, incubated for 30 minutes and washed. Finally, Luminex detection system monitoring the spectral properties of the beads and the amount of associated R-Phycoerythrin fluorescence was used to determine the concentration of selected cytokines. Calibration curves were constructed by polynomial regression. The obtained results were expressed as an index of positivity (ratio of $PM_{2.5}$

stimulated value to negative control value) enabling reciprocal comparison of individual cytokines with very different concentrations in blood cultures.

## 3  Results

### 3.1  IPC-MS

Chemical analysis of PM suspension was aimed at detection of selected metals. Significant amount of elements present in fly ash from combustion process was detected (lead, zinc, cadmium, arsenic) [8].

### 3.2  Chemiluminescence assay

The results, depicted in Figure 1, indicate that the presence of airborne particles did not significantly enhance cpm values. The no effect hypothesis was statistically confirmed using pared Student's t-test. Significant difference between negative control and the PM sample was recorded only in concentration 3,000 pg/ml after 30 minute incubation. With regard to the other negative results, the response cannot be attributed to the action of airborne particles. Likewise, the results of one-way ANOVA confirmed no stimulant effect of the PM sample.
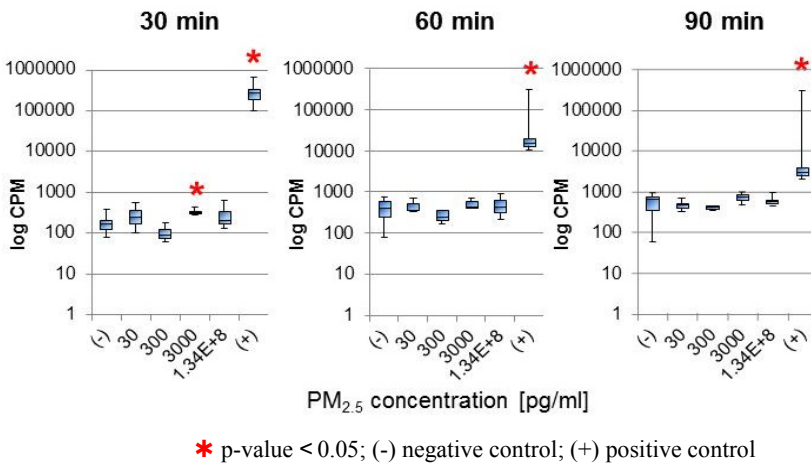


* p-value < 0.05; (-) negative control; (+) positive control

Figure 1:    *In vitro* generation of reactive oxygen species by neutrophils in whole blood after stimulation with $PM_{2.5}$ suspension. Boxplots represents average assay values obtained from blood of three donors. Results were read at 30-minute intervals.

### 3.3  Lymphocyte proliferation assay

Slight growth in cpm was recorded in concentrations of 300 pg/ml and 134 μg/ml, as seen in Figure 2. Pared Student's t-test as well as one-way ANOVA confirmed significant stimulant effects on p-value < 0.05.
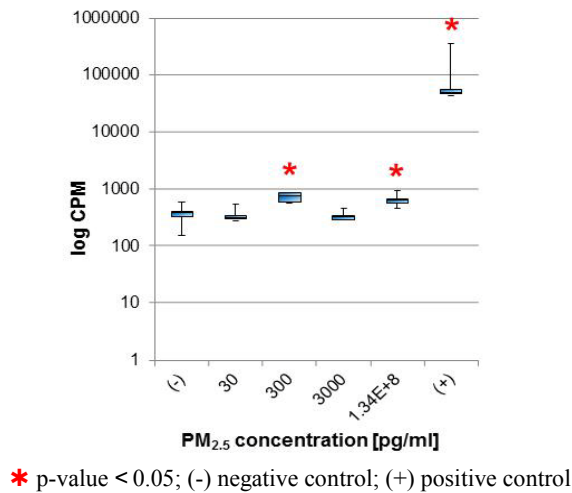
* p-value < 0.05; (-) negative control; (+) positive control

Figure 2:    *In vitro* lymphocyte activation in whole blood after stimulation
             with $PM_{2.5}$ sample. Boxplots represents average values of three
             donors.

## 3.4  Basophil degranulation assay

15% of activated basophils were established as the cut-off limit between negative
and positive response against inhalation allergens. As seen in Figure 3, none of
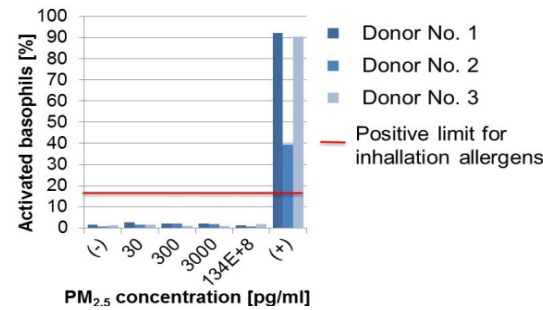the PM concentrations exceed the limit.



