



Inflammatory effects on human lung epithelial cells after exposure to diesel exhaust micron sub particles (PM_{1.0}) and pollen allergens

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ABSTRACT

Asthma is currently defined as a chronic inflammatory disease of the airway. Several evidence indicate that vehicle emissions in cities is correlated with the allergic respiratory diseases.

In the present study, we evaluated in the A549 cells the production and release of IL-4, IL-5 and IL-13 after treatment with sub-micron PM_{1.0} particles (PM_{1.0}), *Parietaria officinalis* (ALL), and PM_{1.0} + ALL together.

Our data demonstrated that PM_{1.0} + ALL together exhibited the greatest capacity to induce A549 cells to enhance the expression of IL-4 and IL-5 compared with the only PM_{1.0} or ALL treatment. Interestingly, IL-13 that is necessary for allergen-induced airway hyper responsiveness, is increased in cells treated with PM_{1.0} + ALL together, but is higher expressed when the cells are treated only with the allergen.

Our data support the hypothesis that the urban environment damage the acinar lung units and activates cells of the immune system.

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1. Introduction

Asthma is currently defined as a chronic inflammatory disease of the airway. Current studies have shown that asthma is the result of an interaction between genetic and environmental factors.

The genetic factors plays a key role in the development of this disease, but its development can only be made in the presence of specific environmental factors (Warner et al., 1996).

Atopic asthma appears to be a complex genetic disorder in which the activity of the gene regulating the immune response, the production of IgE and other peculiar phenomena of the asthma is modulated by non-genetic factors. In particular, as non-genetic factors, it is intended the frequency and mode exposure to many environmental allergens, not only to allergens to which an individual is sensitized, but also to a range of nonspecific stimuli, such as air pollutants and cold air (Daniels et al., 1996; Holgate, 1997).

There is some evidence to indicate that high levels of vehicle emissions in cities and an urban lifestyle are correlated with the rising trend in allergic respiratory diseases (D'Amato et al., 2005, 2002).

The massive increase in emissions of air pollutants, due to economic and industrial growth, causes the more incidence of respiratory diseases, such as rhinosinusitis and bronchial asthma (Burr et al., 1989; Burney, 1993; Woolcock and Peat, 1997).

The most abundant components of air pollution in urban areas are nitrogen dioxide, ozone, particulate matter and aeroallergens by fungal spores or by plant-derived particles, that are an important risk factor for the development of allergic asthma.

Particulate matter is a mixture of organic and inorganic solid and liquid particles of different origins, size, and composition.

It was recently reported that the organic fraction of the particles to induce gene expression of CYP1A1 and its expression might influence the particulate matter induction of IL-6, IL-8 and COX-2 pro-inflammatory mediators (Totlandsdal et al., 2010).

Penetration of the tracheobronchial tract is related to particle size and the efficiency of airway defense mechanisms.

Though it was also demonstrated that the particulate matter exposure the dynamic cell growth, that simulates a mechanical environment similar to normal breathing condition in the lung, to induce the distinct differences in the profiles of inflammatory mediators (IL-8, CPR, and ROS) than the static cell growth system (Patel et al., 2011).

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Inhalable particulate matter that can reach the lower airways is classified in 3 sizes: PM₁₀, PM_{2.5}, and PM₁ (Annesi-Maesano et al., 2007; Mazzarella et al., 2007).

Diesel exhaust particles (DEPs) represent the greatest part of particulate matter (up 90%) given the increase in the number of new cars with diesel engines in industrialized countries (Diaz Sanchez et al., 1997).

Aeroallergens, such as those derived from pollen grains, help to facilitate allergic sensitization of the airways and lead to bronchial obstruction in predisposed individuals (Gilmour et al., 2006).

Therefore the increase in allergic respiratory diseases appears to be paralleled by increasing atmospheric concentrations of gases and respirable particulate matter and pollen allergy is one of the models most frequently used to study the relationship between air pollution and respiratory allergic disease (D'Amato et al., 2005).

