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DOCTORAL THESIS

EFFECT OF ENVIRONMENTAL POLLUTION ON ASTHMA DUE TO SOYBEAN

Efecto de la contaminación ambiental en el asma por soja

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ABBREVIATIONS

AHR	Airway hyperresponsiveness
AhR	Aryl hydrocarbon receptor
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCL20	C-C motif chemokine 20
CCR6	C-C chemokine receptor type 6
CI	Confidence interval
DCs	Dendritic cells
DEP	Diesel exhaust particles
EH-TLV	Environmental High Threshold Limit Value
EIA	Enzyme immunoassays
ELISA	Enzyme Linked Immunosorbent Assay
EL-TLV	Environmental Low Threshold Limit Value
GM-CSF	Granulocyte macrophage colony-stimulating factor
GST1	Glutathione S-transferase-1
HDM	House dust mite
HSP	Hydrophobic soy protein
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
LFIAs	Lateral flow immunoassays
LOD	Limit of detection
NFκB	Nuclear factor kappa B
OD	Optical density
OVA	Ovalbumin
PAH	Polycyclic aromatic hydrocarbons

PBS	Phosphate buffered saline
PM	Airborne particulate matter
R	Airway resistance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SHE	Soybean hull extract
SHLMWE	Soy hull low molecular weight extract
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TSLP	Thymic stromal lymphopoietin

SUMMARY

Summary

Soybean is one of the most valuable crops in the world, a major source of vegetable protein and oil, but also, a cause of asthma. In cities with ports where soybean is loaded or unloaded, community outbreaks of asthma have been described and attributed to inhalation of soybean dust. Measuring soybean aeroallergens levels with highly sensitive methods is essential in the assessment of health risks due to these airborne substances. Currently, soybean aeroallergens exposure in the environment is monitored using ELISA assays which must be evaluated in a specialized laboratory by skilled personnel. Cost-efficient, less labor-intensive technological procedures for monitoring soy allergens levels can be a very useful tool. Consequently, the development, performance and suitability of a rapid test for the detection of soybean aeroallergens in environmental samples was investigated.

The rapid test developed detected a wide range of soybean aeroallergen concentrations with a substantial agreement in visual assay interpretations between independent evaluators, a good concordance with ELISA results, a strong correlation with densitometry results, and adequate sensitivity and specificity. The strip assay developed is rapid, simple, and has considerable potential in the environmental monitoring field for screening soy aeroallergens levels in port cities where allergen measurements are not currently performed. Due to its simplicity, the test will improve the management of soy allergic patients by controlling environmental allergen exposure without the need for apparatus or skilled personnel.

Although the understanding of allergic asthma pathogenesis has increased substantially, and the effect of aeroallergen assessment shows to be effective, our current understanding about soybean asthma has some knowledge gaps, as is to know how pollution can interact or modify the effect of soybean allergens. Diesel exhaust particles (DEP) are the solid fraction of the complex mixture of diesel exhaust, and one of the most prevalent anthropogenic pollutants worldwide. Research over recent decades has provided very informative results, and has suggested that DEP can enhance allergen-driven asthmatic immunopathology, but the ability of DEP to do so appears highly dependent on a wide range of variables, and the underlying mechanism remains unclear.

The experimental modelling of asthma, particularly the development of murine models, besides being very useful to further investigate the pathogenesis of the disease, can contribute to a deeper understanding of the effects of DEP over asthma. The development of a murine model of combined exposure to soybean and DEP can provide new insights to increase our

understanding of the contribution of DEP to asthma development, exacerbation and the mechanisms underlying these processes.

This study with the standardization of a novel murine model of asthma and the assessing of the combined effect of soybean aeroallergens and DEP, shows that the continuous administration of soybean allergens at a certain concentration is capable of triggering an asthmatic response. In addition, it also demonstrates that the coexposure to soybean allergens and DEP results in a stronger asthmatic response, increasing airway hyperresponsiveness and pulmonary inflammation even when the concentration of soybean allergen is incapable of promoting an inflammatory response by itself. This mouse model provides evidence that the mechanism underlying soybean asthma is a mixed Th2/Th17 response, and also that DEP is capable of enhancing the allergenic effect of soybean through a Th17-mediated mechanism. These findings suggest that particulate matter monitoring as a surrogate of DEP exposure may be a useful addition to the allergen monitoring in the attempt to prevent new asthma outbreaks.

Resumen

La soja es uno de los cultivos más valiosos del mundo y una importante fuente tanto de proteína vegetal como de aceite, pero también, es una causa de asma. En ciudades con puertos donde se carga o descarga soja, se han descrito brotes epidémicos de asma por soja. La medición de los niveles de aeroalérgenos de soja con métodos altamente sensibles es esencial en la evaluación de los riesgos para la salud que estos implican. En la actualidad, los aeroalérgenos de soja se cuantifican mediante ensayos ELISA que deben ser realizados en un laboratorio por personal especializado. Metodologías menos laboriosas, baratas y sencillas para medir los niveles de alérgenos de soja podrían ser una herramienta extremadamente útil. Por lo tanto, se ha investigado el desarrollo y rendimiento de un test rápido para la detección de aeroalérgenos de soja en muestras ambientales.

El test rápido desarrollado detectó un amplio rango de concentraciones de aeroalérgenos de soja cuya interpretación visual tenía un grado de acuerdo substancial entre evaluadores independientes, una buena concordancia con los resultados obtenidos mediante ELISA, una buena correlación con los resultados de densitometría y una sensibilidad y especificidad adecuadas. El test desarrollado es rápido, sencillo y tiene un potencial considerable en el campo de la monitorización ambiental de los niveles de aeroalérgenos de soja. Debido a su simplicidad, este test rápido puede ayudar a mejorar el manejo de pacientes alérgicos a la soja, permitiendo controlar la exposición a los alérgenos ambientales sin la necesidad de tecnología o personal especializado.

Aunque la comprensión de la patogénesis del asma alérgico ha aumentado sustancialmente, y la monitorización de los niveles de aeroalérgenos ha demostrado ser una medida eficaz, todavía existen pequeñas lagunas en nuestra comprensión del asma por soja. Todavía no se conoce cómo la contaminación puede interactuar, o modificar el efecto, de los alérgenos de soja. Las partículas diésel (DEP) son la fracción sólida de la mezcla compleja que genera un motor diésel y uno de los contaminantes antropogénicos más prevalentes en todo el mundo. La investigación en las últimas décadas ha proporcionado resultados que sugieren que las DEP puede incrementar la respuesta asmática producida por alérgenos, pero la capacidad de las DEP para hacerlo parece depender de una amplia gama de variables y el mecanismo subyacente todavía no se conoce en profundidad.

El desarrollo de un modelo murino de exposición combinada a soja y DEP puede aumentar nuestra comprensión de la contribución de las DEP al desarrollo y exacerbación del asma, así como, de los mecanismos subyacentes a estos procesos.

Este estudio con la estandarización de un nuevo modelo murino de asma y la evaluación del efecto combinado de los aeroalérgenos de soja y las DEP, muestra que la administración continua de alérgenos de soja a una cierta concentración es capaz de desencadenar una respuesta asmática. Además, también demuestra que la coexposición a los alérgenos de soja y las DEP puede producir una mayor respuesta asmática, aumentando la hiperreactividad de las vías respiratorias y la inflamación pulmonar incluso cuando la concentración de alérgeno de soja es incapaz por si sola de provocar una respuesta inflamatoria. Este modelo proporciona nuevas evidencias que apoyan que el mecanismo subyacente en el asma por soja es una respuesta mixta Th2/Th17, y también que las DEP son capaces de potenciar el efecto alergénico de la soja a través de un mecanismo mediado por Th17. Estos hallazgos sugieren que la monitorización de partículas como alternativa a la valoración de la exposición a las DEP puede ser una adición útil a la monitorización de alérgenos dentro de las estrategias de prevención de nuevos brotes de asma.

INTRODUCTION

Asthma

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by a history of respiratory symptoms such as wheezing, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation[1]. Although originally believed to be a single disease associated with atopy and allergic reactions, there is increasing evidence that asthma is a multifactorial heterogeneous condition comprising a complex of multiple phenotypes, each one with its own natural history, characteristics and severity[2,3].

It is estimated that around 300 million people of all ages and ethnic backgrounds suffer from asthma[4]. The disease causes 250,000 deaths annually and its burden on governments, health care systems, families, and patients is rising steadily all over the world[4]. Although the mortality rate has fallen dramatically with the development and regular use of new treatments, the global impact of asthma remains high and the prevalence of the disease seems to be increasing[2].

The susceptibility to asthma and its severity are due to the numerous interactions between genetic profiles[5], environmental exposure (to aeroallergens[6], pollution[7,8] and viruses[9]) at different stages of life, lifestyle (diet[10], smoking[11]) and other comorbid conditions (obesity[12] and atopic dermatitis[13]). However, the exact causes of asthma remain uncertain. Traditionally, on the basis of the factors just mentioned, asthma has been subdivided into two phenotypes: allergic and nonallergic asthma[14]. People with allergic asthma develop the disease early in life; they are atopic, (specific immunoglobulin E (IgE) production to identifiable allergens) and have identifiable allergic triggers, and other allergic diseases such as rhinitis or a family history of allergic disease[15,16]. Nonallergic asthma develops later in life, is not associated with allergic sensitization and by definition does not present IgE reactivity to allergens in serum[14,15]. Despite the convenience and usefulness of this division in clinical practice, clinicians have realized that the categorization of asthma into just two clinical forms is an oversimplification that may create biases. For the last twenty years, asthma has increasingly been seen as a syndrome rather than a single disease[15], and clinical guidelines reflect its heterogeneity by defining multiple levels of severity and by dividing patients into categories or phenotypes[17]. These asthma phenotypes have been defined using cluster analysis on the basis of clinical or physiological characteristics (severity, age of onset and resistance to treatment), by asthma triggers (exercise, allergens and aspirin) or on the basis of the type of inflammation (eosinophilic, neutrophilic or paucigranulophilic)[14,16,17]. Although these efforts to identify phenotypic clusters have helped to distinguish different subtypes of patients, precise definitions of these clusters remain elusive.

Phenotyping by clusters has not yet led to the development of differential treatment strategies[2] and it is unable to predict future outcomes such as exacerbations[16]. However, with recent advances in the understanding of the different molecular mechanisms underlying asthma and the development of specific biologic agents, it is hoped that endotyping will lead to better and more useful classifications.

The description of endotypes aims to define asthma entities according to identified or suspected pathophysiological mechanisms associated with, and putatively leading to, the disease. They include parameters such as clinical characteristics, biomarkers, genetics, histopathology, lung physiology and response to therapy[18]. Allergic asthma is probably the most frequent endotype[16,18]. Since the development of pulmonary immunology, asthma has been seen as the hallmark T helper 2 (Th2) disorder of the lungs. However, the pathogenesis of allergic asthma is characterized by different patterns of cytokine-based airway inflammation involving not just Th2 cells but also other subtypes of T cells and B cells, eosinophils, neutrophils, mast cells and dendritic cells (DCs), as well as structural cell types including epithelial and mesenchymal cells[15,19] (Fig. 1).
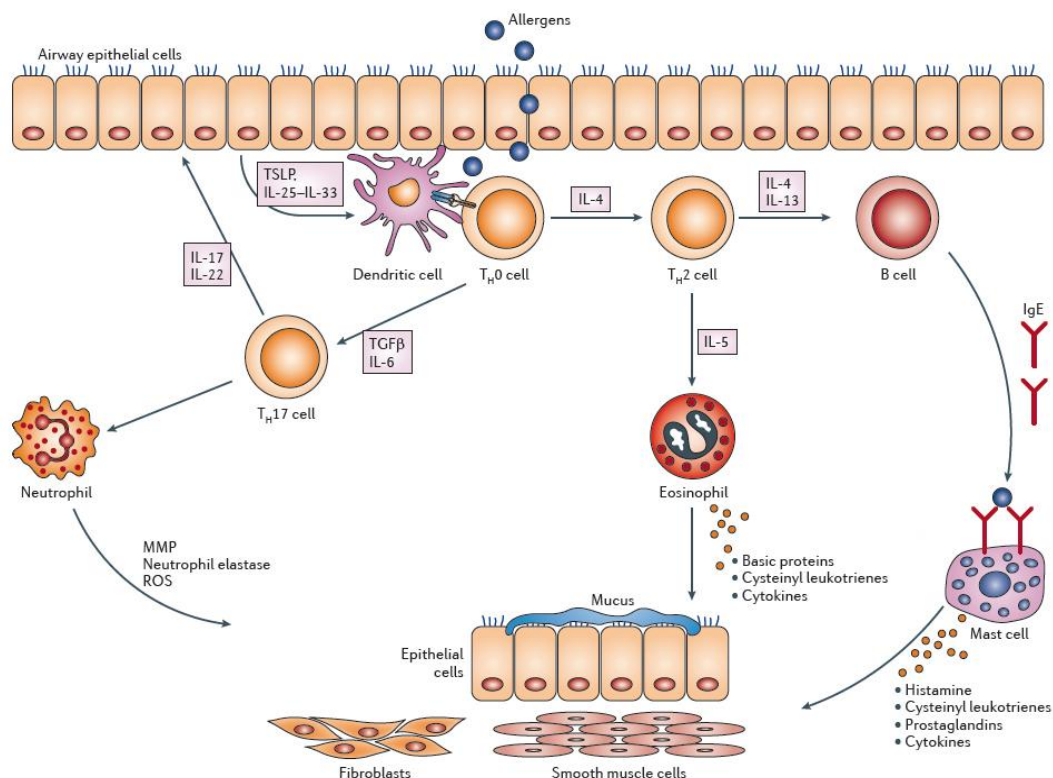

Figure 1. Overview of allergic asthma immunologic mechanisms. Figure adapted from Pelaia, G., Vatrella, A. & Maselli, R. The potential of biologics for the treatment of asthma. Nat. Rev. Drug Discov. 11, 958–72 (2012).

Airway epithelial cells are the main cells that form a barrier to aeroallergens, pollutants and infectious agents. Contact with aeroallergens can stimulate various types of pattern recognition receptors, such as Toll-like receptor 4, generating an epithelial activation that leads to the production of different cytokines, chemokines and danger signals that recruit innate immune cells such as DCs and eosinophils[20]. Like airway epithelial cells, DCs can also be exposed to the external environment and act as antigen-presenting cells that can be directly stimulated by surface binding of allergens or indirectly stimulated by airway epithelial cells (by mediators such as interleukin(IL)-25, IL-33, thymic stromal lymphopoietin (TSLP), and granulocyte macrophage colony-stimulating factor (GM-CSF)[21] and affect T cell differentiation by presenting inhaled allergens to CD4+ cells when they migrate to the draining mediastinal lymph nodes[21]. After the differentiation process, Th2 polarized cells can produce cytokines that are important for asthma pathogenesis such as IL-4, IL-5, and IL-13[15]. IL-4 and IL-13 are required to drive the isotype switch of B cells into plasmocytes producing allergen-specific IgE antibodies[22]. The allergen-specific IgE antibodies can bind to circulating mast cells when crosslinked by aeroallergens, causing mast cells to degranulate and release their mediators (histamine, prostaglandins and leukotrienes, enzymes, cytokines and chemokines)[20]. IL-5 is crucial for B cell survival and maturation and also for the stimulation of eosinophils, bone marrow-derived granulocytes that play a central role in asthma. Differentiation of eosinophils in the bone marrow is mediated by IL-5 and GM-CSF[23] and their recruitment is mediated by IL-13, histamine, prostaglandin type 2, and eotaxins[24]. In addition to releasing toxic granular proteins such as eosinophilic cationic protein and eosinophil-derived neurotoxin, eosinophils secrete dozens of cytokines and chemokines, which promote inflammation through the Th2 pathway and also cause airway epithelial damage[24,25]. T helper 17 (Th17) cells, a CD4 positive subset of T cells that express IL-17, also play a role in some patients with asthma. The Th17 pathway is unusual and differs from the classic Th2 response in that neutrophils are its primary effector cells[26]. Neutrophils probably play a role in certain asthma endotypes. Recruited through Th17 pathways, neutrophil numbers are increased in several patients with asthma, especially in those who are relatively unresponsive to inhaled steroids[26,27].

Thus, in the past decades, our understanding of allergic asthma pathogenesis has substantially improved. The Th2 dominant immune response leading to tissue inflammation and remodeling has long been regarded as the underlying mechanism[28]. However, new evidence has also been reported of important roles for structural cells, and additional types of T cell responses[2,28]. At present, our understanding of the molecular bases and immune mechanisms underlying asthma is incomplete.

Asthma due to soybean

Soybean (*Glycine max*) is one of the most valuable crops in the world, a major source of vegetable protein and oil both for consumption and for industrial applications. However, soybean dust is a well-known aeroallergen, identified for the first time as a cause of asthma in 1934[29].