Figure 3:    *In vitro* basophil activation with $PM_{2.5}$ sample. Each column
             represents percentage of activated basophils for one donor and one
             PM concentration. (-) negative control; (+) positive control.

## 3.5  Cytokine release evaluation

Slight stimulation effects were observed at IL-6, IL-8, TNF-α, eotaxin, MIP-1α,
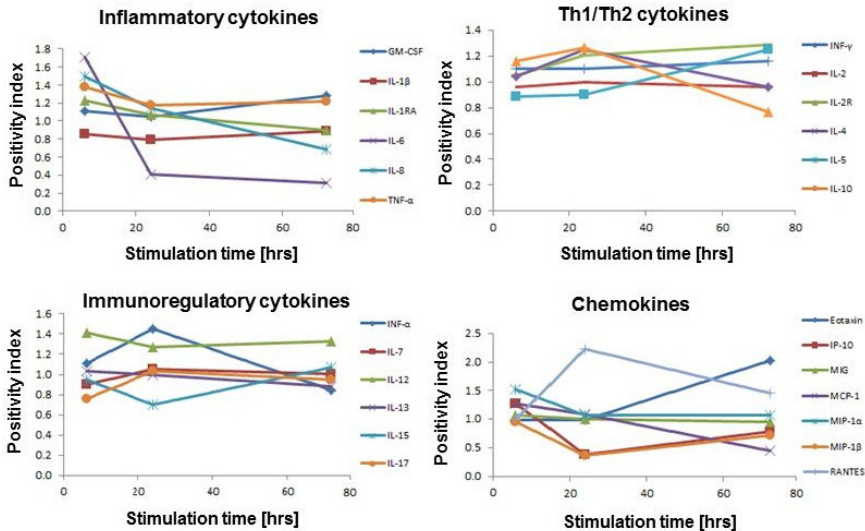RANTES as illustrated in Figure 4

Figure 4:  *In vitro* cytokine levels in whole blood after stimulation with PM$_{2.5}$ sample. Results were expressed as a positivity index (ratio of PM$_{2.5}$ stimulated values to unstimulated values). Plots were constructed for particular stimulation times as an average of values of three donors.

## 4  Discussion

Data from number of epidemiological studies have indicated that heavy airborne PM pollution considerably contributes to morbidity and mortality, above all from pulmonary and cardiovascular causes [1, 9]. It has been established that numerous adverse health effects associated with ambient particle exposure originate in particle-mediated changes of the immune system functions. Heavy and/or long-term exposure to ambient PM can disturb protective and integrating functions of the immune system and consequently lead to development of various cell and tissue damages [2]. This study was aimed at screening evaluation of the harmful potential of fine PM sample, using laboratory immunological assays employed in clinical practice to evaluate various immunological disorders. Set of the selected *in vitro* immunological assays enables to monitor changes in both the humoral and cell-mediated components of the innate and acquired immune response which can be caused by exposure to airborne PM and can result in adverse health consequences.

### 4.1  Chemiluminescence assay

The chemiluminescence assay enables the evaluation of phagocytic functions. Stimulation of phagocytes involves generation of reactive oxygen species (ROS)

in a process termed respiratory burst. The return of the excited chemical groups to the basic state is accompanied by foton emission – chemiluminescence [4].

We have used the chemiluminescence assay to assess the impact of the PM sample on the oxidative burst of phagocytic cells represented in the whole blood mainly by polymorphonuclears. Enhancement of phagocytic activity induced by presence of airborne particles could support particle clearance [10]. Despite this possible benefit, high reactivity of ROS together with their nonspecific attacking of biomolecules represent a danger of inflammation, cell and tissue damage leading to development of serious respiratory disease, such as chronic obstructive pulmonary disease, asthma, etc. [10]. Therefore, the measurement of the rate of ROS generation can serve as a screening tool for evaluation of possible PM associated oxidative damage. Based on experiments dealing with particle-related inflammation, Antonini *et al*. [11] have regarded luminol-dependent chemiluminescence as a suitable method to monitor the earliest events in the inflammatory process.

In this study, stimulation effects of PM on ROS generation by phagocytes were not observed. Only in the concentration of 3,000 pg/ml after 30-minute incubation PM sample significantly increased cpm values ($p < 0.05$). With regard to the negative results of the other particle concentrations, the increase most likely was not induced by the presence of PM sample. Likewise, one way ANOVA did not confirm the stimulation effect. The negative results could be caused by absence of ligands on the particle surface able to bind on the phagocyte receptors. Such ligands are typical for biological components of PM [12]. However, the presence of biological components was not investigated in this study.