It has been hypothesized that urban fine particulate matter (PM₁₀) can penetrate deep into the airways and induce alveolar inflammation, which is responsible for variation in blood coagulability and release of mediators favoring acute episodes of respiratory and cardiovascular diseases.

Pollen particles, in high environmental humidity conditions, can be subject to osmotic shock, resulting in the release of microparticles or paucimicronic particles that may contain allergenic proteins (Suphioglu et al., 1992).

In the same way, through physical contact with the pollen particles, diesel exhaust particles can disrupt the former, leading to the release of paucimicronic particles and transporting them by air thus facilitating their penetration of the human airways (Churg and Brauer, 1997).

The first study that revealed the linkage of five markers in chromosome 5q31.1 with a gene controlling total serum immunoglobulin E (IgE) concentration was carried in 170 individuals from 11 Amish families. This study suggest that IL-4 or a nearby gene in 5q31.1 regulates IgE production. (Marsh et al., 1994).

Another genetics study that represents a major step in mapping genes important in the regulation of allergic responses and the pathogenesis of asthma was conducted by Meyers et al. (1994). They have shown the linkage of the IgE phenotype to 5q31.1 where there are multiple candidate genes that may influence the control of IgE and inflammation. (Meyers et al., 1994).

Therefore, it has consolidated the scientific evidence that the linkage between chromosome 5q and asthma or atopic phenotypes is a more reproducible linkage among all those reported in the literature and concerning environmental pollution and asthma phenotype.

Drawing from background we decided to establish a linkage between asthma and a gene cluster on the long arm of chromosome 5 (region 5q31–q33), which includes genes coding for IL-4, IL-13, IL-3, IL-5, IL-9 (Holgate, 1999) involved in inflammatory effect on A549 human lung cells after treatment with sub-micron PM₁₀ particles, *Parietaria officinalis* (ALL), and PM₁₀ + ALL together.

2. Materials and methods

2.1. Particulate sampling

According to EU regulations (Directives 91/441/CE, 94/12/CE and 98/69/CE), Diesel PM is sampled by filtering air-diluted diesel car exhaust fumes at temperatures not higher than 52 °C. A dilution tunnel connected with a NOVA-MMB Messtechnik PDP-CVS unit has been used to produce the air/diesel combustion gases mixture; this configuration cleverly resembles conditions at which diesel exhaust PM is released from vehicles into the atmosphere.

PM was collected at the exhaust of a primary brand automobile equipped with a 2000 cm³ turbo-diesel engine, compliant to Euro IV emission regulations (Euro IV emission limits for cars not exceeding 2.5 tonnes laden being reported in Table 1). The car was tested over the New European Driving Cycle (NEDC) chassis dynamometer.

Diesel particulate is composed of elemental carbon particles, which agglomerate and adsorb other species to form structures of complex physical and chemical

Table 1

Euro IV emission limits for cars not exceeding 2.5 tonnes laden.

Fuel	Directive	Limit Values (g KM)				
		CO	HC	NOx	HC + NOx	PM
Gasoline	98/69/EC	1.00	0.10	0.08	—	—
Diesel	98/69/EC	0.50	—	0.25	0.30	0.025

properties. PM has a bimodal size distribution, with particles in nuclei mode and in accumulation mode, as shown in Fig. 1.

PM nuclear particles are very small with diameters between approximately 7 and 40 nm and they constitute the majority of particle number (about 90%), although they only represent a small part of total PM mass. Accumulation mode particles normally size less than 1 µm, with particle diameter most often ranging from 30 to 500 nm with a maximum concentration around 0.1–0.2 µm.

PM₁₀ was collected as described previously (Mazzarella et al., 2007). Briefly the particulate sampling filters were conditioned, then weighted; after sampling, filters were weighted again, and 5.5 mg of DEP-PM were placed in a beaker containing HPLC-grade Et-OH (Aldrich CHROMASOLV®). The suspension so obtained was sonication-treated for 30 min on a Misonix XL-2020 device, at a frequency of 20 kHz. Most Et-OH was subsequently removed from sample using a vacuum rotary evaporator, in order to get stock samples with PM concentrated to 900 mg/mL. Aliquots so prepared were stored at 2 °C upon usage.