The valued portion of the soybean plants is the seed, which contains about 40% protein and 20% oil[30,31]. Although soybean has many uses, most of the bean is discarded. Most of the soybean produced undergoes a dehulling process, followed by pressing in order to obtain oil and soybean meal. Soybean hulls are further processed for animal feed or for use as fiber additives for breads, cereals and snacks[30].

Thanks to its versatility, soybean is among the world's most widely grown agricultural products. World soybean production increased by 4.6% annually from 1961 to 2007, by which time it had reached an average annual production of 217.6 million tons; its rates of production are still rising and are predicted to reach 311 million tons by 2020. Nevertheless, soybean production is concentrated in particular areas, and in fact 95% is produced in only seven countries[31]. As a consequence, large amounts of soybean must be handled and transported from production regions to overseas harbors, mainly via maritime routes.

In cities with ports where soybean is loaded or unloaded, community outbreaks of asthma have been described and attributed to the inhalation of soybean dust[32–36]. The first reported epidemic outbreaks took place in Barcelona, Spain, due to the release of soybean dust during unshipping to silos located in the harbor[32]. Other epidemic asthma outbreaks related to the presence of soybean have been documented in other Spanish cities such as Cartagena[35], Valencia and A Coruña[33], and also in Naples (Italy)[33] and New Orleans (USA)[34]. The characterization of the allergens responsible for soybean asthma epidemics shows that the serum of affected patients reacts specifically to proteins mainly present in the soybean hull[37]. Two soy hull proteins were shown to be the main allergens responsible for these epidemics[38], and were found to be isoallergens with a high degree of homology with the hydrophobic soy protein (HSP) described by Odani*et al*[39].

The dose-response relationship involved in sensitizing a person or in eliciting symptoms in those already sensitized is unknown; nevertheless, several studies have shown the importance of reducing soy emissions to prevent asthma attacks[32,40,41].

In the city of Barcelona, considerable efforts have been made to improve the control of soybean dust released during harbor activities. After the first epidemic outbreaks of asthma related to soybean, in 1987, corrective measures were taken in the harbor's unloading facilities in order to prevent the dispersion of soybean dust. Regular measurement of dust emissions during the unloading, and the installation of sleeve filters in the silos, achieved a drastic reduction in the dissemination of soybean dust and avoided the emergence of new outbreaks for several years[42]. Nevertheless in 1994 and 1996, new asthma outbreaks were recorded. These outbreaks, together with occasional increases in environmental aeroallergen levels, persistent sensitivity to soybean in affected patients and occasional hospital admissions of patients with acute asthma provided evidence of significant leakages of soybean dust into the atmosphere during unloading[43]. After the 1996 outbreak, new measures were adopted and all aspects of the handling and processing were revised, leading to a temporary suspension of unloading operations. Subsequently, unloading was authorized on a boat-to-boat basis under special restrictions related to the loading/unloading flow, the timetable of activity, and weather conditions. In addition, the filter sleeves were replaced with better ones, and sleeve rupture detectors were installed[42]. To assess the effectiveness of these new corrective measures, several new risk indicators were adopted including allergen levels in the city, allergen emissions by the soybean unloading or processing companies operating in the harbor, the aggregate numbers of asthma cases treated at the city's emergency services, and the health status of a panel of asthma patients allergic to soybean[40].

These new indicators demonstrated the effectiveness of the new measures in controlling allergen emissions and in decreasing the environmental soybean aeroallergen concentrations[40,42]. In fact, since 1996, no further epidemic asthma outbreaks have been detected in the city. However, the evolution over time of the patient panel's health status has shown that in given circumstances, small increases in comparatively low allergen concentrations may suffice to produce mild symptoms in some patients allergic to soybean[40].

Avoiding or reducing exposure to soybean aeroallergens is crucial in order to prevent the associated adverse respiratory outcomes. The use of highly sensitive methods for measuring soybean aeroallergen levels is essential to evaluate the health risk associated with the exposure,

to assess the efficacy of exposure reduction measures, and to establish whether further additional measures are needed.

In Barcelona, two threshold values were determined empirically based on the levels of soybean aeroallergens recorded on epidemic and non-epidemic days: the Environmental High Threshold Limit Value (EH-TLV), and the Environmental Low Threshold Limit Value (EL-TLV). When the EL-TLV is exceeded, soybean facilities and meteorological conditions are assessed and a report is produced, and when the EH-TLV is reached an inspection of the soybean processing facilities is performed[44]. Soy aeroallergen exposure in the city was monitored with a sandwich enzyme linked immunosorbent assay (ELISA)[45], which defined the EH-TLV as 19 ng/m^3 and the EL-TLV as 6 ng/m^3.

Monitoring of soybean aeroallergen levels in the city was a crucial indicator for demonstrating the effectiveness of the measures adopted to prevent new outbreaks from 1997 onwards. This monitoring was also very useful for examining the exposure-response relationship in the asthmatic population.

Simple, fast, reliable and sensitive techniques are essential for monitoring soybean aeroallergens. Samples for monitoring are obtained by large-volume automated air samplers containing glass microfiber filters. Aeroallergens are extracted from the sampling filters and yield a 24-hour average measurement. In the city of Barcelona, soybean aeroallergen levels were initially measured by a radioallergosorbent technique inhibition method[46]. Based on this technique, an amplified ELISA inhibition technique was implemented[47] which uses a soybean hull extract as antigen and reference, and a pool of sera from allergic patients with specific IgE to soybean. Despite the value of this method, its use of human antibodies is a major drawback; the availability of suitable human sera is limited, and the manipulation of human blood samples presents a health risk. What is more, IgE inhibition methods have potential long term reproducibility problems due to the use of heterogeneous antibody pools[48], and the fact that different laboratories use their own pool of sera makes comparison of absolute measurements difficult. To overcome some of these limitations, a specific sandwich immunoassay (ELISA) was developed for measuring airborne soybean hull allergens based on polyclonal rabbit antibodies, as an alternative to the human inhibition method[49] and has been in use in Barcelona since April 2012. The sandwich ELISA using polyclonal antibodies has a high sensitivity and specificity and has demonstrated its value for measuring soybean aeroallergens, even at relatively low

concentrations. Moreover, as the method uses only rabbit antibodies at relatively high dilutions, it does not require human sera[49]. A monoclonal antibody-based method to quantify soybean aeroallergens has also been reported[50]; however, using polyclonal antibodies is cheaper and easier, the method is less disturbed by epitope losses, and may also present advantages in sensitivity when several allergens are present in the same sample[49].

The Barcelona experience shows that strict control criteria including the assessment of soy aeroallergen levels are fundamental for the safe management of soybean transport near urban settings. The identification of similar problems in port cities with soybean harbor facilities in Spain[33,35] and in other countries[33,34] highlights the importance of the monitoring process. However, no country has developed legislation to regulate this environmental risk[40], due in part to the lack of a widely available assay that allows the monitoring of soybean aeroallergen levels. There is a clear need now for cost-efficient, less labor-intensive technological procedures to monitor soy allergen levels that do not require specialized laboratory staff.

Rapid immunoassays

Diagnostic technologies have improved substantially in recent decades[51]. Despite these advances, most diagnostic laboratory technologies are centralized and need highly trained staff and specialized facilities; the equipment is generally expensive and needs regular maintenance by skilled technicians. Consequently, most standard laboratory-based tests are prohibitively expensive and are inaccessible in certain circumstances and certain countries. Testing with simple, rapid devices at or near the site where a sample is obtained can provide results without having to wait hours or even days for sample transport and laboratory processing[51,52]. Furthermore, rapid devices have advantages in cost, ease-of-use, speed of detection, spectrum of application, and disposability[52].

The rapid testing era began in 1962 with the development of a new, rapid method to measure blood glucose concentration, and was bolstered with the introduction in 1977 of a rapid pregnancy test. Rapid device testing gained substantial traction in the early 1990s with the introduction of small, portable devices for hospitals emergency rooms, and many rapid tests have been developed since then[51]. These devices can be separated into various groups according to their practical use, sensor characteristics, complexity, measuring mode, underlying detection principle and sample matrix[53]. One of the simplest designs for rapid devices are the strip immunoassay based methods such as the rapid dipstick immunoassays or lateral flow immunoassays (LFIA) (Fig.2).

Since their development in the 1970s, rapid immunoassays have been applied in a wide range of settings including medical diagnostics, food safety, and environmental control[54]. These immunoassays are based on the principle of immunochromatography in which the specific interaction of antigen and antibody takes place in a membrane[55,56]. Rapid immunoassays are similar in design to ELISA, and although they have not superseded the ELISA format, they overcome its main drawbacks and provide a portable one-step analysis test with low operational cost, high specificity, comparable or better sensitivity, and long-term stability in a user-friendly format[55].

Rapid immunoassays combine a number of variants, such as formats, biorecognition molecules, labels and detection systems, but they all share a strip-based structure with the same components. The basic components of a rapid immunoassay are the antibodies, the labels, the membrane and the pads[55,56].

The antibody is the most basic component of all the immunochemical applications. Depending on the assay design and format, antibodies may be used as a capture reagent, as a conjugate to the label, or both. In the case of rapid immunoassays, antibody sensitivity and specificity are especially important because the interaction time between antigen and antibody is limited, and is usually very short[56]. Labels are conjugated with recognition antibodies and provide a signal that is read as the result of the assay. Today many different labels can be used in rapid immunoassays, but the most common are nanoparticles. In the majority of cases these nanoparticles are based on gold, but other materials like colloidal carbon or colored latex particles can also be used[54–56].

The membrane is the structural base of the strip. Usually made of porous polymeric nitrocellulose, the membrane plays a critical role in the sensitivity of the assay and also provides support and good binding to the capture antibodies[55,56]. Three different pads can be added to the strip structure based on the membrane. The sample pad can be placed before the membrane provides a surface for the application of the sample, and promotes its homogeneous transportation. The conjugate pad can work as a dry storage area for the conjugate label that will dilute in the sample fluid, interacting with it in the chromatographic process. Finally, the absorbent pad can be added at the end of the membrane to drain the fluid, maintain the flow rate, and stop the sample from back flowing[55,56].
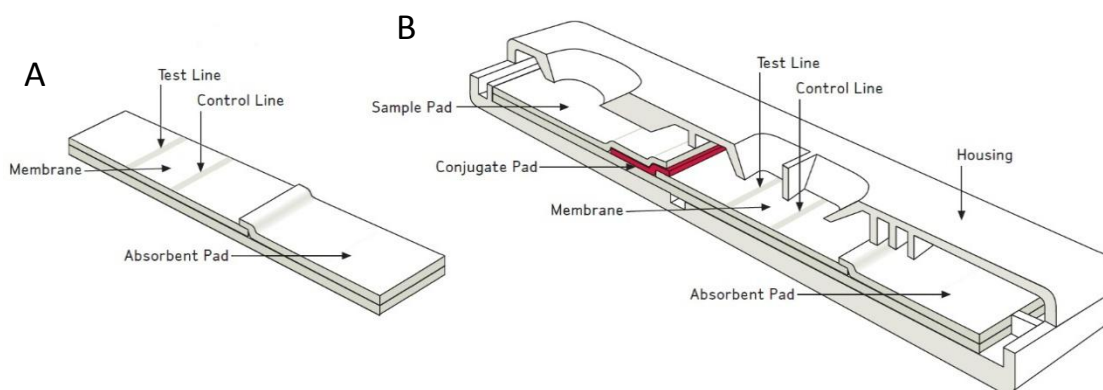
Figure 2. Schematic diagram of a rapid dipstick immunoassay (A) and a lateral flow immunoassay (B). Figure adapted from Millipore. Rapid Lateral Flow Test Strips: Considerations for product development. EMD Millipore Corp. Billerica, 1–42 (2008).

With the combination of these elements, various strip designs can be constructed with varying levels of complexity, and suitable for different kinds of analytes, samples, applications and environments. In fact, in the research environment it is quite common to work with basic strip designs that consist only of a membrane with immobilized capture antibodies, and sometimes an absorbent pad (Fig. 2A). However, most of the strip designs for large-scale commercialization are much more complex and include complicated sample pads, durable and stable conjugate pads, attractive housings and even multiplexing in order to measure different analytes in the same strip[56] (Fig. 2B).

Although different formats of rapid immunoassays have been developed, the most frequently used, especially for high molecular mass analytes like proteins, is the sandwich format. In this assay format, a primary antibody against the target analyte is immobilized over the membrane in a line disposition called the test line. A secondary antibody against the labeled conjugate is immobilized in the membrane as a control line. The label, usually a gold nanoparticle coated antibody, is mixed with the sample solution or immobilized in the conjugate pad if there is one in the strip construction. The sample alone or the combination of sample and conjugate is applied to the sample pad, or the membrane is directly submerged in the mixture promoting the migration through the strip. When the sample conjugate complex reaches the test line, the analyte/conjugate complex can be captured by the primary antibody immobilized in the membrane forming a labeled complex over the line. The excess conjugate will be captured by the secondary antibody immobilized in the control line, while the buffer or excess solution goes to the absorption pad. The intensity of the test line should correspond to the amount of the target analyte in the sample, and the appearance of color in the control line ensures the proper functioning of the test.

Rapid immunoassays can provide fast qualitative or even semi-quantitative results within minutes with very good analytical sensitivity. Furthermore, the characteristics of their design and construction make them especially suitable for field testing and for use by non-laboratory-skilled personnel[54,55]. Therefore, if the reduction or cessation of the exposure to allergens is key to the prevention and management of asthma[57], and since the best and fastest allergen monitoring have been shown to be highly beneficial for diminishing exposure[54,57], especially in the case of aeroallergens, a rapid immunoassay for the assessment of soybean aeroallergens can be a very useful and versatile tool. Consequently, one of the main objectives of this thesis was to develop a rapid test for the detection of soybean aeroallergens in environmental samples (Chapter 1).

Pollution, diesel exhaust particles and asthma

Our understanding of the pathogenesis of allergic asthma has increased substantially in recent times, and aeroallergen assessment has proved to be effective. Nonetheless, several gaps in our knowledge remain: for example, how pollution can interact or modify the effect of soybean allergens.

Air pollution is a serious problem worldwide, and the growing use of fuels is one of the primary drivers of atmospheric pollution. Exposure to air pollutants has been correlated with an increase in the incidence and severity of asthma, and its implication in the exacerbation of pre-existing asthma is supported by a body of evidence which has accumulated over several decades[7,58–60]. Furthermore, evidence of the effect of air pollution as a cause of new asthma onset has recently emerged[7,61–63]. Although air pollution almost always occurs as a mixture, air quality regulation and the majority of experimental studies have focused on individual pollutants. While epidemiological studies inherently involve exposure to mixtures of pollutants, they are useful tools for identifying the individualized effects, and for distinguishing the ones that have the greatest repercussions for human health. Numerous epidemiological studies have shown that airborne particulate matter, a major component of air pollution, is associated with several deleterious effects[64].

Airborne particulate matter (PM) is a complex mixture of small particles and liquid droplets composed of organic chemicals, acids, metals, soil and dust[64]. PM is usually categorized on the basis of its aerodynamic diameter, a parameter with very important implications because it determines where the PM will deposit in the human airways when inhaled. Due to the massive

internal surface of the human respiratory tract, the importance of efficient mechanisms for avoiding the entrance and deposition of particulate pollutants becomes evident. Sneezing and coughing are the main mechanisms in the nasopharyngeal and upper tracheobronchial regions, but once the lower tracheobronchial region is reached the particles may be trapped and retained in the mucus present in the airway epithelium[65]. The filtration process of PM has different degrees of efficiency depending on the aerodynamic diameter of the particle and the region of the respiratory tract involved. As a consequence, different regions of the airway will be exposed to different size ranges of particles.

Based on its aerodynamic diameter, PM is usually classified as Coarse, Fine or Ultrafine. Coarse PM includes particles with an aerodynamic diameter between 2.5 to 10μm, and is mainly deposited in the upper respiratory tract and large conducting airways. Fine PM comprises particles with a diameter between 0.1 and 2.5μm, and deposits throughout the respiratory tract, particularly in small airways and alveoli. Particles with a diameter inferior to 0.1μm are classified as ultrafine; like fine PM, ultrafine PM deposits mainly in the alveoli, but these smaller particles may also be retained in the upper respiratory tract (Fig.3).

Particles with a larger diameter than coarse PM can be considered irrelevant because they have a relatively small suspension half-life and are largely filtered out by the nose and upper airways. PM can also be defined as PM10, PM2.5 and PM0.1, in which PM10 includes the all three particle fractions, PM2.5 fine and ultrafine PM, and PM0.1 only the ultrafine fraction[7,64].