No effect results obtained in the present study cannot exclude possible adverse effects of the PM arise from the enhanced ROS generation. Prolonged particle action as well as pre-existing diseases, mainly those accompanied by the increase of ROS levels in the organism, can significantly influence effects of airborne particles on phagocytic activity.

## 4.2  Lymphocyte proliferation assay

Proliferative activity of T-cells is an important parameter in evaluating the status of the immune system. Enhanced lymphocyte proliferation reflects more effective immune response and possible more intensive elimination of particles from the organism. However, excessive lymphocyte proliferation is related to inflammatory and autoimmune diseases. Contrarily, the decreased lymphocyte proliferation is related to immunodeficiency. Worsened T-cell activity can be caused by oxidative stress resulting in apoptosis of T-cells [13]. Lymphocyte proliferation is triggered by mitogens or by antigens/allergens binding to T-cell receptor. Quantitation of $^{3}$H-thymidine incorporated into newly synthetized lymphocyte DNA allows to detect proliferative capacity of T-cells [5].

Slight increase in cpm compared with negative control value was recorded with particle concentrations 300 pg/ml and 134 µg/ml. However, the intermediate concentration 3,000 pg/ml did not exhibit enhanced proliferative activity. Thus, another evaluation is needed to make a decision on the possible

stimulation properties of the airborne particles on the lymphocyte proliferation. There is a possibility that PM as other less potent stimulants needs longer incubation time [14].

### 4.3  Basophil degranulation assay

Environmental pollution, particularly polluted air, is considered to be an important factor contributing to the recent increase in incidence and prevalence of allergies. PM can serve as a vector carrying allergens (pollen grains, mites, moulds) into the respiratory tract, where they can be deposited and can trigger allergic response [15]. Besides the vector role, PM may enhance the susceptibility to common environmental allergens and trigger sensibilization to neoallergens [16].

Basophil degranulation assay represents a simple tool for *in vitro* investigation of immediate allergy, based on the simulation of contact between allergens and basophils, as the cells responsible for allergic symptoms. Basophils upon allergen challenge degranulate, release inflammatory mediators and expose activation markers on their surface [17]. Basophil degranulation assay monitors exposition of CD63 transmembrane protein on the surface of activated basophils [6].

The obtained results have showed that the PM sample did not stimulate significant basophil degranulation since application of the airborne particle suspension did not elicit exceeding of 15% of activated basophiles established as a positivity cut-off limit. The negative results can be caused by the absence of antigens in the tested sample. However, airborne PM can enhance the release of allergic inflammatory mediators from basophils in the presence of antigen, thus exposure to particulate air pollution can have an adjuvant effect. In the study of Schober *et al.* [18], synergy effects between organic extracts of urban aerosol and major allergen of birch pollen grains (rBet v 1) on basophil activation in birch pollen-allergic donors has been proven. Samples from healthy controls did not show upregulation of CD63 by rBet v 1. In the present study, assay was performed on the whole blood cultures of healthy donors. In future work, the engagement of allergic donors could be useful.

Finally, basophil degranulation assay enables to evaluate allergic properties triggered through basophil activation after the allergen is bound to IgE on the basophil surface. But other mechanisms can be involved in mediating of the allergy response [19].

### 4.4  Cytokine release evaluation

Cytokines represents important molecules regulating and mediating immune response, inflammation and hematopoiesis. Cytokines are secreted by immune cells in response to stimuli. Great number of cytokines with various immune functions is known and new ones are discovered [20]. For this pilot study, commerce kit Human Cytokine 25-Plex Panel was employed to evaluate changes in cytokine levels in whole blood cultures cultivated with the PM sample. To detect dynamics in cytokine release, cytokine levels were measured at 6, 24 and

72-hour incubation time. Using pared Student's t-test, slight stimulation effects were observed at inflammatory cytokines (IL-6, IL-8, TNF-α) and chemokines (eotaxin, MIP-1α, RANTES). However, the ultimate cytokine levels were low. Therefore, we do not suppose that observed changes in cytokine release could have any impact in a real organism.

## 5  Conclusion

The present study deals with effects of the fine airborne particles ($PM_{2.5}$) on the human immune cells' functions. We have selected parameters of both the humoral and cell-mediated components of the innate and acquired immune response, which can be affected by exposure to PM. Parameters of immune responses were monitored in whole blood cultures using selected *in vitro* immunological assays. In conclusion, observed changes in evaluated immune parameters were not high enough to take effect in a real organism. Results of our experiments are related to short-term action of the real airborne particle mixture incubated in whole blood cultures of healthy donors. However, obtained data cannot exclude health risks of long-term exposure to airborne particles, especially for individuals with genetic predisposition to certain diseases or with already existing disease. Age, co-pollutants and compromised health status can modify the immune response to PM exposure.

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## References

[1]  Pope, C.A. III., Burnett, R.T., Thurston, G.D., Thun, M.J., Calle, E.E., Krewski, D. and Godleski, J.J., Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation,* 109, pp. 71–77, 2004.