2.2. Epithelial cell culture

We used the A549 cell line and was cultured as described previously (Mazzarella et al., 2007). Briefly, A549 cells were cultured in RPMI-1640 (Sigma, Poole, UK) medium supplemented with 10% fetal bovine serum (Sigma) and 10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 mg/mL amphotericin B (Sigma), in humidified atmosphere of 5% CO₂ 95% air at 37.5 °C to confluence. At this stage, A549 monolayers were gently detached using ready prepared Trypsin solution (Sigma), cells were rinsed and pelleted; pellets were resuspended in serum-free RPMI-1640 medium only supplemented with antimicrobial agents and plated onto "Multi-G-Lass" chambers (Iwaki Asahi Techno Glass); incubation took place at 36.5 °C in humidified atmosphere containing 5% CO₂ for 48 h, where cells normally reached semiconfluence.

At this time (our "starting time") PM₁₀ (0.1 mg/mL) and pollen extract *Parietaria officinalis* (0.5 DBU/mL) (Diagnostic Biological Units by Lofarma). One DBU is one hundredth of the potency of one extract inducing a wheal equal to that induced by histamine chloride 10 mg/mL, were added, separately and in combination, to cell cultures.

Escherichia coli-derived lipopolysaccharide (LPS, 10 ng/mL) was added to positive control cell samples. Negative control cell samples only received fresh medium. ELISA assays and immunocytochemistry experiments were performed at: starting time 24, 48, and 72 h following exposure to PM₁₀. Experiments always were performed as triplicates.

2.3. Immunocytochemistry and intracytoplasmic cytokines assessment

The A549 cells were first fixed with 4% paraformaldehyde in PBS for 30 min, and then with ice-cold methanol for 30 min. Then antigen retrieval was performed with 1% SDS for 3 min, followed by three steps of washing in 1x PBS for 5 min. Then slices were incubated with blocking buffer, 1x PBS containing 6% bovine serum albumin, for 1 h before application of the primary antibody.

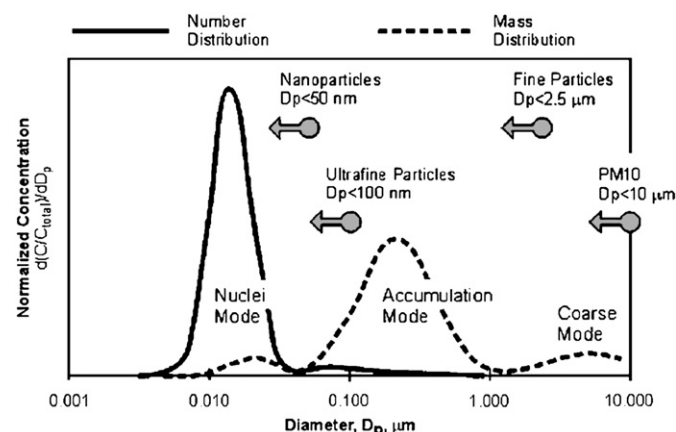


Fig. 1. Schematic drawing showing typical PM composition.

To confirm the epithelial-like nature of the A549 cell line we used an anti-(α) cytokeratin (IgG) monoclonal antibody as described previously (Mazzarella et al., 2007). Detection of intracytoplasmic IL-4, IL-5 and IL-13 was performed as follows: endogenous peroxidase activity was blocked by 10 min incubation with peroxidase blocking reagent (Dako).

Cells were incubated for 32 min at 40 °C with either α -IL-4 (STAT 6), α -IL-5 (H-85) and α -IL-13 (IL-13) primary Abs (Chemicon) diluted 1:25 (IL-4), 1:50 (IL-5) and 1:10 (IL-13) in phosphate-buffered saline (PBS). Streptavidin-biotin peroxidase complex (Dako) was added for 30 min, followed by 5 min incubation in 3,3' diaminobenzidine tetrahydrochloride in PBS. Nuclei were counterstained with Mayer's hematoxylin. Entellan (MERCK)-mounted slides were analysed for intracellular IL-4, IL-5 and IL-13 positivity on a LEIKA IMM-500 image analyser.