Some of the constituents of PM have the potential to produce oxidative stress and also many of the phenotypic changes associated with asthma. Among them, polycyclic aromatic hydrocarbons (PAH) and environmentally persistent free radicals are very common and their association with asthma has been stressed[7].
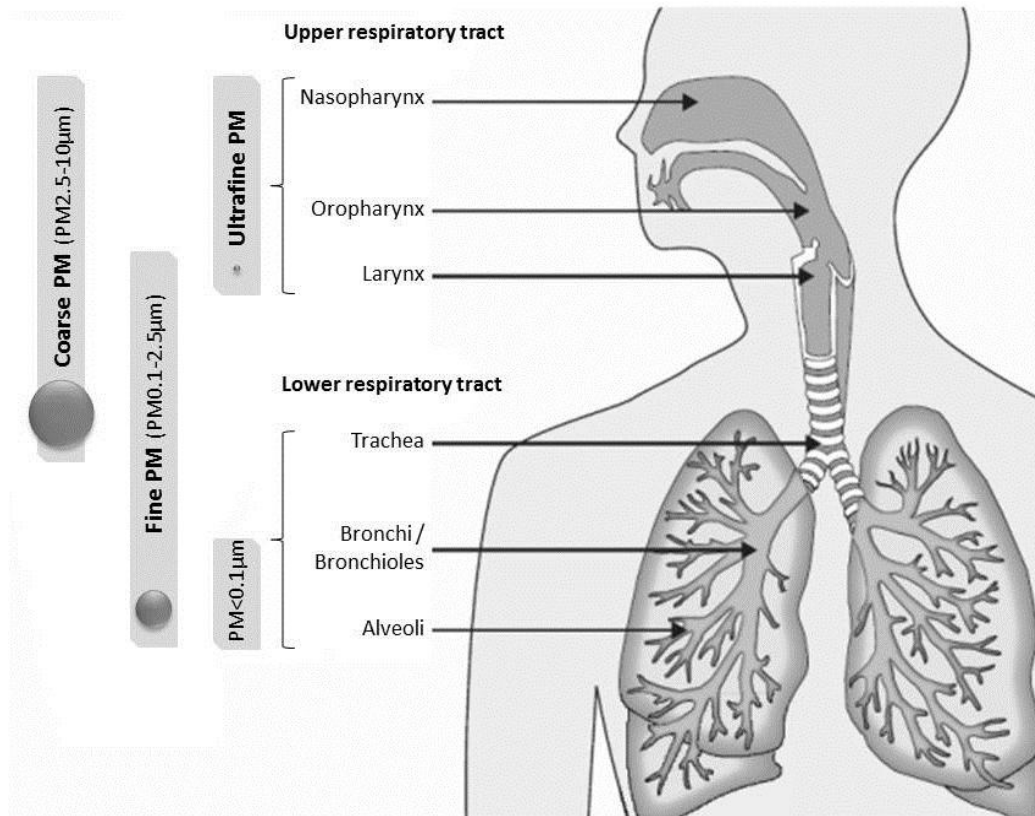
Figure 3. Diagram of the compartmental deposition of Particulate Matter (PM). Figure adapted from Guarnieri, M. &Balmes, J. Outdoor air pollution and asthma. Lancet 383, 1581–1529 (2014).

The composition and size distribution of PM vary according to the source of its production[7,66], which may be either natural or anthropogenic. Natural sources of PM include volcanoes, forest fires and soil erosion, while anthropogenic sources include industrial processes such as electricity generation, mining and welding but the most important of all are traffic emissions[66]. Diesel exhaust particles (DEP) are the main contributors to vehicle traffic PM [8,67].

Diesel Exhaust Particles (DEP)

Diesel engine emissions are among the most prevalent anthropogenic pollutants worldwide[66]. Since the invention of the diesel engine in the late 1890s, this type of internal combustion engine has established itself as the prominent technology for heavy duty applications in marine, railroad, military and stationary sectors. In agriculture the diesel engine replaced the gasoline engine in the 1960s, but this technology did not reach the private transportation sector until the 1980s, with the adaptation of turbochargers and the development of common rail injection systems. Since then, its popularity has grown steadily in most of the world's car markets, especially in the European Union and India[65,68]. The large-scale implementation of diesel engines

in the private transport sector brought their emissions into urban centers, inevitably resulting in a continuous and sometimes high level exposure for a large part of the population.

DEP are the solid fraction of the complex mixture of diesel exhaust. They are composed by a central core, whose main component is elemental carbon, and a variety of organic compounds adsorbed to this core[65,69] (Fig. 4A). DEP mainly comprises fine and ultrafine particles whose central core appears to be generated mainly via a pathway that involves the pyrolyzation of unburnt fuel and lubrication oil, followed by polymerization reactions, ring closure and the stacking of the resulting polycyclic, graphite-like sheets in a turbostrated structure[65]. These primary particles can agglomerate generating aggregates classified as coarse particles, which are the main cause of the macroscopic black appearance of diesel exhaust[65,67] (Fig. 4B).
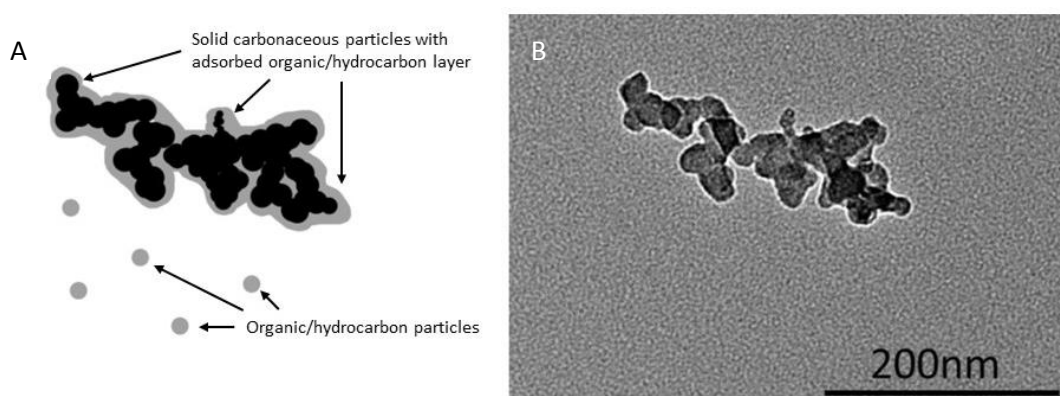

Figure 4. Diesel exhaust particles (DEP) from an unfiltered diesel passenger car. (A) Schematic diagram of DEP, (B) irregularly shaped elongated DEP aggregates of 200nm length. Primary particles are clearly identifiable. Figure adapted from Anderson, J. O., Thundiyil, J. G. & Stolbach, A. Clearing the Air: A Review of the Effects of Particulate Matter Air Pollution on Human Health. J. Med. Toxicol. 8, 166–175 (2012).

In addition to elemental carbon, the core of DEP can contain other minor components like metal and metal-oxides originating from lubrication and fuel additives, and also from engine wear. Fuel and lubrication oil additives contain metals as functional components, such as zinc, magnesium, cerium, iron, manganese, platinum and copper. However, the highly biopersistent nature of elemental carbon, the most prevalent element of the core, means that whatever chemical compounds the cores contain their dissolution into biofluids will be very limited. As a consequence, even though the composition of the core of DEP is known to play a role in defining the toxicity of the particles, it is not the main factor; the main factors are rather the adsorbed compounds of the surface, and the surface properties of the particles[8,65,67,69]. The final composition of both the particle core and the surface-adsorbed chemicals depends heavily on the engine type, the operation mode, fuel, lubrication oil, and additives[65,67].

Numerous organic compounds that are found adsorbed to the particles' surface are individually known to have pernicious effects on human health, and many of them have been defined as mutagenic and carcinogenic. Epidemiological studies have shown a positive correlation between levels of particulate pollution, including DEP, and adverse health effects[70,71] including increases in rates of pulmonary disease[72]. Based on the proven genotoxicity of their constituents, DEP have been judged as mutagenic and carcinogenic to humans by the World Health Organization[73]. The causal connection between DEP and adverse health effects is still not fully understood, but certain molecular and cellular mechanisms are assumed to play a key role.

The best described cellular responses to interaction with diesel exhaust are the induction of pulmonary oxidative stress and inflammation, which are both involved in the onset and/or exacerbation of respiratory diseases. In the 1990s it was already recognized that the adverse health effects caused by particulate air pollution like DEP rely on pulmonary inflammation[60]. Oberdorster *et al.*[74] demonstrated that ultrafine particles produced significantly stronger inflammatory responses in mice than the identical mass of large particles, suggesting that the particles' surface area is a relevant toxicology factor. Subsequent research identified the induction of oxidative stress and the formation of reactive oxygen and nitrogen species (ROS/RNS) as the main factors determining particle toxicity. These two findings corroborate the notion that particle toxicity increases with the particle surface area, since ROS and RNS are known to form on particle surfaces[65].

A mechanism that has been proposed as a possible explanation of the effects of particle inhalation[65] and the connection between inflammatory processes and oxidative stress is the following[75]. ROS formed on the surfaces of particles cause either the activation of antioxidant defense mechanisms (at low levels of oxidative stress), or inflammatory responses (at intermediate levels of oxidative stress) or cytotoxic effects (at high levels of oxidative stress). Besides, and more importantly, an additional promoter of the impact of the smaller fraction of particles is their retention time in the alveolar region and their ability to translocate across the respiratory epithelium and/or enter cells, where they may accumulate over time. Due to the extremely low solubility of DEP, they may remain there for years and exert adverse effects[7,65].

While oxidative stress seems to be the trigger for the induction of pulmonary inflammation, more recent studies have shown that PAH, organic compounds especially abundant on the surface of DEP[65,67,69], may initiate inflammatory reactions without the formation of ROS or RNS

on their own[76]. This was found to rely on the activation of inflammatory signaling cascades via highly specific interactions between PAH and the aryl hydrocarbon receptor (AhR) present in certain cell types. The activation of the AhR signaling pathway triggers immunologic cellular responses and leads to the intracellular formation of ROS[61,65,77–79], which play an active role as secondary messengers, regulating cellular events downstream from the AhR. One of these events is the activation of nuclear factor kappa B (NFκB), one of the key inducers of inflammatory responses[15,66,78]. Inflammatory stimulation by complex aerosols such as DEP is therefore likely to be the result of the combined action of particle-mediated ROS formation and ROS-independent mechanisms[65].

Diesel exhaust particles and asthma

For several decades, along roadways with high traffic density the prevalence of cedar pollinosis was seen to be increased, even though the pollen levels remained essentially unchanged[8]. This classic observation, reported on several occasions, is one of the first descriptions of the relationship between traffic-related pollution and the exacerbation of allergic disease. Since the 1990s many epidemiological studies have described an increased incidence of asthma in urban regions, and a higher prevalence of asthma symptoms among people who live closer to major roads[80–82]. In fact, even short-term exposure to high diesel traffic has been associated with a reduction of the airway function in asthmatic patients[83]. Furthermore, several birth cohorts have demonstrated positive correlations between exposure to traffic pollution and allergic sensitization[84,85]. Traffic pollution and DEP exposure have also been associated with increased asthma severity in asthmatic children[86]. This body of epidemiological data suggests that traffic-related pollution and DEP are associated with increased asthma prevalence and severity and also represent a risk factor for allergic sensitization[87,88].

A considerable number of experimental approaches including animal and human nasal models have provided evidence of the stimulant effect of DEP over allergic responses[89,90]. The results of some of these studies support the notion that DEP can promote changes in the immune response and in airway inflammation[91,92]. However, experimental exposure to DEP in isolation has produced inconsistent and sometimes even contradictory results[86,89,93–97]. Some controlled human exposure studies have suggested that DEP may trigger pulmonary and systemic inflammation. A controlled exposure study in healthy volunteers demonstrated increased neutrophils and B cells in bronchoalveolar lavage (BAL), an influx of neutrophils, mast cells, and T cells into the bronchial wall, and elevated neutrophils in the peripheral blood, thus supporting the idea of systemic inflammation[98]. These results partly agree with an investigation of healthy

volunteers exposed to a slightly lower concentration of DEP which demonstrated increased sputum neutrophils but no evidence of a systemic inflammation[99]. Another healthy volunteer study showed evidence of lung inflammation with increased numbers of eosinophils in the BAL, while yet another found activation of an inflammation pathway in peripheral white blood cells[96]. In contrast, several controlled human exposure studies have reported no biological effects of diesel exhaust and DEP exposure[96,100,101]. Like controlled exposure, the nasal challenge has repeatedly been used in human studies and provides similarly conflicting data[96,97].

Unlike exposure to DEP alone, in most studies experimental exposure to DEP concomitant with or after sensitization to a variety of allergens has produced different levels of oxidative stress, increased airway hyperresponsiveness (AHR), enhanced neutrophilic and eosinophilic airway inflammation, and a modified immune response[7,8,65].

The first described effect of DEP when combined with an allergen was its action as a mucosal adjuvant to amplify the allergic response[93]. After that, several human nasal studies suggested that the combination of DEP with an allergen can promote allergic responses[90,102]. Some authors propose a mechanism to explain this adjuvant effect which involved DC maturation[103], production of allergen-specific IgE[93,104], and a Th2 polarization of cytokine production[90,104]. Nonetheless, the effect observed differed according to the circumstances of the exposure[8].

The apparent variability in the effect of co-exposure of DEP and allergens may indeed be real in this context, since factors such as genetic and epigenetic variations may modify the outcome of the exposure. For example, the induction of phase II drug metabolizing enzymes can block the enhancement of IgE production[105], and the null allele of the glutathione S-transferase-1 (GST1) significantly increases the risk of IL-5 elevation due to the combined exposure of allergen and DEP[8]. The relevance of epigenetic changes in the mechanism underlying the effect of the co-exposure allergen-DEP has recently emerged, and significant changes in microRNAs in the airway epithelium and blood have also been described[106]. Besides epigenetic and genetic factors, the timing of measurements may also account for the variability found across studies. Other factors such as the sequence and duration of exposure and the concentration, size and detailed composition of DEP may also contribute to the variability.

Research over recent decades has provided very informative results and suggests that DEP can enhance allergen-driven asthmatic immunopathology. However, its ability to do so appears to be highly dependent on a wide range of variables. To the extent that this enhancement occurs,

the augmentation of the Th2-related response seems a common element, but the implication and the mechanism through which the co-exposure allergen-DEP mediates Th2, other T cells, IgE and epithelial response (among other effects) remains unclear.

Mouse models of asthma and DEP

Epidemiological and controlled human exposure studies can be very useful tools for evaluating the effects of DEP on asthma onset and exacerbations, but they are limited by a large number of practical and even ethical issues. Experimental modeling of asthma, particularly the development of murine models, is very useful for investigating its pathogenesis and can provide a deeper understanding of the effects of DEP on the disease.

Asthma is a uniquely human disease. With the exception of cats (eosinophilic bronchitis) and horses (heaves)[107], no animals exhibit an asthma-like syndrome similar to the human disease. Several species and study designs have been used to try to model human asthma, but although no generally accepted model has been devised, the mouse has emerged as the animal of choice[108]. Mice are easy to breed, maintain and handle and a large selection of specific reagents and devices are available for the analysis of the cellular, mediator and physiological responses. Furthermore, genetically engineered transgenic or gene-knockout mice for modeling airway diseases are easy to obtain[107,109].

Because mice do not spontaneously develop asthma, in order to investigate the mechanisms underlying the disease an asthma-like reaction has to be artificially induced. Many different mouse models have been developed and a large number of sensitization and challenge protocols have been used. The inflammatory response of these models varies according to the choice of mouse strain, the allergen, and the sensitization and challenge protocols[109,110].

Although other mouse strains (C57BL/6 and A/J) have been successfully used for antigen challenge models, the strain most commonly used is BALB/c. These mice have a genetic immunologic bias to develop a Th2 immune response upon allergic sensitization and challenge[110]. Ovalbumin (OVA) has been the allergen of choice for many years, and it is still frequently used in traditional murine model protocols [110,111]. Exposure of animals to OVA, usually combined with a potent adjuvant, produces an airway inflammation model that exhibits a number of human asthma-like cellular and pathophysiological features such as increased AHR, cellular inflammation and elevated levels of inflammatory cytokines in BAL[109–111]. However, OVA is not associated with asthma in humans; it requires systemic administration protocols, adjuvants and the prolonged exposure can induce tolerance, resulting in reduced airway

inflammation and AHR[111]. Furthermore, OVA-based protocols using antigen sensitization with adjuvant followed by an inhaled antigen challenge result in an acute asthma-like phenotype which focuses on the inflammatory mechanisms linked to manifestations of established asthma in humans, but fails to model the etiology and natural history of human asthma, which develops over time through multistep processes[107]. All these shortcomings significantly limit the applicability of OVA mouse models.