[2]  Gilmour, I.M., Stevens, T. and Saxena, R.K., Effect of particles on the immune system, ed. Donaldson, K. and Borm, P., *Particle Toxicology,* CRC Press: NY, pp. 245–258, 2007.

[3]  Klener, P., *Vnitřní lékařství*, Galén: Praha, 2006.

[4]  Lochmanová, A., *Polymorphonuclear Metabolic Activity Evaluation Using Chemiluminescence Assay SOP 25.47 CKL ZÚ Ostrava*, 2005.

[5]  Lochmanová, A., *Lymphocyte proliferation Evaluation using Lymphocyte proliferation Assay with $^{3}H$ thymidin incorporation SOP 25.46 CKL ZÚ Ostrava*, 2005.

[6]  EXBIO, *BasoFlowEx® Kit Cat. No: ED7036.*

[7]  Invitrogen Corporation, *Human Cytokine 25-Plex Pane. Cat. No: lhc0009 User manual*, 2010.

[8]  Meij, R., Janssen, L.H.J.M and van der Kooij, J., Air pollutant emissions from coal fired power stations. *KEMA Sci. Tech. Rep.*, 4(6), pp. 651–669, 1986.

[9]  Pope, C.A., III. and Dockery, D.W., Health effects of fine particulate air pollution: lines that connect. *J. Air Waste Manag. Assoc.*, 6, pp. 709–742, 2006.

[10] Donaldson, K., Slight, J. and Bolton, J.E., The effect of products from bronchoalveolar–derived neutrophils on oxidant production and phagocytic activity of alveolar macrophages. *Clin. Exp. Immunol.,* 74(3), pp. 477–482, 1998.

[11] Antonini, J.M., Dyke, K.V., Ye, Z., Dimatteo, M. and Reasor, M.J., Introduction of luminol–dependent chemiluminescence as a method to study silica inflammation in the issue and phagocytic cells of rat lung. *Environ. Health. Perspect.*, 102 (Suppl. 10), pp. 37–42, 1994.

[12] Donaldson, K., Borm, P.J.A., Castranova, V. and Gulumian, M., The limits of testing particle–mediated oxidative stress *in vitro* in predicting diverse pathologies, relevance for testing nanoparticles. *Part. Fibre Toxicol.,* 27, pp. 6–13, 2009.

[13] Yang, M., Butterworth, L., Munson, A.E. and Meade, B.J., Respiratory Exposure to Diesel Exhaust Particles Decreases the Spleen IgM Response to a T Cell–Dependent Antigen in Female B6C3F1 Mice. *Toxicol Sci.*, 71, pp. 207–216, 2003.

[14] Burchiel, S.W., Lauer, F.T., Dunaway, S.L., Zawadzki, J., Mcdonald, J.D., and Reed, M.D., Hardwood Smoke Alters Murine Splenic T Cell Responses to Mitogens Following a Six Month Whole Body Inhalation. *Exposure. Toxicol App Pharmacol.*, 202(3), pp. 229–236, 2005.

[15] Jin, C., Shelburne, C.P., Li, G., Potts, E.N., Riebe, K.J., Sempowski, G.D. and Foster, M.A., Particulate allergens potentiate allergic asthma in mice through sustained IgE–mediated mast cell activation. *J. Clin. Invest.,* 3, pp. 941–955, 2011.

[16] Diaz–Sanchez, D., Tsien, A., Fleming, J. and Saxon, A., Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed–specific IgE and skews cytokine production to a T helper cell 2–type pattern. *J. Immunol.,* 5, pp. 2406–2413, 1997.

[17] Boumiza1, R., Debard, A.L., and Monneret G., The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives. *Clinical and Molecular Allergy,* 3, pp. 1-9, 2005.

[18] Schober, W., Lubitz, S., Belloni, B., Gebauer, G., Lintelmann, J., Matuschek, G., Weichenmeier, I., Eberlein–Konig, B., Buters, J. and Behrendt, H., Environmental polycyclic aromatic hydrocarbons (PAHs) enhance allergic inflammation by acting on human basophils. *Inhal. Toxicol.*, 19(S1), 151–156, 2007.

[19] Chen, E.Y., Garnica, M., Wang, Y.C., Mintz, A.J., Chen, C.S., and Chin, W.C., A mixture of anatase and rutile $TiO_2$ nanoparticles induces histamine secretion in mast cells. *Part Fibre Toxicol*., 19, pp. 9–22, 2012.
[20] Largelius, M., Jones, P., Franck, K. and Gaines, H., Cytokine detection by multiplex technology useful for assessing antigen specific cytokine profiles and kinetics in whole blood cultured up to seven days. *Cytokine,* 33, pp. 156–165, 2006.