For each specimen, an HSCORE value was derived by summing the percentages of cells/areas that stained at each intensity and multiplying this value by the weighted intensity of the staining. An average of 22 fields was observed for each specimen by two observers at different times as described previously (De Falco et al., 2004) and the average score was used. All values were expressed as the mean $\pm$ SEM, and differences were compared by using Student's *t*-test.

2.4. Proinflammatory cytokines detection in cell supernatants

A549 cells were exposed to PM_{1.0} at the final concentration of 0.1 mg/mL. After 24, 48, and 72 h of treatment, supernatant aliquots were taken. IL-4, IL-5 and IL-13 were measured using commercially available enzyme-linked immunoabsorbent assay (ELISA) kits (Bender MedSystems BMS225INSTCE, BMS278INST and BMS231INST, respectively). ELISA analysis was performed according to manufacturer's instructions. Experiments always were performed as triplicates.

3. Results

A549 cell line treated with PM_{1.0} and pollen extract *Parietaria Officinalis* (ALL) was examined at 24, 48, and 72 h and compared with control cells.

A morphological comparison among the different points showed that untreated cells appeared moderately large and exhibited different kind of shapes. Most of the cells appears separated from one another nicely and neatly with a shape often hexagonal or spindle-like.

Instead treated cells appear with clearly atypical nuclei and moderately big. The cytoplasmic compartment showed granular inclusions with a diameter around 3.5 μ m. Morphological changes were observed also in cells treated with LPS (positive control).

Morphological comparison of A549 cell vitality at 72 h showed that the exposure to PM_{1.0} has greater toxic effect compared to treatment with pollen extract (ALL), instead treatment with both, the combination of PM_{1.0} and ALL, showed a toxic effect even greater.

Next we investigated the production of IL-4, IL-5 and IL-13 in the A549 cells treated and untreated. The A549 cells were first fixed on slides and later on were performed by immunocytochemistry for IL-4, IL-5 and IL-13 antibody. Immunocytochemistry for IL-4, IL-5 and IL-13 on A549 cells untreated showed a very small number of immunopositivity cells, instead treated cells showed a characteristic pattern of expression.

Immunocytochemistry for IL-4 revealed that at 24 h from PM_{1.0} stimulation, 50% of A549s would express intracytoplasmic IL-4, and 65% both at 48 and 72 h. ALL stimulation led to IL-4 intracytoplasmic expression in 45% of A549s at time 24 h, and 65% both at 48 and 72 h. A549 treated cells with PM_{1.0} and ALL together led to IL-4 intracytoplasmic expression in 60% of cells at time 24 h, and 65% both at 48 and 72 h.

Immunocytochemistry for evaluating the intracytoplasmic IL-5 revealed that at 24 h from PM_{1.0} stimulation, 25% of A549s would express intracytoplasmic IL-5, and 70% both at 48 and 72 h. ALL stimulation led to IL-5 intracytoplasmic expression in 30% of A549s at time 24 h, and 60% both at 48 and 72 h. PM_{1.0} and ALL together treated cells led to IL-5 intracytoplasmic expression in 50% of A549s at time 24 h, and 70% both at 48 and 72 h.

Immunocytochemistry for evaluating the intracytoplasmic IL-13 revealed that at 24 h from PM_{1.0} stimulation, 40% of A549s would express intracytoplasmic IL-5, and 70% both at 48 and 72 h. ALL stimulation led to IL-13 intracytoplasmic expression in 25% of A549s at time 24 h, and 60% both at 48 and 72 h. PM_{1.0} and ALL together treated cells led to IL-13 intracytoplasmic expression in 50% of A549s at time 24 h, and 70% both at 48 and 72 h.