In order to better simulate the chronic nature of human asthma, longer duration models avoiding the use of adjuvants and with more physiologically relevant antigens, such as house dust mite (HDM), cockroach or grass pollen, have been developed[109]. These models are based on the repeated exposure of the airway, for longer periods of time, to low levels of allergens via physiological routes like inhalation or intranasal instillation[109,110,112]. These chronic models have been shown to reproduce some of the hallmarks of human asthma better than acute models, such as allergen-dependent sensitization, a Th2-dependent allergic inflammation characterized by eosinophilic influx, sustained AHR and even airway remodeling[109,110,113,114]. Because of the better reproduction of human disease and the higher physiological relevance of the allergens used, chronic allergen exposure now appears to be the model of choice for studying the role of specific cell types, inflammatory cytokines and mediators in the processes involved in chronic inflammation and even some of the structural changes to the airways[109,110].

A common feature and a shortcoming of both traditional acute and chronic animal models of asthma is that their design is based on the concept that allergen-driven Th2 inflammation is the underlying abnormality in asthma. This could be the reason why, although some models have been very useful in identifying the pathways possibly related to human asthma, they have failed to reproduce important features of the disease. Epidemiological studies[115], birth cohort studies in asthma-susceptible families[116] and experimental results[26,117] suggest that the Th2-type allergen concept may not be the primary mechanism, or at least not the only one, in disease development and progression. Instead, infection, diet, tobacco smoke and especially air pollution with agents such as DEP and their interplay with genetic factors are being increasingly recognized as important risk factors for the development of asthma, its persistence, and its sudden exacerbations[2,28].

Many animal studies have examined the effects of DEP in isolation and have reported alterations in inflammatory endpoints. Several studies support the effect of DEP on the induction of airway

inflammation[8,97,108], but while some of them report the presence of eosinophils, neutrophils and lymphocytes in BAL, others report increases in alveolar macrophages and neutrophils but not in eosinophils[91,96]. Increased expression of proinflammatory cytokines, hypertrophy of mucous cells and mucus hypersecretion, alveolar type II epithelial cell hyperplasia and increased AHR have also been reported as the effects of the exposure of DEP in murine models but, much as in human studies, the results are inconsistent and contradictory[8,65,91,97].

Muranaka and coworkers were the first to show the effects of DEP combined with an allergen using an OVA-based murine model[93]. Since then, many authors have described the effects of DEP on asthma, using different immunization routes, allergens and protocols. The earliest studies were based on traditional OVA acute asthma protocols with simultaneous administration of different concentrations of DEP, mainly using outbreed mouse strains[8,93,118,119]. Despite the limitations of these acute protocols, the early OVA studies served to confirm that the combination of DEP and allergen is able to increase allergen-specific IgE[93,118,120]. Furthermore, these models showed that combined exposure with DEP can promote increases in AHR[118] and in the numbers of eosinophils and neutrophils in BAL, and can also raise levels of antigen-specific IgG[8,108,118,120].

To overcome the limitations of OVA acute models, the majority of recent studies that have used murine models to study the relationship between DEP exposure and asthma apply chronic protocols with physiologically relevant allergens like HDM and inbreed strains, mainly BALB/c. The studies using chronic DEP exposure models confirmed some of the previous findings reported with OVA models and provided new insights into the effect of DEP on asthma.

The results obtained from the chronic protocol models of combined exposure to HDM and DEP confirmed that this coexposure leads to increases in AHR[86,89,94,95,121] and eosinophil and neutrophil counts, and elevated levels of IgE[89,94,108,121]. Mouse models of exposure to DEP and HDM have also been able to reproduce important functional features of allergic asthma which mimic the human disease and were not seen in previous models.

The greatest advantage of chronic models for the study of the development and exacerbation of asthma and the combined effect of DEP and allergens is their better reproduction of the immunological mechanisms observed in the disease in humans. Chronic models showed that DEP and allergen can synergistically increase the expression of some Th2-related cytokines, like IL-4, IL-5 or IL-13[86,89,121], and in some cases the expression of Th17 cytokines[86,121] and the modulation of the T helper 1 (Th1)-related response (Fig. 5)[89,104,122].

Research in recent decades has suggested that DEP may increase allergen-driven airway immunopathology, but that this is highly dependent on a wide range of variables. The increase in Th2 response seems to be a common element, but a range of other immune and genetic mechanisms have also been implicated. Acknowledging the considerable gap between observational perspectives and experimental evidence, the development of chronic mouse models of combined exposure to physiologically relevant allergens and DEP can provide powerful tools for increasing our understanding of the contribution of DEP to asthma development and exacerbation and the mechanisms underlying these processes. Studies until now using mouse models of allergic asthma combined with DEP have been based on allergens which are physiologically relevant but are mostly of indoor type (such as HDM).
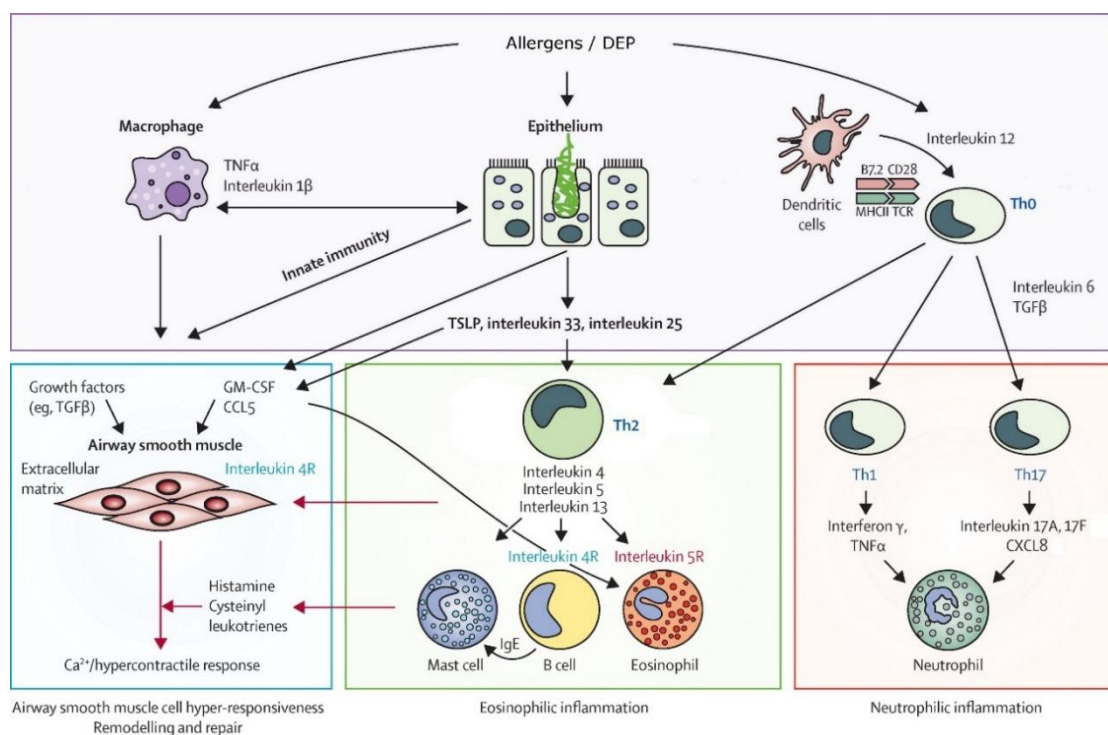

Figure 5. Immunological asthma mechanisms related to exposure to allergens and DEP. Figure adapted from Chung, K. F. Targeting the interleukin pathway in the treatment of asthma. Lancet 386, 1086–1096 (2015).

The development of a murine model of asthma due to soybean, a physiologically relevant outdoor aeroallergen, may help to fill the gaps in our knowledge of soybean asthma. Equally, the study of the combination of DEP and soybean allergens may help to unravel how DEP can interact with allergens or modify their effect. Therefore, one of the objectives of the present thesis was to develop a murine model of asthma due to soybean and to use it to assess the effect of DEP on the induction or exacerbation of soybean asthma (Chapter 2).

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HYPOTHESIS AND

OBJECTIVES

Hypothesis and objectives

Hypothesis:

- The development of a rapid test would allow the environmental monitoring of soybean aeroallergens without the need for expensive equipment or specific skills.

- Asthma can be induced in a murine model by repeated instillation of soybean allergens.

- The exposure to diesel exhaust particles enhances the induction or exacerbation of asthma due to soybean, inducing changes in the immunopathogenesis.

- The environmental pollution levels must be taken into account to set safe threshold values for soybean aeroallergens

Objectives:

- To develop a rapid, simple and inexpensive test for the detection of soybean aeroallergens in environmental samples.

  - To evaluate the performance of the rapid test using environmental samples

- To study how the combination of soybean allergens and diesel exhaust particles can affect the induction or exacerbation of asthma.

  - To develop and standardize a novel murine model of asthma due to soybean

  - To assess the effect of DEP on that murine model regarding airway hyperresponsiveness, lung inflammation and immunological response.

CHAPTER 1. A rapid test for soy aeroallergens exposure assessment

Daniel Álvarez-Simón, María-Jesús Cruz, María-Dolores Untoria, Xavier Muñoz, Joan R. Villalbí, Ferran Morell, Susana Gómez-Ollés.

Abstract

Background: Abstract Determining soy aeroallergens levels is extremely important in the assessment of health risks due to these airborne substances. Currently, soy aeroallergens exposure in the environment is monitored using enzyme immunoassays (EIA) which must be evaluated in a specialized laboratory by skilled personnel.

Objective: To describe the development and performance of a rapid immunochromatography assay for the detection of soy

Methods: aeroallergens in environmental samples. A test strip using gold labeled anti-soy hull low molecular weight extract (SHLMWE) antibody for the rapid detection of soy aeroallergens in environmental samples was developed. One hundred nineteen airborne samples were analysed in parallel by the strip assay and the anti-SHLMWE sandwich EIA. The assay results were visually analysed by three independent observers who ranked samples as: -, + or ++. Strips were also scanned and analysed by densitometry.

Results: The rapid test detected a range of concentrations from 6.25 to 25 ng/mL. Agreement in strip assay interpretations between evaluators was substantial (Kappa=0.63; CI 0.544–0.715). Visual interpretation also gave a good concordance with EIA results, with sensitivity ranging from 77.3 to 100 and specificity from 65 to 83.5 depending on the observer. Furthermore, a strong correlation was observed between densitometry results of strip assay and EIA determinations.

Conclusions: The strip assay developed is rapid, simple, and sensitive and does not require expensive equipment or specific skills. It has considerable potential in the environmental monitoring field for screening soy aeroallergens levels in port cities where allergen measurements are not currently performed. Due to its simplicity, the test will improve the management of soy allergic patients by controlling environmental allergen exposure without the need for apparatus or skilled personnel.

Introduction

Soy dust is a well-known aeroallergen. In cities with ports where soybeans are loaded or unloaded, community outbreaks of asthma have been recorded and attributed to inhalation of soy dust [1–5]. In addition, exposure to soy dust in the workplace has been identified as a cause of occupational asthma [6–8] and hypersensitivity pneumonitis [9].

Avoiding or reducing exposure to inhaled soy allergens is crucial in order to prevent the adverse respiratory outcomes associated with soy exposure. Measuring soy aeroallergens levels is vital in order to assess the health risks and the efficacy of current exposure reduction measures, and to establish whether further additional measures are needed. A striking example is the city of Barcelona, Spain, where considerable efforts have been made to improve the control of soybean dust released during harbor activities. Control measures adopted include the assessment of the emission and dispersion of the allergen, the identification of allergen concentration levels compatible with health, the reduction of allergen emission levels, and the definition of complementary safety measures. Thanks to this strategy, this important economic activity is compatible with the strict requirements the city maintains for public health [10].

One of the measures adopted in Barcelona is daily soy monitoring in a district close to the harbor using a large-volume automated air sampler, as a means of determining the exposure in the population [11]. From autumn 1997 to May 2012 these measurements were performed by an enzyme immunoassay (EIA) inhibition assay using a serum pool with specific immunoglobulin E (IgE) antibodies from subjects allergic to soybean as a detector antibody [10,11]. Levels of soy aeroallergens should not exceed two threshold values: the Environmental High Threshold Limit Value (EH-TLV, set at 480 U/m^3), and the Environmental Low Threshold Limit Value (EL-TLV, set at 160 U/m^3). These threshold values were determined empirically based on the levels reached during epidemic and non-epidemic days [12]. When the EL-TLV is exceeded, soy facilities and meteorological conditions are assessed and a report is produced, and when the EH-TLV is reached an inspection of the soy facilities' processes is performed [12]. Since April 2012, monitoring of soy aeroallergens exposure in the city has been performed by a sandwich EIA described previously [13] and the EH-TLV and EL-TLV were redefined as 19 ng/m3 and 6 ng/m3 respectively. This method of environ- mental monitoring requires the use of a specialized laboratory staffed by skilled personnel.

The Barcelona experience shows that industrial soybean plants may be safely located near urban settings if strict control criteria are applied, including assessment of soy aeroallergens levels. The identification of similar problems in port cities with soybean harbor facilities in Spain [2,4,5], and in other countries [3] highlights the importance of the monitoring process. However, to date, no country has developed legislation to regulate this environmental risk [10], partly due to the lack of a widely available assay to monitor soy aeroallergens levels. Clearly, cost- efficient and less labor-intensive technological procedures for monitoring soy allergens levels are needed.

The aim of the present study is to describe the development and performance of a rapid immunochromatography assay for the detection of soy aeroallergens in environmental samples. The result of the test appears as a distinct color band in the ''test line'' of the soy strip. The test is simple and straightforward, the results are easily interpretable, and neither expensive equipment nor specific skills are required.

Materials and Methods

Preparation of immunoassay reagents

a) Preparation of Soy Hull Low Molecular Weight Extract (SHLMWE).

As previously described [13] the SHLMWE was obtained from soy hull with a chromatographic process (CM-cellulose and DEAE-cellulose chromatography). The SHLMWE contains low molecular weight allergens responsible for the asthma outbreaks in Barcelona [13]. The protein concentration in the extract was 1 mg/mL, as determined by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL, USA) following the manufacturer's instructions.

b) Production of anti-SHLMWE polyclonal antibodies and colloidal gold labeling.

Anti-SHLMWE polyclonal antibodies were produced as previously described [13]. The IgG fraction of the polyclonal antiserum was isolated on an immobilized Protein A–agarose column (Pierce), and then eluted onto an Excellulose column (Pierce) to desalt and exchange the buffer to Phosphate buffered saline (PBS). The protein concentration of the IgG fraction was 3.3 mg/mL, as determined by the Bicinchoninic acid (BCA) method (Pierce). No cross-reaction was observed with other legumes or cereals tested (data not shown). A portion of Anti-SHLMWE polyclonal antibody was colloidal gold labeled by BBI Solutions (British Biocell International, Cardiff, UK). Briefly, the antibody was conjugated to a 40 nm gold colloid using passive adsorption and was stable at least for 10 months. After incubation with rabbit Anti-SHLMWE

polyclonal antibodies, the conjugate was blocked with bovine serum albumin. The resulting conjugate was concentrated by ultrafiltration to give a final optical density (OD) (520 nm) of 10 and suspended in a 2 mM disodium tetraborate buffer pH 7.2, containing 0.095% sodium azide.

Rapid immunochromatographic test strip

a) Membrane blotting and assembly of the immunostrip.

The rabbit anti-SHLMWE (1200 ng/strip) capture antibody was applied to direct cast backed nitrocellulose nitrate membranes (Unisart CN95 19910 Sartorius Stedim Biotech, Goettingen, Germany) in a line format at 3 mm from the application end of the strip (Test line). At 6 mm, a goat anti- rabbit IgG antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) was bound at 600 ng/strip to provide a positive control (Control line). Membranes were dried for 1 h at 37⁰C, and assembled as follows: on an adhesive support an absorbent pad was layered and the membrane was placed overlapping it by 2 mm and cut into individual strips of 4 mm in width (see schematic diagram in figure 1A).

b) Rapid immunochromatographic test procedure.

After optimization, environmental samples were diluted 1:100 in sample diluent buffer (PBS,1% BSA, 0.5% Tween 20). Twenty microliters of diluted samples or SHLMWE standards were mixed 1:1 in a microtiter plate well with a gold labeled anti-SHLMWE antibody solution with an OD of 2. During brief gentle mixing, the gold-labeled antibody was allowed to react with soy allergens in the sample to form a complex. The test strip was dipped in the well and allergen-antibody complexes diffused across the nitrocellulose membrane and reacted with the specific anti-SHLMWE antibodies immobilized on the test line forming a red-purple line, or migrated further and reacted with the goat anti-rabbit IgG antibody in the control line. Figures 1B and 1C provide a schematic diagram of the rapid immunochromatographic test procedure.

Strip assay results were read by three independent observers 30 minutes after application of sample. A visible red-purple band had to appear at the control line for the test to be valid. After the liquid reagent flowed through the lines, the changes in the color on the test lines could be observed with the naked eye. Readers ranked sample results as negative, positive or double positive by comparing the color intensity of the test line with the test line of a 3-point standard curve: 1.56 ng/ml (2), 6.25 ng/ml (+) and 25 ng/ml (++) (Fig. 2). In parallel, test lines were scanned

with a flatbed scanner (Epson Expression 1600) and the line intensities were analysed by densitometry with ImageJ 1.45s (NIH, USA).