The Fig. 2 shows the immunostaining of IL-4, IL-5 and IL-13 in the A549 cells treated and untreated at 72 h.

Furthermore in order to further evaluate the production of these three IL on A549 human lung cells we employed ELISA to actually measure the amounts of IL-4, IL-5 and IL-13 produced by A549s (and by these released into the medium) following stimulation with either fresh medium alone (neg. control), or fresh medium complemented with LPS (pos. control), or fresh medium containing PM_{1.0} [0.1 mg/mL], or fresh medium containing ALL [0.5DBU/ml], or fresh medium containing PM_{1.0} [0.1 mg/mL] and ALL together.

ELISA analysis gave us the following values at 24 h: LPS stimulation resulted in the production of about 7 pg/mL of IL-4, 4 pg/mL of IL-5 and 1 pg/mL of IL-13; PM_{1.0} treatment led to the release of about 30 pg/mL of IL-4, 25 pg/mL of IL-5 and 6 pg/mL of IL-13; ALL treatment led to the release of about 20 pg/mL of IL-4, 22 pg/mL of IL-5 and 5 pg/mL of IL-13; PM_{1.0} and ALL together treatment led to the release of about 41 pg/mL of IL-4, 30 pg/mL of IL-5 and 11 pg/mL of IL-13 at the same time point.

ELISA analysis gave us the following values at 48 h: LPS stimulation resulted in the production of about 20 pg/mL of IL-4, 11 pg/mL of IL-5 and 4 pg/mL of IL-13; PM_{1.0} treatment led to the release of about 53 pg/mL of IL-4, 31 pg/mL of IL-5 and 9 pg/mL of IL-13; ALL treatment led to the release of about 43 pg/mL of IL-4, 28 pg/mL of IL-5 and 10 pg/mL of IL-13; PM_{1.0} and ALL together treatment led to the release of about 62 pg/mL of IL-4, 34 pg/mL of IL-5 and 14 pg/mL of IL-13 at the same time point.

ELISA analysis gave us the following values at 72 h: LPS stimulation resulted in the production of about 47 pg/mL of IL-4, 31 pg/mL of IL-5 and 11 pg/mL of IL-13, PM_{1.0} treatment led to the release of about 93 pg/mL of IL-4, 44 pg/mL of IL-5 and 16 pg/mL of IL-13, ALL treatment led to the release of about 58 pg/mL of IL-4, 34 pg/mL of IL-5 and 21 pg/mL of IL-13, PM_{1.0} and ALL together treatment led to the release of about 106 pg/mL of IL-4, 54 pg/mL of IL-5 and 19 pg/mL of IL-13 at the same time point.

Figs. 3, 4 and 5 showed the ELISA reading for IL-4, IL-5 and IL-13 immunopositivity in the A549 cells treated and untreated.

4. Discussion

The chronic asthma is mostly due to a deregulation of the cytokine network and T-cell activation that lead to the development of persistent inflammation (Robinson et al., 1993; Bodey et al., 2005).

Morphologically in chronic asthma areas without epithelium have been observed, presenting a detachment of the basal cells than suprabasal, with a permanent epithelial damage. Morphological and histological alterations that characterize the asthma phenotype could be the resulting to the combined effect of both components, genetic predisposition and environmental factors.

The genes implicated in the pathogenesis of respiratory diseases belong to two classes; genes involved directly in antigen recognition and genes that regulate the antibody response and allergic inflammation. In the second group of the genes, the analysis of molecular biology, have established a linkage between asthma and a gene cluster on the long arm of chromosome 5 (region 5q31-q33), which includes genes coding for IL-3, IL-4, IL-5, IL-9, IL-13 (Holgate, 1999).

In the present study we evaluated in the A549 cells the production and release of IL-4, IL-5 and IL-13 after treatment

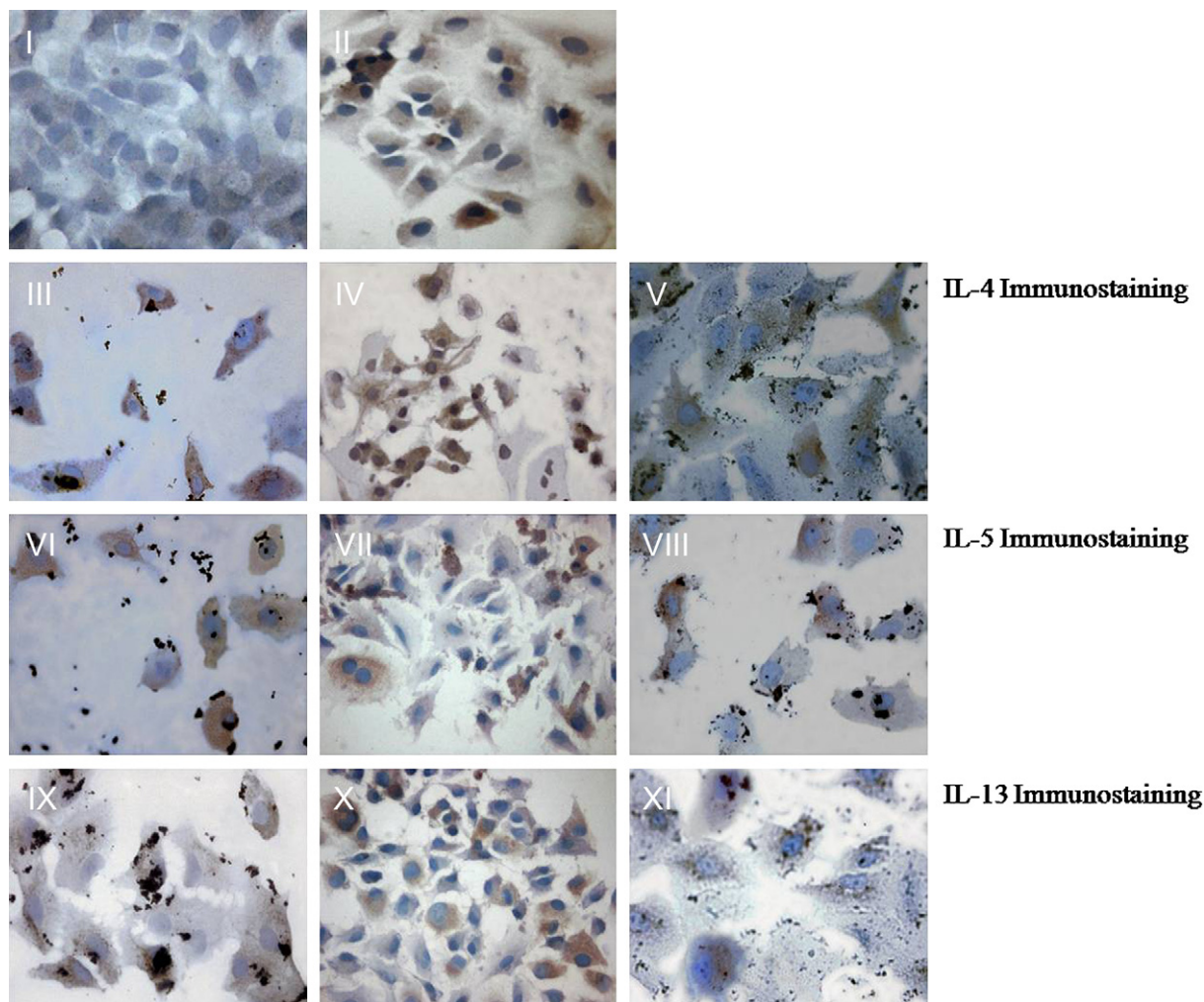


Fig. 2. Immunostaining of IL-4, IL-5 and IL-13 at 72 h. Pannel I and II: positive controls and LPS treatment for 72 h. Pannel III, IV and V: PM_{1.0}, ALL, PM_{1.0} and ALL treatment for 72 h respectively. Pannel VI, VII and VIII: PM_{1.0}, ALL, PM_{1.0} and ALL treatment for 72 h respectively. Pannel IX, X and XI: PM_{1.0}, ALL, PM_{1.0} and ALL treatment for 72 h respectively.

with sub-micron PM_{1.0} particles (PM_{1.0}), *P. officinalis* (ALL), and PM_{1.0} + ALL together. We used the particles with a size of 1.0 μ m or less and at a concentration of 0.1 mg/ml derived from filters to diesel engine exhausts. The concentration used was 1/10 compared to that which causes chronic inflammation of the airways.