To determine the limit of detection of the strip assay, SHLMWE was used in a series of concentrations from 1.56 to 50 ng/mL and tested in triplicate (Fig. 3).
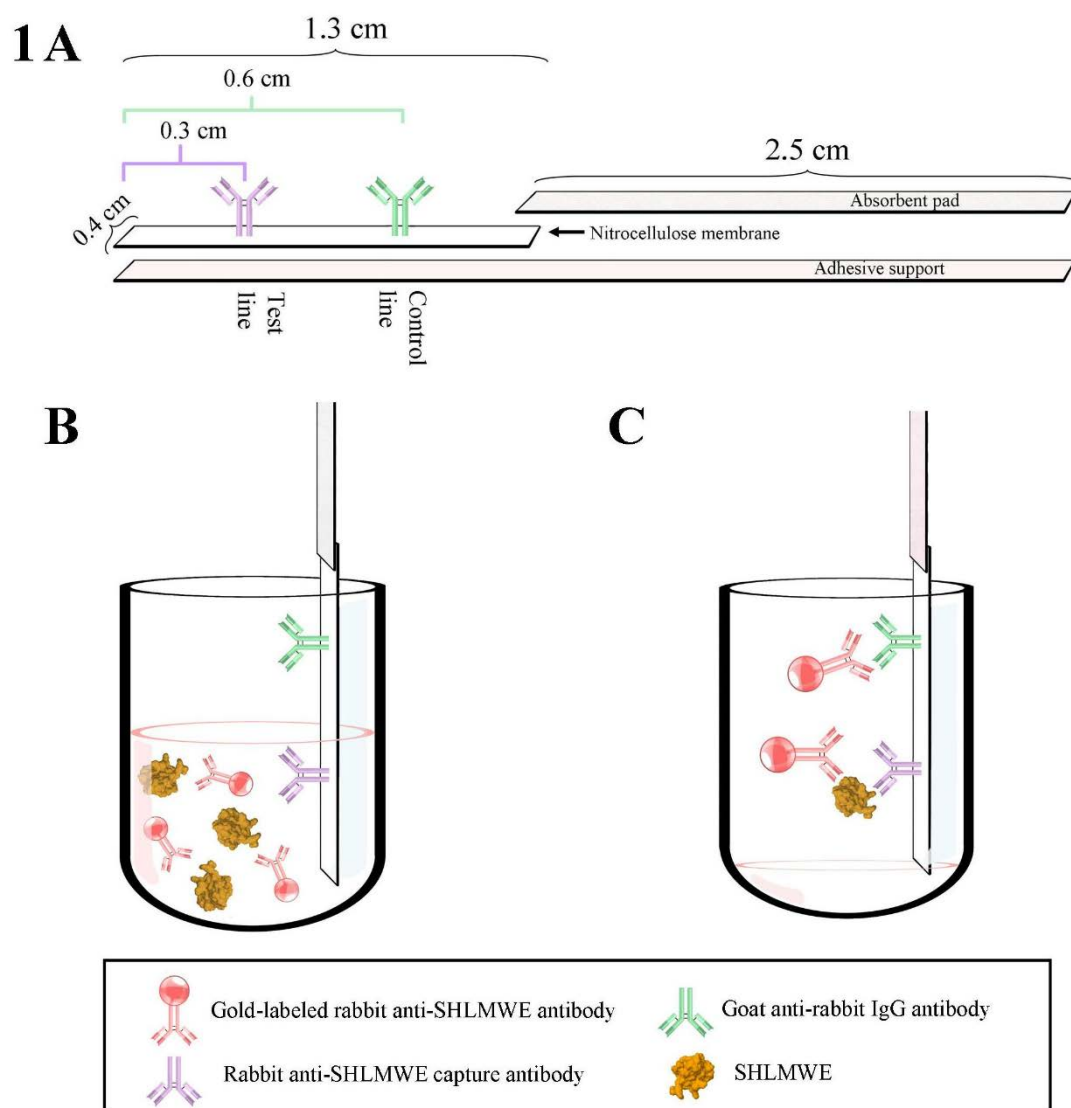

Figure 1. Schematic diagram of the strip assay: 1A) Assembly of the immunostrip; 1B) Dipping of the strip; 1C) Binding of gold labeled antibody and gold labeled antibody-SHLMWE complex to control and test line respectively.

Anti-SHLMWE sandwich EIA

Soy aeroallergens levels were measured by the anti-SHLMWE sandwich EIA described previously [13]. Results directly obtained by the EIA are expressed in nanograms per milliliters, referring to the protein content of the standard preparation. However, results of soy environmental levels

are expressed as nanograms per cubic meter (ng/m^3) of air. As previously mentioned, for surveillance of soy aeroallergens exposure in the city of Barcelona using the sandwich EIA, an EH-TLV of 19 ng/m3 and a EL-TLV of 6 ng/ m3 were defined, corresponding to 627 ng/ml and 198 ng/ml respectively for an average air volume of 165 m^3.
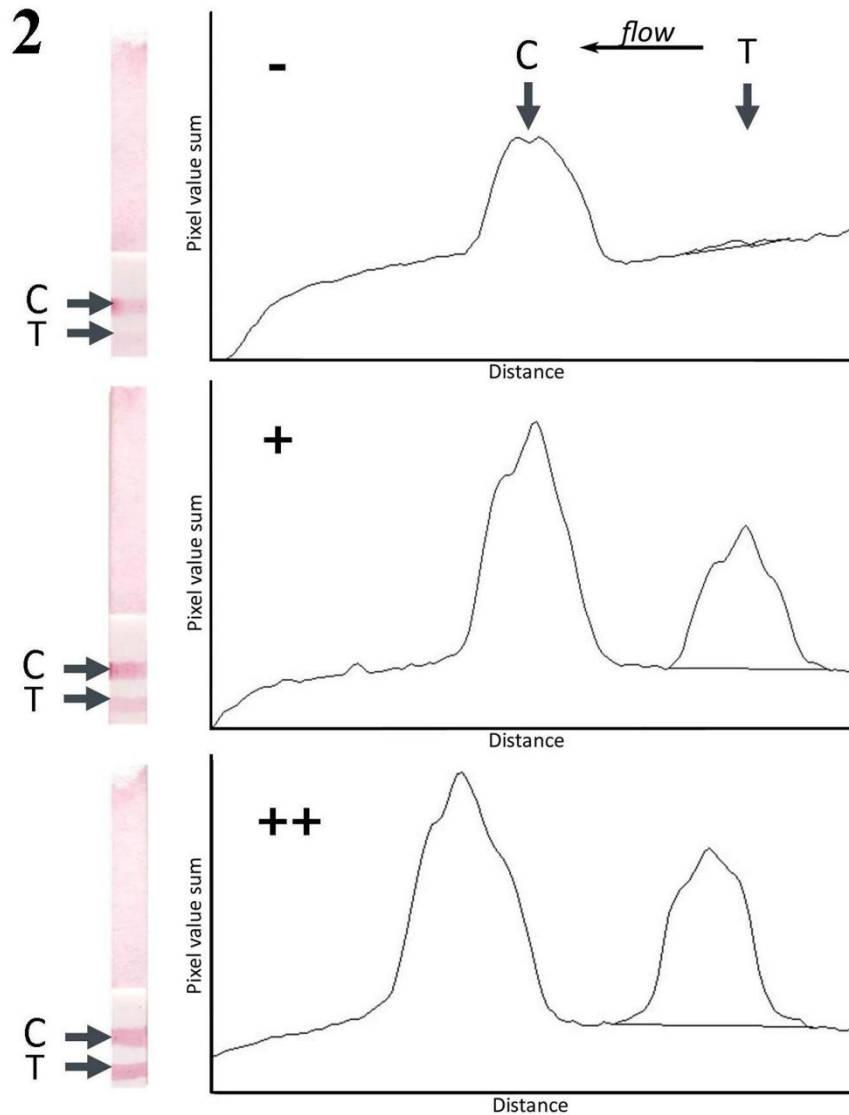

Figure 2. Three-point standard curve of SHLWE used as a source for comparison to rank the line seen on the strips as negative, positive or double positive. The figure shows scanned strips and density analysis of the strips.

Sample collection and analysis

One hundred nineteen samples routinely used for the daily monitoring of soy aeroallergens in the city were studied. Briefly, samples were collected from the air of the city of Barcelona with a large-volume automated air sampler (CAV-A/HF, MCV, SA, Barcelona, Spain) containing glass

microfiber filters with a 1mm pore size (Whatman International Ltd., UK), installed near the harbor and changed daily as previously described [12,14]. Dust was collected at a flow of 55 m3/h. Soy aeroallergens were extracted from a eighth of the filters in 5 mL PBS/0.2% BSA/ 0.1% Tween 20 (pH 7.4) overnight at 4°C. Filters were discarded and the eluates stored at 220 °C. All the eluates were analysed in parallel by the anti-SHLMWE sandwich EIA and the rapid immunochromatographic strip test.

Statistical analysis

The percentage of samples higher than the settled EH-TLV and the median and range of soy aeroallergens levels analysed by the EIA were calculated. A one-sample Kolmogorov-Smirnov test to assess normality was calculated for soy aeroallergens levels measured by EIA. The Kolmogorov-Smirnov test showed a non-normal distribution; therefore, the correlation between airborne soy allergen levels and strip assay density values were analysed using Spearman's rank correlation coefficient (r_s). Differences between density values of samples categorized by the EIA assay as higher as or lower than the EH-TLV were analysed by the Mann-Whitney test. Differences were considered significant at a p-value of ≤0.05.

The assay results were visually analysed by three independent observers. Agreement between the three evaluators was calculated using the Fleiss' Kappa test and percentage of agreed interpretations. Sensitivity, specificity and percentage of agreement of visual interpretation of the strips versus EIA results was calculated for each reader and differences between soy allergen levels of samples visually categorized as negative, positive or double positive were analysed by the Kruskal-Wallis test followed by Dunn's multiple comparison test.

Statistical analyses were performed using GraphPad Prism version 4.01 for Windows, (GraphPad Software, San Diego, California, USA) and a freely-available Microsoft Excel spread- sheet that estimates the generalized kappa statistic based on equations presented in Fleiss et al. [15].

Results

Lower and upper limit of detection

The rapid test detected a range of concentrations from 6.25 to 25 ng/mL (Fig. 3). The lower limit of detection (LOD) of the strip assay was determined as the minimum amount of SHLMWE producing a clearly visible red-purple band at the test line (6.25 ng/ml, Fig. 3). The upper LOD of 25 ng/ml was determined as the maximum amount of SHLMWE producing a more intense red-purple band than the preceding concentration at the test line (Fig. 3).

Sample dilution

In the EIA, the median (range) (ng/ml) was 274.5 (1.18–1848), 22 (18.5%) samples being higher than the settled EH-TLV of 627 ng/ml. In the strip assay, samples were diluted 1/100 to match the lower LOD of the strip assay of 6.25 ng/ml with the environmental settled high threshold value. Thus, a positive or double positive result in the strip assay would indicate that the sample has a soy aeroallergens concentration equal to or higher than the settled EH-TLV.
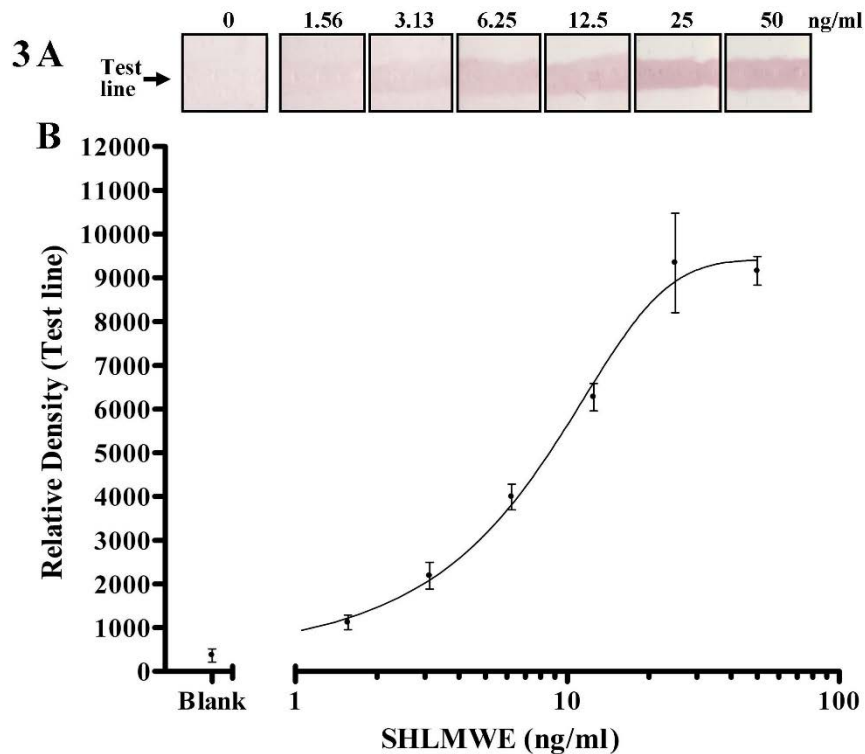

Figure 3. A typical strip assay standard curve to determine the limit of detection of the assay: 3A) Scanned strips at test line level; 3B) Graph of densitometry results (means±SEM) at the indicated concentrations of SHLMWE using a four parameter logistic curve fit.

Inter-reader agreement and comparison between the EIA and the strip assay

The three observers agreed on the interpretation of 71.4% of the samples, and in all the samples at least two readers agreed. Ranking samples as negative, positive or double positive, agreement in strip assay interpretations between evaluators was substantial, with a kappa index of 0.63 (CI 0.544–0.715). The kappa index was higher (0.701, CI 0.598–0.805) when positive and double positive results were considered as one category. In addition, strips were scanned and the color intensity of the test line was analysed by densitometry. The values calculated by ImageJ are essentially arbitrary and only have meaning within the context of the set of peaks that are

analysed together. Thus, all samples were analysed at the same time. A strong correlation was observed between the densitometry results of strip assay and EIA determinations (rs= 0.887 [CI 0.839–0.921]; p<0.0001) (Fig 4A).
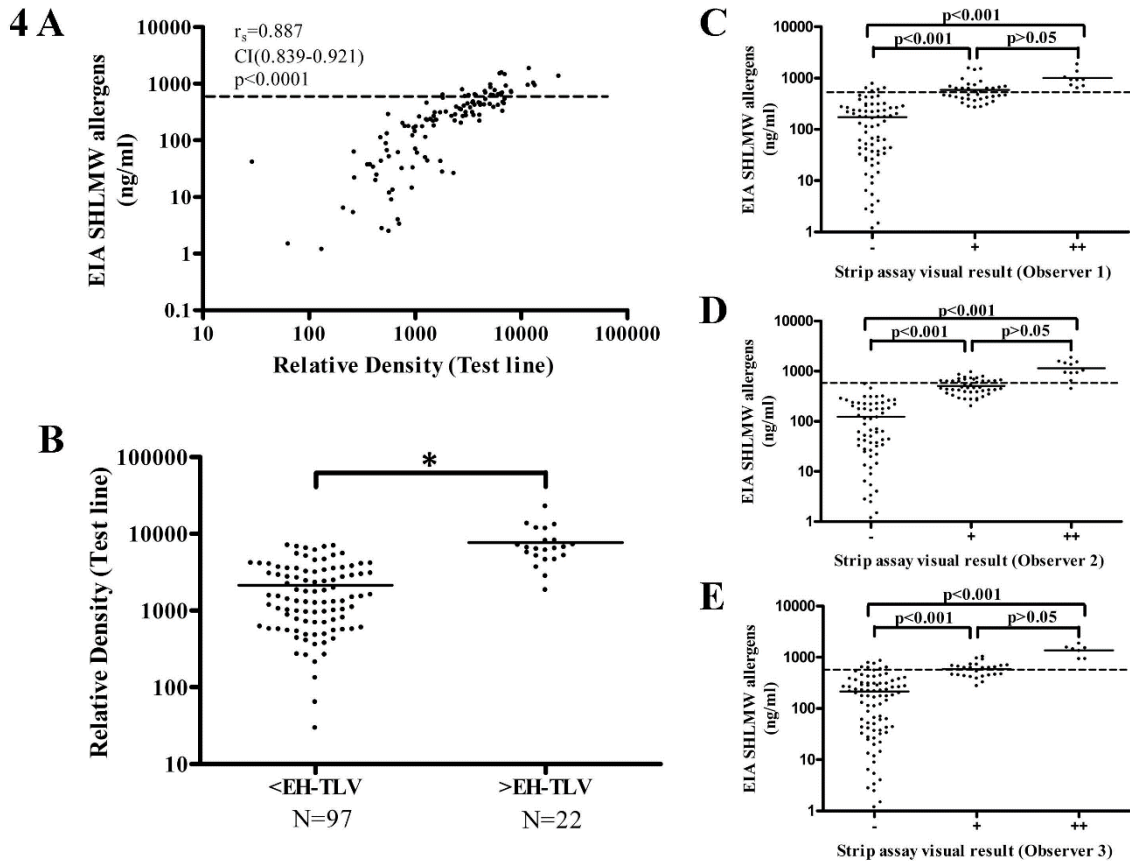

Figure 4. Densitometry results of strip assay and EIA determinations and EIA results by strip assay visual interpretation category. The dashed lines indicate the EH-TLV of 627 ng/ml. 4A) Scatter plot showing the correlation between SHLMW allergens concentrations and density values of strip assay; 4B) Densitometry results by EIA result category (higher or lower than the EH-TLV).* p<0.0001; EIA results by strip assay visual interpretation category: 4C) Observer 1; 4D) Observer 2; 4E) Observer 3.

Besides, intensity of the line analysed by densitometry was significantly higher (p<0.0001) in the samples categorized by the EIA assay as higher than the EH-TLV (Fig 4B). Visual interpretation of the strip assay also had a good concordance with EIA results, with sensitivity ranging from 77.3 to 100 and specificity from 65 to 83.5 depending on the observer (Table 1). The percentages of agreement were 75.63%, 71.43% and 82.35% for observers 1, 2 and 3 respectively. Samples visually categorized as negative by strip assay had significantly lower soy allergen levels by sandwich EIA and samples categorized as double positive had significantly higher levels than those categorized as negative or positive by all readers (Figures 4C, 4D and 4E). The three readers visually detected SHLMW allergens in a concentration range from 266 to 1848 ng/mL, 200.4 to 1848 ng/mL and 274.5 to 1848 ng/ mL.

Table 1. Sensitivity and specificity of strip assay estimated from the visual interpretation of three independent observers.

	Sensitivity; %(CI)	Specificity; %(CI)
Observer 1	86.4 (65.1–97.1)	73.2 (63.2–81.7)
Observer 2	100 (84.6–100)	65 (54.6–74.4)
Observer 3	77.3 (54.6–92.2)	83.5 (74.6–90.3)

Discussion

Surveillance of soy aeroallergens levels in the harbor areas of cities where soybean is loaded or unloaded and/or processed is crucial in order to prevent asthma outbreaks [10,11,16]. Thus, there is a clear need for broadly available, fast, easy-to-use devices for detection of high levels of soy aeroallergens in the air of port cities. To address this need, we developed a strip assay able to measure soy aeroallergens at levels as low as 6.25 ng/ml.

The LOD of the strip assay is quite similar to those described for other immunochromatographic assays developed for allergens from house dust mites with a sensitivity of 1–2 ng/ml [17], for fungal alpha-amylase, in which the LOD was 1–10 ng/ml [18] or for rodents, in which the LOD was 31 pg/ml for both mouse and rat urinary allergens, though a LOD of 4 ng/ml for rat urinary allergens has been indicated as more realistic for real field samples [19].

Surprisingly, the readers detected soy aeroallergens concentrations below the LOD of the assay determined using a buffer system. The actual LOD of the assay with field samples ranged from 2 to 2.7 ng/ml depending on the reader. This observation has also been reported in other studies. For example, in some samples Tsay et al. [17] graded line intensity as medium or high although mite group 2 levels analysed by ELISA were below the theoretical sensitivity of the rapid test. The same occurred in the Bogdanovic et al. [18] study, where some samples with a-amylase levels below the sensitivity of the rapid test were classified as positive.

In a buffer system with a concentration of SHLMW allergens above 1,600 ng/mL the assay signal, i.e., the gold colloid line intensity, decreased as a result of the prozone or high-dose hook effect (results not shown). This effect appears in one-step immunoassays where sample and labeled antibody are added simultaneously and the antibodies are saturated by a very high concentration of sample antigen binding to all available sites and preventing the sandwich-

formation. Thus, the hook effect causes false-negative results [20]. To detect the hook effect, samples are often tested undiluted and after dilution. Unfortunately, this approach increases labor and reagent costs for assays that only rarely encounter extremely high antigen concentrations. Fortunately this effect will not have any significance for the control of environmental samples near the harbor of Barcelona as in recent years the highest concentration reached was 1,848 ng/ml. This is also the case of the samples analysed for this study; the highest SHLMW allergen concentration was 1,848 ng/ml, and it was tested at 1:100 dilution. Thus, in this study we did not observe the hook effect. However, for each new sampling site without previous recordings of soy aeroallergens levels, we recommend performing an initial evaluation with the sandwich EIA assay and the subsequent follow up evaluations with the strip assay at an adequate dilution based on the EIA results.

Visual interpretation of the strip assay is inherently subjective as it depends, among other factors, on the reader's perception of color. Thus, it is important to assess inter-rater agreement. In this study agreement between readers was substantial according to the classification recommended by Landis and Koch [21]. Despite the limitations of visual interpretation, the results of the rapid test presented high agreement with the EIA results, and strip assay visual interpretation showed a high sensitivity and specificity. Furthermore, line intensity, analysed by densitometry (an objective measurement) of samples categorized as negative was significantly lower than the line density of positive and double positive samples (data not shown).

Environmental monitoring to assess airborne allergens is a time- consuming process that commonly includes three steps: sampling aeroallergens on a filter, elution of the allergens, and determination of allergen levels by an allergen-specific EIA. The strip assay replaces the EIA, speeding up the last step of the process. The strip assay's advantage, its speed, is not decisive at present for airborne samples as the time needed to obtain the sample is still too long. In fact, this speed will be more useful for industrial hygiene monitoring than for environmental monitoring, as some of the sampling procedures widely used in occupational hygiene take only a short time (for example, surface wipe sampling, dust sampling or bulk sampling).

In conclusion, the strip assay described is rapid, simple, sensitive and does not require expensive equipment or specific skills. Thanks to its simplicity, this method has considerable potential in the field of environmental monitoring for screening soy aeroallergens levels in port cities with soybean harbor facilities where allergen levels are not currently measured.

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CHAPTER 2. Effects of diesel exhaust particle exposure on a murine model of asthma due to soybean

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Abstract

Background: Exposure to soybean allergens has been linked to asthma outbreaks. Exposure to diesel exhaust particles (DEP) has been associated with an increase in the risk of asthma and asthma exacerbation; however, in both cases the underlying mechanisms remain poorly understood, as does the possible interaction between the two entities.

Objective: To investigate how the combination of soybean allergens and DEP can affect the induction or exacerbation of asthma in a murine model.

Methods: BALB/c mice received intranasal instillations of saline, 3 or 5 mg protein/ml soybean hull extract (SHE), or a combination of one of these three solutions with DEP. Airway hyperresponsiveness (AHR), pulmonary inflammation in bronchoalveolar lavage, total serum immunoglobulin E and histological studies were assessed.

Results: A 5 mg protein/ml SHE solution was able by itself to enhance AHR (p=0.0033), increase eosinophilic inflammation (p=0.0003), increase levels of IL-4, IL-5, IL-13, IL-17A, IL-17F and CCL20, and reduce levels of IFN-γ. The combination of 5 mg protein/ml SHE with DEP also produced an increase in AHR and eosinophilic inflammation, but presented a slightly different cytokine profile with higher levels of Th17-related cytokines. However, while the 3 mg protein/ml SHE solution did not induce asthma, co-exposure with DEP resulted in a markedly enhanced AHR (p=0.002) and eosinophilic inflammation (p=0.004), with increased levels of IL-5, IL-17F and CCL20 and decreased levels of IFN-γ.

Conclusions & Clinical Relevance: The combination of soybean allergens and DEP is capable of triggering an asthmatic response through a Th17-related mechanism when the soybean allergen concentration is too low to promote a response by itself. DEP monitoring may be a useful addition to allergen monitoring in order to prevent new asthma outbreaks**.**

Introduction

Several asthma epidemics due to soybean dust inhalation have been described in Spain and elsewhere [1–3]. The first reported epidemics occurred in Barcelona where soybean dust released during the unloading of these legumes from ships to silos caused asthma epidemics among residents of the neighborhoods closest to the harbor [1,2,4]. In this city, the control measures adopted to avoid these outbreaks included the reduction of allergen emission levels by the installation of filter bags, the establishment of threshold values compatible with health, and the daily assessment of the emission and dispersion of the allergen to keep levels below these thresholds [5]. However, in the determination of the threshold values the possible effect of pollution in combination with soybean allergens was not taken into account.

It is known that airborne particulate matter (PM), a major component of air pollution, may have direct effects on the pulmonary system, including the induction of inflammatory responses. Airborne PM has been related to an acute increase in the incidence of asthma in urban areas, particularly the fine and ultrafine particles emitted by vehicular traffic [6,7]. Diesel exhaust particles (DEP), the main contributor to traffic PM [6], have a potential enhancing effect on responses to inhaled allergen exposure, and may also induce sensitization to neoallergens in human and animal models [8]. Several mechanisms through which DEP could enhance sensitization to aeroallergens have been proposed [6,8,9]. Animal model studies suggest that exposure to DEP provokes allergic inflammation with Th2 and Th17 phenotypic differentiation, and that, in this differentiation, a specific role is played by environmentally persistent free radicals and polycyclic aromatic hydrocarbon fractions [7,8,10]. Attempts to explain the participation of DEP in the pathogenesis of asthma have suggested a role for oxidative stress and immune dysregulation, but at present the mechanisms involved remain poorly understood.

The experimental modeling of allergic airway inflammation, particularly in murine models, has made a significant contribution to our understanding of asthma pathogenesis. Traditional protocols used ovalbumin combined with a potent adjuvant, but this approach results in an acute asthma-like phenotype that does not model the etiology and natural history of human asthma [11,12]. In order to achieve a better simulation of the chronic nature of human asthma, longer duration models have been developed that avoid the use of adjuvants and include more physiologically relevant antigens, like house dust mite (HDM) [11–13]. The repeated exposure of the airway to low levels of allergens via inhalation or intranasal instillation has been shown

to perform better than acute models with regard to the reproduction of some of the hallmarks of human asthma, such as allergen-dependent sensitization, a Th2-dependent allergic inflammation characterized by eosinophilic influx, sustained airway hyperresponsiveness (AHR), and even airway remodeling [11,13–15].

The aim of the present study was to develop and standardize a novel murine asthma model with repeated exposure of the airway to low levels of soybean, a physiologically potent and relevant outdoor aeroallergen, and to assess the effect of DEP on this murine model with regard to the development of AHR, lung inflammation, and immunological response.

Material and Methods

Animals

Female BALB/c mice (20 g, 6 weeks old) were obtained from ENVIGO (Udine, Italy). Mice were housed in filter top cages in a conventional animal house with 12 h dark/light cycles and received slightly acidified water and pelleted food (Teklad 2014, Harlan Laboratories, IN, USA) ad libitum. All experimental procedures were approved by the Ethical Committee for Animal Experiments of Hospital Universitari Vall d'Hebron.

Soy hull extract

Soy hull extract (SHE) was obtained as previously described [16]. In brief, soybean hull proteins were extracted in 0.1 M NH4HCO3 buffer. The eluate was filtered, dialyzed in a 3.5 kDa cut-off membrane and lyophilized. The protein concentration in the extract was 33% as determined by the bicinchoninic acid (BCA) method (Pierce Chemical Co., Rockford, IL, USA) following the manufacturer's instructions. All the experiments were carried out with the same SHE batch.

Diesel exhaust particles

Diesel exhaust particles (Standard Reference Material (SRM) 2975) were purchased from National Institute of Standards Technology (NIST) (Gaithersburg, MD, USA).The reported mean diameter of these particles was 11.2±0.1μm by area distribution, and the surface area, as determined by nitrogen gas adsorption, was 0.538±0.006m3/cm^2.

Experimental design

Six experimental groups were created. Soy hull extract was resuspended in sterile saline (0.9% NaCl, Fresenius Kabi, Barcelona, Spain) at two different concentrations of 3 and 5 mg protein/ml. Mice were exposed during five consecutive days over three weeks, under light anesthesia with isoflurane (Forane, Abbott Laboratories, Madrid, Spain), to either 20µl of saline, 20µl of SHE at 3 mg protein/ml concentration (Soy3), 20µl of SHE at 5 mg protein/ml concentration (Soy5), or a combination of one of these three solutions with 150 µg of DEP depending on the experimental group. In the groups receiving DEP, the supplement was administered three days a week for three weeks. A chart of the intranasal instillations of the six groups is shown in fig 1. Each group comprised eight mice.
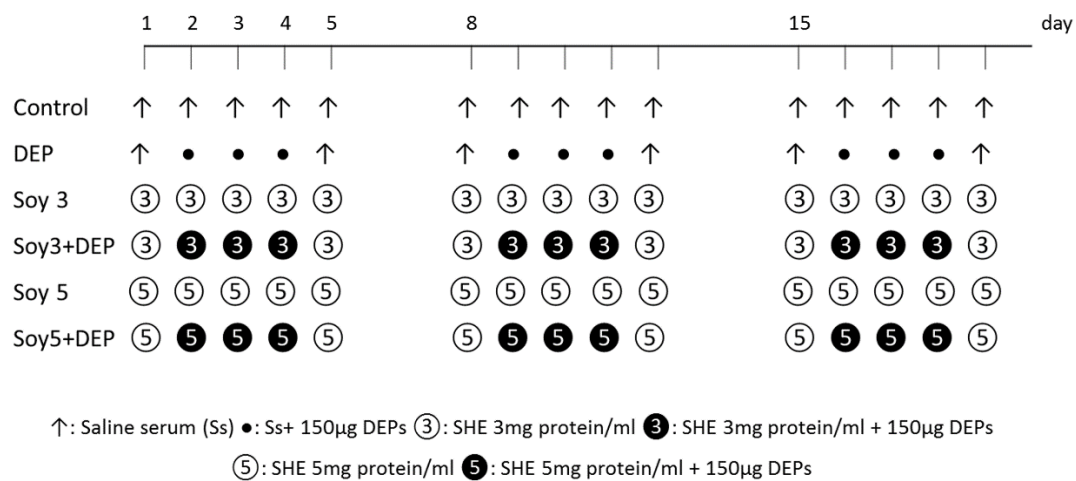

↑: Saline serum (Ss)　●: Ss+ 150µg DEPs　③: SHE 3mg protein/ml　❸: SHE 3mg protein/ml + 150µg DEPs
⑤: SHE 5mg protein/ml　❺: SHE 5mg protein/ml + 150µg DEPs

Fig 1. Schematic diagram of the experimental protocol and experimental groups. Six experimental groups were created. Female BALB/c mice received intranasal instillations of saline, 3 or 5 mg protein/ml soybean hull extract (SHE) on five consecutive days for three weeks. Solutions were administered three days a week, either alone or in combination with 150 µg of DEPs.

Airway hyperresponsiveness

One hour after the last intranasal instillation, reactivity to methacholine was assessed invasively using a forced oscillation technique with the FlexiVent system (Flexivent, SCIREQ; Montreal, Canada). Mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (70 mg/kg) (Nembutal, Abbot Laboratories, Spain). The trachea was exposed and, after tracheotomy, was connected to a computer-controlled ventilator. Airway resistance (R) was

measured with a ''snapshot'' protocol and plotted against methacholine concentration (from 0 to 20 mg/ml), and the area under the curve (AUC) was calculated [17].

Total serum immunoglobulin E (IgE)

After assessment of the methacholine test, blood was taken by cardiac puncture. Blood was centrifuged and serum samples were obtained and stored at -80ºC for further analyses. Total serum IgE was measured using the Mouse ELISA IgE kit (Bethyl Laboratories, Inc., Montgomery, USA). Measurements were performed according to the manufacturer's instructions.

Bronchoalveolar lavage: cell count

After blood sampling, bronchoalveolar lavage (BAL) was performed. The lungs were lavaged three times with 0.7 ml of sterile saline (0.9% NaCl). The first fraction recovered was stored separately and the following two fractions were pooled. The volume recovered was recorded. Total cells were counted using a hemocytometer and the BAL fluid was centrifuged (1000 g, 10 minutes, 4°C). The supernatant was frozen (-80°C) until further analyses. For differential cell counts, 100 ⏄l of the resuspended cells (600000 cells/ml; 1400 g, 6 minutes) were spun (Cytospin 3, Shandon Thermo Scientific, Runcorn, Cheshire, UK) onto microscope slides, air-dried and stained with May-Grünwald for 5 min (QCA; Tarragona, Spain) and Giemsa for 15 min (Merck, Darmstadt, Germany). Cell counts were performed in 400 cells from each sample to determine the number of macrophages, eosinophils, neutrophils and lymphocytes.

Bronchoalveolar lavage: cytokines and chemokine

Levels of interferon-gamma (IFN-γ), interleukins-4 (IL-4), IL-5, IL-10, IL-13, IL-17A, IL-17F, IL-33, IL-31, IL-21, IL-22, IL-23 and the chemokine CCL20 were measured in the first fraction of undiluted BAL fluid by a mouse cytokine magnetic bead panel according to the manufacturer's instructions (Bio-Plex X plex Custom Mouse Cytokine Assay, Bio-Rad Laboratories S.A.; Madrid, Spain).