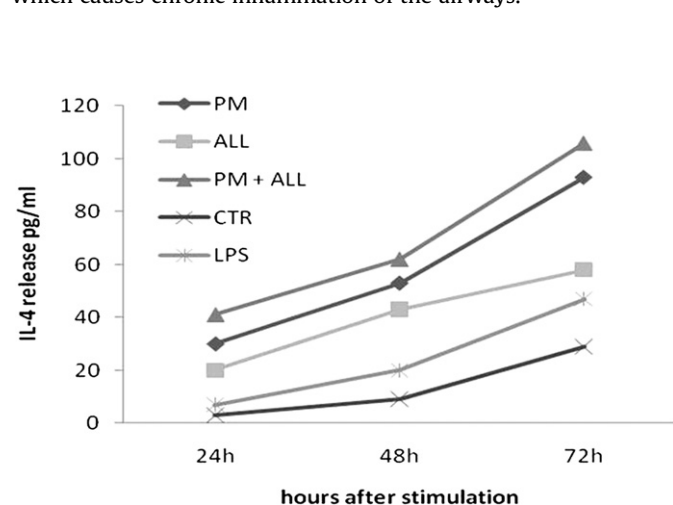


Fig. 3. ELISA reading of LPS, PM_{1.0}, ALL and PM_{1.0} + ALL together induced IL-4 secretion. Ordinate: IL-4 release pg/ml. Abscissa: Time points after stimulus.

In particular, we have observed the strong production of IL-5 in A549 cells treated with ALL and PM_{1.0} together compared with only ALL or PM_{1.0} treated. These data support the hypothesis that IL-5 high production enhances the activation and survival of

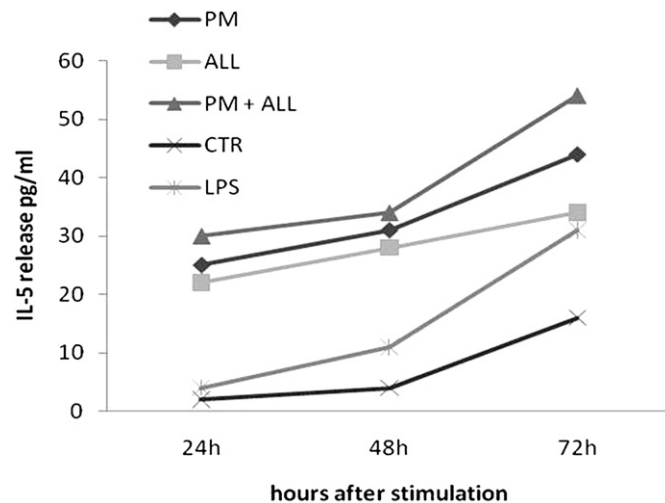


Fig. 4. ELISA reading of LPS, PM_{1.0}, ALL and PM_{1.0} + ALL together induced IL-5 secretion. Ordinate: IL-5 release pg/ml. Abscissa: Time points after stimulus.