Lung pathology

After BAL, lungs were instilled with formaldehyde 3.7–4.0% until all lobes were deemed to be fully inflated by visual inspection. The tissues were formalin-fixed, paraffin embedded and cut in sections that were placed on slides. Evaluation of lung injury on slides stained by hematoxylin and eosin (H&E) was performed by an experienced pathologist in a blinded manner. A semi-quantitative scoring system was used to grade the severity and extent of inflammation on stained sections. Interstitial inflammatory infiltrate, peribronchial lymphoid activation and

perivascular infiltrate were graded: 0 (normal) = absence of inflammatory cells; 1 (mild) =1–2 layers of inflammatory cells; 2 (moderate) = 3–5 layers; 3 (severe) = more than 5 layers.

Data analysis

All data are presented as medians with interquartile range, and were analyzed using the Kruskal-Wallis test with Dunn's multiple comparison posttest (Graphpad Prism 6.0, Graphpad Software Inc, San Diego, USA). A level of $p \leq 0.05$ (two-tailed) was considered significant.

Results

Airway hyperresponsiveness to methacholine

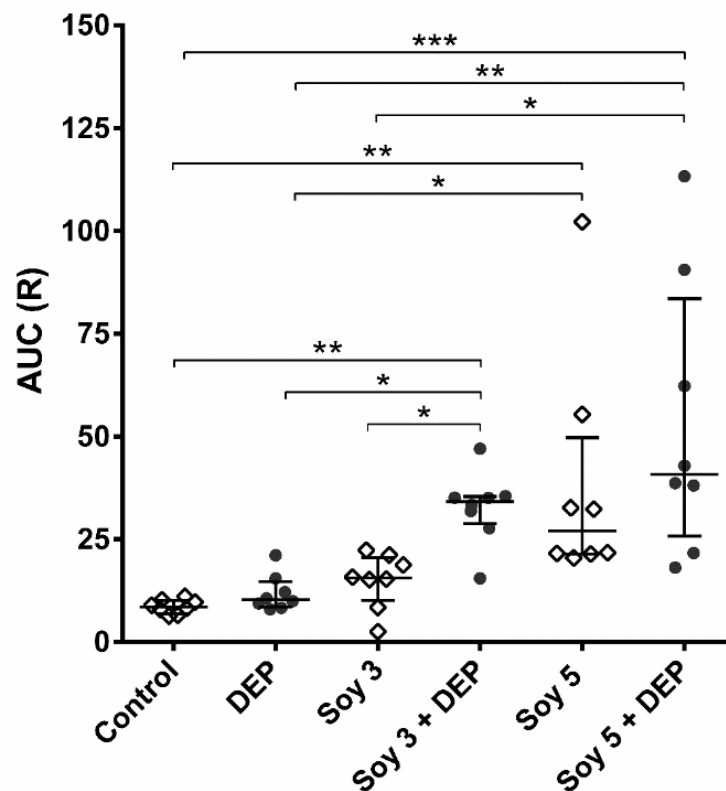To assess AHR to methacholine, AUC was calculated for each individual mouse in each experimental group (Fig 2).

Fig 2. Airway hyperresponsiveness (AHR) to methacholine expressed as individual values of area under the curve (AUC) of resistance (R). Experimental groups are the same as in Fig 1. Median and interquartile range of individual values of AUC measured one hour after last intranasal instillation by the forced oscillation technique to increasing concentrations of methacholine. *p<0.05, **p<0.01; ***p<0.001.

Exposure to DEP alone or to Soy3 did not induce any changes in AHR when compared with the control group; however, the combination of Soy3+DEP resulted in a markedly enhanced AHR compared to control, DEP and Soy3 groups (p=0.002, p=0.0116 and p=0.048 respectively). Soy5 by itself significantly enhanced AHR compared with control and DEP (p=0.0033 and p=0.0277 respectively), while Soy5+DEP significantly increased AHR compared with control, DEP and also Soy3 (p=0.0001, p=0.0041 and p=0.04 respectively). No significant differences were found between Soy3+DEP, Soy5 and Soy5+DEP.

Total serum immunoglobulin E

Total serum IgE in the six experimental groups are shown in Fig 3. Exposure to DEP, Soy3 or Soy3+DEP did not increase serum IgE levels, while Soy5 and Soy5+DEP groups showed significant increases in total serum IgE compared to control, DEP, Soy3 and also Soy3+DEP groups (Soy5: p=0.0043, p=0.0043 and p=0.043 respectively; Soy5+DEP: p=0.0043, p=0.0043 and p=0.043 respectively).
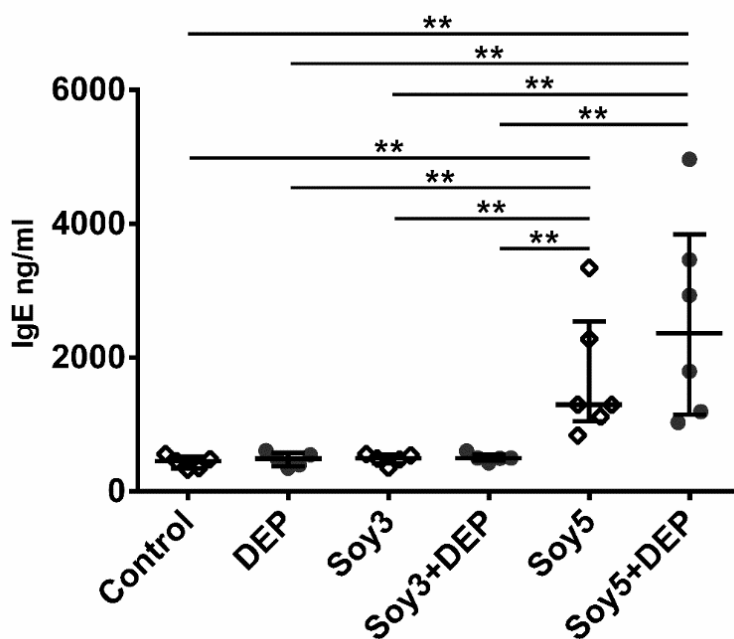

Fig 3. Total serum immunoglobulin (Ig)-E. Experimental groups are the same as in Fig 1. Median and interquartile range of total serum IgE. **p<0.01.

Bronchoalveolar lavage: cell count

Fig 4 shows the inflammatory cells in BAL. No differences were found in the total cell count when mice were exposed to DEP alone or Soy3 in comparison with control group. However, a significant increase in total cell count was found between Soy3+DEP and the control group (p=0.0302). Soy5 alone and Soy5+DEP also showed increased total cell counts compared with control, DEP and Soy3 groups (Soy5: p=0.0006, p=0.0054 and p=0.0252; Soy5+DEP p<0.0001; p=0.0008 and p=0.0044 respectively) (Fig 4A). Cell counts in the BAL showed differences in eosinophils and neutrophils between groups (Fig 4B and 4C). Similarly to total cell count, the DEP and Soy3 groups did not show any changes, but the Soy3+DEP showed increased numbers of eosinophils (p=0.0004, p=0.0015 and p=0.04 respectively) and neutrophils (p=0.002 and p=0.0475 and p=0.014 respectively) compared with control, DEP and Soy3 groups. Soy5 and Soy5+DEP groups also showed a significant increase in eosinophils (p=0.0003, p=0.0001 respectively), and neutrophils (p=0.008, p=0.0001) when compared both to controls, and to DEP (Eosinophils: p=0.0007, p=0.0003; Neutrophils p=0.0486, p=0.0066) and Soy3 (Eosinophils: p=0.0106, p=0.0047; Neutrophils p=0.048, p=0.0013).
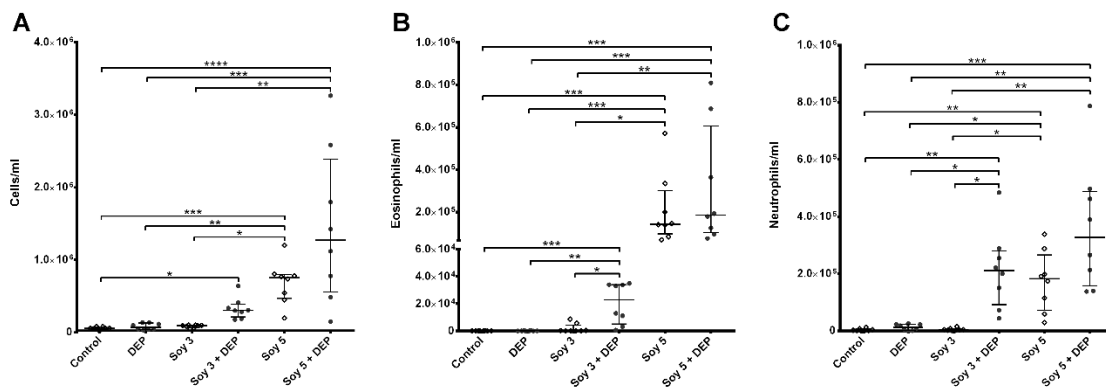

Fig 4. Number of total cells, eosinophils and neutrophil in one milliliter of BAL. Experimental groups are the same as in Fig 1. Median and interquartile range of total cell count (A), eosinophils (B) and neutrophils (C). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Bronchoalveolar lavage: cytokines and chemokine

Measurement of the cytokines previously mentioned in BAL fluid revealed that the levels of IL-4 were increased in Soy5 and Soy5+DEP in comparison with control, DEP and Soy3 groups (Soy5:

p=0.001, p=0.001, p=0.001; Soy5+DEP: p=0.0022, p=0.0022, p=0.0022 respectively), as well as compared with Soy3+DEP in the case of Soy5 (p=0.0375) (Fig 5A).
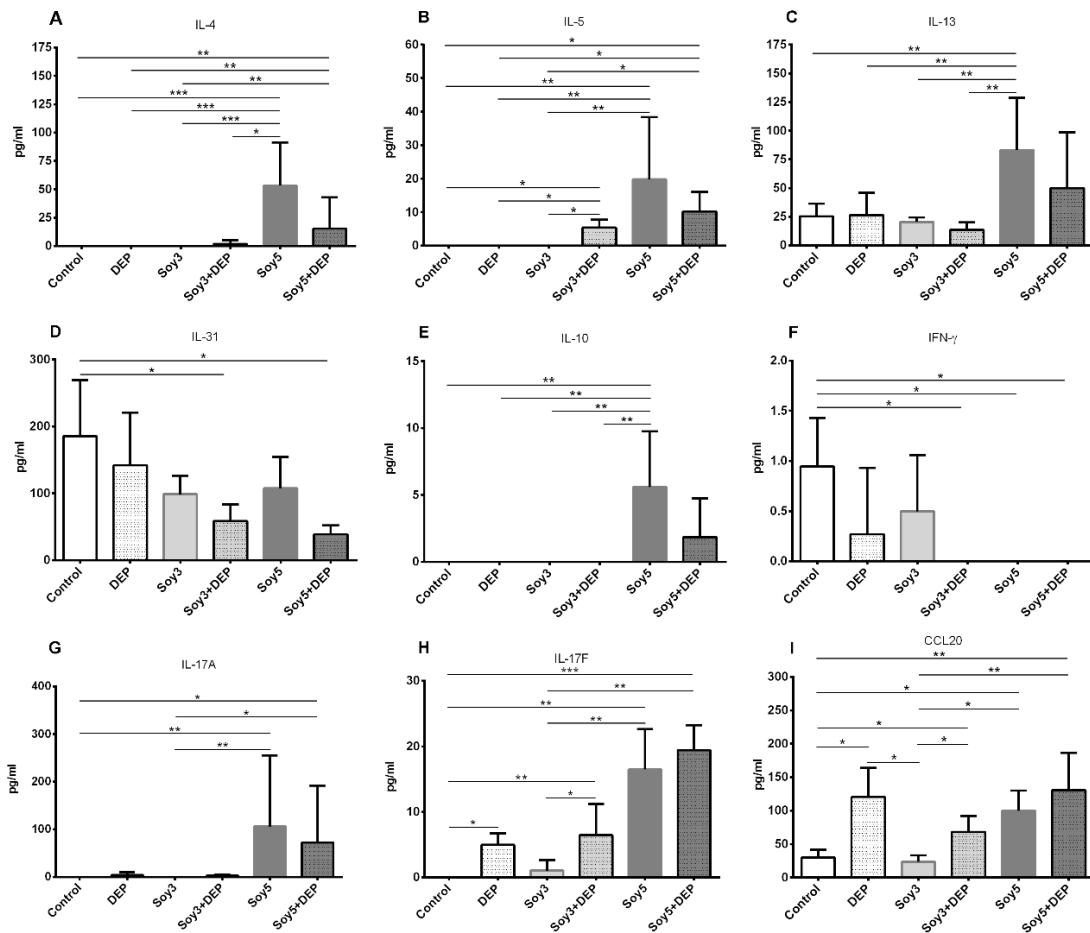

Fig 5. Levels of interleukin (IL)-4, IL-5, IL-13, IL-31, IL-10, IFN-γ, IL-17A, IL-17F and chemokine CCL20 in BAL fluid supernatant. Experimental groups are the same as in Fig 1. Median and interquartile range of(IL)-4 (A), IL-5 (B), IL-13 (C), IL-31 (D), IL-10 (E), IFN-γ (F), IL-17A (G), IL-17F (H) and chemokine CCL20 (I).

Levels of IL-5 (Fig 5B) showed an increase in Soy5 and Soy5+DEP, and also in Soy3+DEP (Soy5: p=0.0082, p=0.0082, p=0.0082; Soy5+DEP: p=0.0198, p=0.0198, p=0.0198; Soy3+DEP: p=0.0495, p=0.0495, p=0.0495 respectively) when compared with control, DEP and Soy3. We only found significant changes in the levels of IL-13 (Fig 5C) and IL-10 (Fig 5E) in Soy5 group, where we found significant increases compared with controls, DEP, Soy3 and Soy3+DEP (p=0.0022, p=0.0087, p=0.0022, p=0.0022 respectively). The levels of IL-31 (Fig 5D) and IFN-γ (Fig 5F) showed decreases in both Soy3+DEP and Soy5+DEP groups and also, in the case of IFN-γ, in Soy3+DEP compared to controls (p=0.0339, p=0.013 respectively). IL-17A showed significant increases only in Soy5 and Soy5+DEP groups in comparison with controls and Soy3 (Soy5: p=0.0029, p=0.0029; Soy5+DEP: p=0.015, p=0.015 respectively) (Fig 5E). In contrast to IL-17A levels, both IL-17F (Fig

5F) and CCL20 (Fig 5G) levels were significantly increased in Soy5 and Soy5+DEP in comparison with controls but also in Soy3+DEP and DEP (Soy5: p=0.0013, p=0.0389, Soy5+DEP: p=0.0003, p=0.0084; Soy3+DEP: p=0.0036, p=0.0358; DEP: p=0.0138, p=0.0168 respectively). Furthermore Soy5 and Soy5+DEP and Soy3+DEP showed a significant increase in IL-17F (Soy5: p=0.0066; Soy5+DEP: p=0.0016; Soy3+DEP: p=0.0311 respectively) in comparison with Soy3, while levels of CCL20 were significantly higher in Soy5 and Soy5+DEP, Soy3+DEP and also DEP (Soy5: p=0.0246; Soy5+DEP: p=0.005; Soy3+DEP: p=0.02; DEP: p=0.0103 respectively) compared to Soy3. Levels of IL-21, IL-22, IL-23, and IL-33 did not show statistical differences between groups.