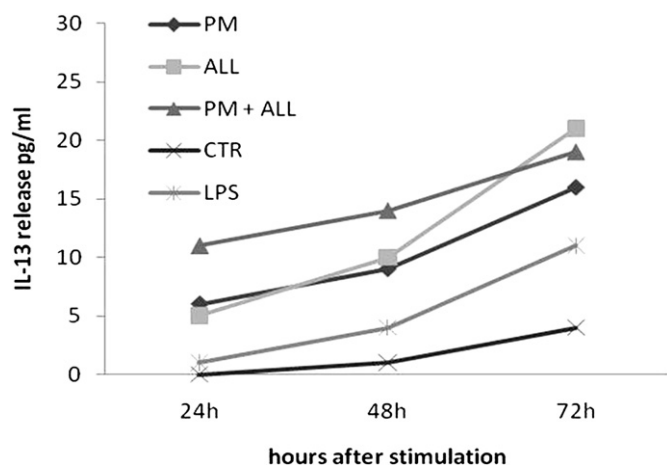


Fig. 5. ELISA reading of LPS, PM_{1.0}, ALL and PM_{1.0} + ALL together induced IL-13 secretion. Ordinate: IL-13 release pg/ml. Abscissa: Time points after stimulus.

eosinophils; several studies have shown that the organism reactions to allergens are mediated by the activation of eosinophils.

The continued recruitment of cells from the circulation, the continuous activation of these cells and their survival are the basis of progressive inflammation in the chronic asthma (Montefort et al., 1994).

In addition PM_{1.0} + ALL together are able to enhance the expression of IL-4 compared to the only ALL treatment. Elevated IL-4 levels are necessary for IgE isotype switching and are able to up-regulate vascular cell adhesion molecule.

The IgE play a key role inducing the allergy, the most common diseases of hypersensitivity affecting the population of industrialized countries. PM_{1.0} particles with *P. officinalis* in the environment could increase the reactivity of the airways in atopic subjects probably because PM_{1.0} particles play a role of immunological adjuvant.

Interestingly, our results point out that, IL-13 that is necessary for allergen-induced airway hyper responsiveness, is enhanced when the A549 cells are treated with PM_{1.0} + ALL together, but is highly expressed when the cells are treated only with the allergen.

The IL-13 and IL-4 cytokine are closely correlated and both are produced by Th2 lymphocytes (Ackerman-Liebrich, 2000).

The IL-13 and IL-4 can direct cells B to produce IgE, but only the IL-4 cytokine can induce differentiation of Th2 cells. This phenomenon of differentiation occurs in the early asthmatic reactions when there is a synergistic effect between the stimulation mediated by allergens and PM.

Several studies have demonstrated the IL-13 key role in the chronic asthma characterized by deposition of collagen in the reticular lamina under the epithelial basement membrane with an increase in myofibroblasts, epithelial damage and airway remodeling (Wills-Karp et al., 1998; Zhu et al., 1999). In addition, Graves et al. (2000) and Liu et al. (2000) have shown the close association between the IL-13 polymorphism and atopy in children. These data make us to suppose that the IL-13 has a key role in the process of chronic asthma due to allergen sensitization (Graves et al., 2000; Liu et al., 2000). So it is possible that IL-13 polymorphisms confer susceptibility for airway remodeling in asthma from persistent and chronic allergic inflammation rather than cooperation between allergens and cars pollutants.

In conclusion, our data show that the PM_{1.0} + ALL together exhibited the greatest capacity to induce A549 cells cytokine responses compared with the only PM_{1.0} or ALL treatment and suggest the hypothesis that the interaction between diesel particulates and

pollen is capable of forming micro compounds that can reach the acinar lung units and are able to activate cells of the immune system. This activation may depend on the interaction between susceptibility genes and environmental factors that trigger the onset of allergic disease. Genetic polymorphisms may determine the immune responses to the factors that direct Th1/Th2 immune responses.

Clinically we can conclude that chronic asthma is due to a deregulation of the Th2 cytokine network that leads to the development of persistent inflammation in the airways structurally altered, which becomes unresponsive to treatment. This deregulation is mostly due to the urban environment with the combination of inhalable air pollution and particulate.

To determine the interaction between genetic and environmental factors will be essential to understand the mechanisms that cause the onset and severity of allergic disease. The clarification of these interactions will provide new opportunities for the preventive measures to reduce the environmental risk of developing allergic disease in susceptible individuals.

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