Lung histopathology

The blinded histopathological examination of lung tissue sections revealed a mild to moderate (grade 1–2) interstitial, perivascular and peribronchiolar inflammatory infiltrate in the Soy5 (Fig 6E) and Soy5+DEP groups (Fig 6F). While no inflammatory infiltrate was observed in Soy3 group (Fig 6C), Soy3+DEP showed a mild to moderate (grade 1–2) interstitial inflammatory infiltrate, and a mild (grade 1) perivascular and peribronchiolar inflammatory infiltrate (Fig 6D). No inflammatory infiltrate was observed in the control group (Fig 6A), while the DEP group (Fig 6B) showed a moderate interstitial inflammatory infiltrate.
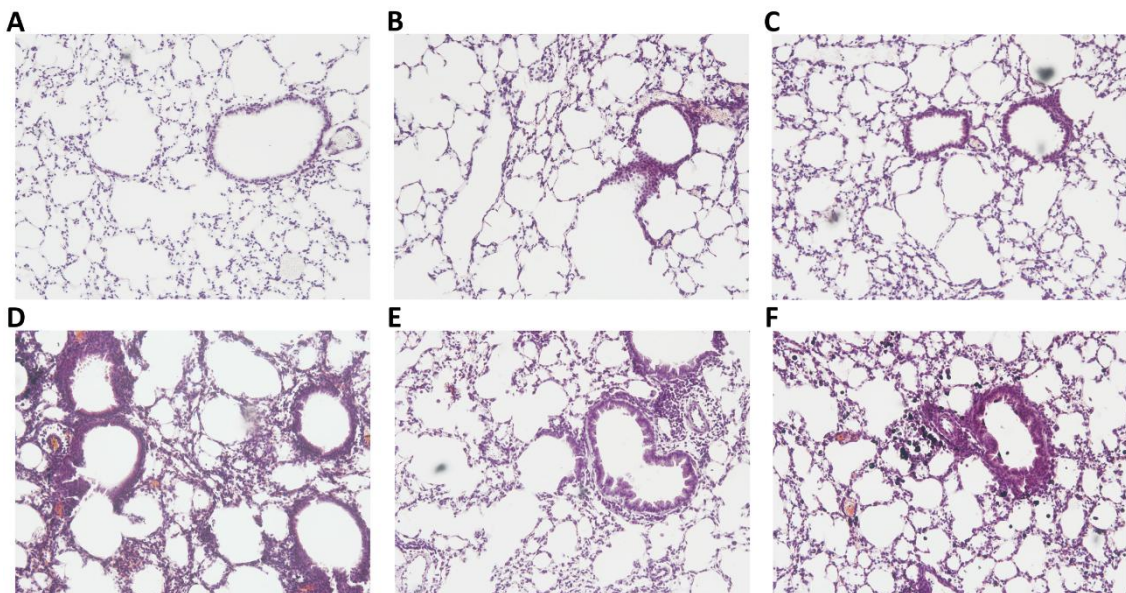

Fig 6. Lung histopathology. Representative images of hematoxylin and eosin stained histological lung sections. Slides photographed at 100x magnification. Experimental groups are the same as in Fig 1. (A) Control, (B) DEP, (C) Soy3, (D) Soy3+DEP, (E) Soy5, (F) Soy5+DEP.

Discussion

In this study, we assessed the effect of air pollution, in particular DEP exposure, on a new soybean asthma murine model. Our results show that the repeated intranasal administration of soybean, in the form of a sufficient concentration of SHE, triggers a severe asthmatic response with increased AHR, a robust inflammatory response in BAL, and increased levels of IgE, Th2-related cytokines such as IL-4 and IL-13, and also of Th17 cytokines. The results also demonstrate that when the concentration of SHE is too low to produce an asthmatic response by itself, DEP can synergize with SHE to produce a response. However, the immunological mechanism underlying the effect of the combination of lower SHE concentrations (3 mg/ml) with DEP seems to be Th17-related.

Asthma caused by soybean is a relatively well known entity, but in spite of its significance as a relevant outdoor allergen, little or no experimental work has been carried out to date to understand its airway immune-inflammatory responses. Nor, to our knowledge, has the interaction between soybean aeroallergens and DEP been adequately assessed: it has been studied mainly in ovalbumin (OVA) and house dust mite (HDM) murine models, but OVA is not a physiologically relevant allergen, and HDM is mainly an indoor allergen.

Our soybean asthma model showed that exposure to soybean aeroallergens in a sufficient concentration (5 mg/ml) leads to increased AHR, pulmonary inflammation with increases in both eosinophil and neutrophil counts and also higher levels of IgE. Nonetheless, our results also show that this response involves not just a Th2-driven inflammation with IL-4, IL-5, IL-13 and IL-10, but a more complex mechanism as well which, besides increases in Th2-related cytokines, also presents increased levels of Th17-related cytokines like IL-17A, IL-17F and the chemokine CCL20. Th17-mediated inflammation is increased in patients with severe asthma [18] and Th17 cells have been reported to be alternative drivers of severe asthma pathophysiology in addition to the Th2 pathway [18,19].

CCL20 is a chemokine derived from bronchial epithelial cells in response to several stimuli such as proinflammatory cytokines and ambient particulate matter, and is involved in the pathogenesis of asthma [20]. It is a functional ligand for CCR6, and via this receptor it is able to attract Th17 cells to the site of airway inflammation [20–22]. It has also been reported that the CCL20/CCR6 system may play a pivotal role in allergic airway responses such as AHR, airway eosinophilia, and production of IL-5 and IgE [20,21,23]. IL-17A and IL-17F are two proinflammatory cytokines, produced mainly by Th17 cells but also by other cells like bronchial epithelial cells [18,21]. They are known to recruit, activate and regulate the migration of

neutrophils [21,22,24] and their increased levels in the lung of asthmatic patients are directly correlated with disease severity (i.e., increased AHR to methacholine) [24]. Animal model studies have also established a causative link between Th17 cells and increased levels of IL-17A and IL-17F with glucocorticoid-insensitive asthma [18,19,21,24].

The effect of the combined administration of an allergen with DEP on asthma and some of its hallmarks such as AHR and pulmonary inflammation has been described in previous studies using other murine models with different allergens and protocols. In acute models based on OVA administration [25] or chronic models with HDM [10,26] it has been observed that exposure to DEP cannot produce an asthmatic response and has no effect over AHR. Nonetheless, the effect of DEP on pulmonary inflammation and, more specifically, on neutrophilic inflammation is a controversial issue. DEP has been reported to cause an increase in pulmonary neutrophils [10,27,28]. However, exposure to DEP without any changes in neutrophil counts has also been described both in human exposure studies [29] and in animal studies [26,30,31].

The combination of DEP with an allergen may provoke a marked increase in AHR compared with exposure to the allergen alone [10,26,28,32]. Several studies have related allergen and DEP exposure with increases in pulmonary inflammation [6,28]. Increases in both neutrophil and eosinophil counts in BAL are a common feature in murine models exposed to DEP combined with allergens [6,26,28,33]. Muranaka et al. were the first to describe the effect of DEP and its combination with an allergen on the production of IgE [34]. Since then, the relationship between DEP exposure and IgE has been a controversial issue in the literature: while some authors observed significant increases in IgE levels [25,26,32,33], others reported no changes [6,28].

In our model, the combined administration of SHE with DEP produced different results depending on the allergen dose administered. The combination of Soy5, a SHE solution capable of producing an asthmatic response by itself, with DEP caused significant increases in AHR, pulmonary inflammation and IgE levels in comparison with the control group. In comparison with Soy5 alone, there were no significant changes in any of these asthma hallmarks, even though the asthmatic response seems to be stronger, with higher AHR, a greater degree of pulmonary inflammation, and a higher level of IgE. Nonetheless, the administration of Soy5+DEP results in a different cytokine profile: Soy5+DEP caused decreases in IFN-γ and IL-31 and increases in levels of IL-4, IL-5 and in all the Th17-related cytokines analyzed, but the levels of IL-13 and IL-10 presented no significant variations compared to controls. The decrease in both

IFN-γ, a Th1 signature cytokine that can act as an inhibitor of Th17 differentiation [18,24], and IL-31, a Th2 cytokine whose production is induced by IL-4 [35], together with the absence of IL-13 and the increases in IL-17A, IL-17F and CCL20 compared to controls, resulted in a mixed Th2/Th17 response. These results, together with the increased levels of IL-17F and CCL20 promoted by exposure to DEP alone, support the activation of the Th17 response related to DEP exposure [10,36,37]. This partial downregulation of the Th2 response may be mediated by the counter regulation mechanism between the Th2 and Th17 pathways described by Choy et al. 2015 [19].

The Soy3+DEP combination produced significant increases in AHR and pulmonary inflammation not just compared to the control group, but also, and more importantly, compared to the Soy3 group. As stated above, the literature suggests that the immune mechanism underlying the effects of soybean aeroallergens on asthma onset and exacerbation and even on asthma outbreaks is an allergic asthma response based on allergen-driven Th2 inflammation, in which interleukins IL-4, IL-5, and IL-13 are the main mediators [38]. However, Soy3+DEP promotes AHR and both neutrophilic and eosinophilic pulmonary inflammation, without increasing the levels of IgE, and more importantly without the involvement of IL-4 and IL-13. The absence of the most important Th2 mediators, and the significant increases in Th17-related cytokines, suggest that a Th17-mediated response might be the mechanism underlying the increases in AHR and in both neutrophilic and even eosinophilic pulmonary inflammation. Th17 cells IL-17A and IL-17F are usually associated only with neutrophilic inflammation [18,21,24], but as our results and previous literature reports suggest, they may have multiple forms of biological activity over bronchial epithelial cells [18,20,21,39], airway smooth muscle cells, fibroblasts, endothelial cells [18,21] and even directly over eosinophils [18,21,40]. Eosinophilia of the airway is a phenomenon associated with Th2 allergen-driven inflammation and with the presence of IL-5 produced by Th2 cells, but in fact, the eosinophils themselves are among the main producers of IL-5 [40]. Besides, some authors propose that Th17 cells and their cytokine milieu are involved in the activation of eosinophil production, maturation, survival and cytolytic activity [19,40], probably through the recently described Th2-independent mechanism of eosinophil activation mediated by the production of GM-CSF by Th17 cells related to DEP exposure [41,42].

This study has some limitations. The first is in relation to the levels of total IgE in serum which were determined as a surrogate of specific SHE IgE levels. We cannot rule out the possibility that there might have been a production of low levels of specific IgE in the soy3 condition. Second, due to sample limitations, other relevant antibodies such as IgG1 or IgG2a were not measured. Finally, the absence of flow cytometry confirmation of the presence and frequency

of Th2/Th17 and other cells like type 3 innate lymphoid cells limits our understanding of the underlying cellular mechanism and our ability to identify the cells responsible for the production of the cytokines measured in BAL. Moreover, information on other cytokines such as IL-6 or IL-1β which play an important role in the immunological pathways involved might provide a better understanding of the mechanisms related to asthma due to soybean and DEP.

To our knowledge, ours is the first study to standardize a murine model of asthma due to soybean and to assess the combined effect of soybean aeroallergens and DEP. Our experiments show that the continuous administration of soybean allergens at a certain concentration is capable of triggering an asthmatic response. In addition, we demonstrate that coexposure to soybean allergens and DEP results in a stronger asthmatic response, increasing airway hyperresponsiveness and pulmonary inflammation even when the concentration of soybean allergen is incapable of promoting an inflammatory response by itself. This mouse model provides evidence that the mechanism underlying soybean asthma is a mixed Th2/Th17 response, and also that DEP is capable of enhancing the allergenic effect of soybean through a Th17-mediated mechanism. These findings suggest that particulate matter monitoring as a surrogate of DEP exposure may be a useful addition to the allergen monitoring in the attempts to prevent new asthma outbreaks.

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GENERAL DISCUSSION

General discussion

To control asthma due to soybean it is important to assess soybean allergens in the air, and also to study the pathophysiology and possible interaction of soybean allergens and pollution by means of an animal model. Environmental monitoring to assess airborne allergens is a time-consuming process that commonly includes three steps: sampling of the aeroallergens in a filter, elution of the allergens, and determination of allergen levels with an allergen-specific ELISA. A rapid immunoassay can replace the ELISA, not only speeding up the last step in the process and can also be performed by non-skilled personnel without the need for specialized laboratory equipment. To address this need, one of the objectives of thi thesis was to develop a rapid, simple and inexpensive strip assay test capable of detecting even low levels of soybean aeroallergens in environmental samples.

Aeroallergen exposure plays an important role in the onset and exacerbation of asthma. Direct, sensitive measurement of aeroallergen levels is essential to study exposure, to assess the levels that can lead to sensitization, to provoke symptoms in sensitized subjects and to evaluate the effectiveness of reduction strategies[1]. Many asthma-related aeroallergens are amorphous, non-viable particles that cannot be quantified by microscope counting or chemical assays[2]. Therefore, objective quantification of these aeroallergens requires measurement of the allergen concentration using other methods; historically, seed aeroallergens such as those related to soybean have been detected using antibody-based assays, or immunoassays[3].

Surveillance of soybean aeroallergen levels in the harbor areas of cities where soybean is loaded or unloaded is crucial in order to prevent asthma outbreaks[4–6]. Better and faster allergen monitoring has shown to be very beneficial for reducing exposure[7], and so highly sensitive methods that allow accurate measurement of soybean aeroallergen levels are essential for evaluating the health risk of the exposure episode. Until now, only ELISA immunoassays have been developed for the measurement of environmental soybean aeroallergen concentrations[8–11]. ELISA is a very useful technique for detecting soluble antigens, and meets most of the main requirements for analyzing soybean aeroallergens (i.e., reproducibliity, good specificity and acceptable sensitivity), but it is a time-consuming process that requires skilled personnel and expensive laboratory equipment. Therefore, cost-efficient, less labor-intensive technological procedures that can be performed by non-specialist laboratory staff are needed in order to monitor soybean allergen levels.

Rapid immunoassays to measure other aeroallergens have shown their ability to indicate the presence of other aeroallergens in specific situations in a short period of time[12]. These methods include a test for determining house dust mite allergens in dust samples[13], another for measuring fungal alpha-amylase[14], and two tests for monitoring rat and mouse urinary allergens[15].

In the case of our rapid immunoassay for measuring soybean aeroallergens, the limit of detection (LOD), determined as the minimum amount of allergen capable of producing a clearly visible signal in the test line of the rapid immunoassay, is quite similar to those reported for the immunochromatographic assays developed for other allergens. Our rapid assay is able to measure soybean aeroallergens at levels as low as 6.25 ng/ml, an LOD comparable to the ones described for other rapid tests like house dust mite allergens, with a sensitivity of 1-2 ng/ml[13], or for fungal alpha-amylase, in which the LOD was 1–10 ng/ml[14]. A similar test developed to measure mouse and rat urinary allergens showed an LOD of 31 pg/ml measuring allergen standards in a buffer system[15]; this notable reduction achieved in the detection limit was probably due to the use of colloidal carbon particles, which can theoretically provide a better sensitivity in controlled buffer systems than colloidal gold[16], but with the drawbacks of the presence of irregularly-shaped large particles and non-specific adsorption of proteins and biomolecules[17]. However, in real field samples, the LOD of these assays are also comparable to the figures determined for our rapid test – i.e., 0.5 ng/ml for mouse allergens and 4 ng/ml for rat allergens.

Unexpectedly, in the visual analysis of our test results, the independent observers detected soybean aeroallergen concentrations below the theoretical LOD of 6.25 ng/ml determined for the assay. Depending on the reader, the actual LOD of the assay with real field samples ranged from 2 to 2.7 ng/ml. This observation has also been reported in other studies. For example, Tsay *et al.*[13] graded the line intensity of some samples analysed as medium or high even lthough the allergen levels analysed by ELISA were below the theoretical LOD of the rapid assay. Similarly in the study by Bogdanovic *et al.*[14], some samples with allergen levels below the sensitivity of the rapid test were classified as positive.

A soybean aeroallergen concentration above 1600 ng/ml in a buffer system produces a decreased test line intensity (i.e., a decreased assay signal) which may be due to the phenomenon called the high-dose hook or prozone effect, which occurs when there is an excess

of antigen. Selby described the hook effect phenomenon in immunoassays as the production of artefactually low results from samples that have extraordinarily high concentrations of antigen, far exceeding the concentration of the upper standard in the assay concerned[18]. Solid phase immunoassays that involve both capture and detection antibodies are susceptible to the hook effect, especially one-step immunoassays where sample and labeled antibody are added simultaneously. In case of antigen excess, both capture and detection antibodies are saturated, thus preventing the sandwich-formation that can trigger the detection signal. Therefore, the hook effect causes false-negative results[19,20]. Several mechanisms may be responsible for this phenomenon. At high analyte concentrations, the antibodies compete with each other for antigen-binding places, which may result in steric hindrance or monovalent binding. This has consequences for the strength of the interaction between the antibody and the solid phase, which may produce a lower detection signal.

To detect the hook effect, samples are often tested before and after dilution. Furthermore, to avoid this phenomenon t the assay format sometimes needs to be redesigned with sequential incubations or by increasing the amount of solid-phase antibody. The application of preventive measures and the modification of the assay format to resolve the problems created by the hook effect increases labor and reagent costs for assays that only rarely encounter extremely high antigen concentrations. Fortunately, this effect is of no relevance to the control of environmental samples near the harbor of Barcelona, since in recent years the highest concentration reached was 1.848 ng/ml. This is also the case of the samples analysed in our study; the highest allergen concentration was also 1.848 ng/ml, and it was tested at a dilution of 1:100. Therefore, in this study we did not observe the hook effect. However, for each new sampling site without previous recordings of soybean aeroallergens levels, an initial evaluation should be performed with an ELISA assay and subsequent follow-up evaluations with the rapid assay at an adequate dilution, based on the ELISA results.

Visual interpretation of rapid assays is inherently subjective, and depends on factors such as the readers' color perception. Therefore, it is important to assess inter-rater agreement. In this study, agreement between readers was graded as substantial, according to the classification recommended by Landis and Koch[21]. Despite the limitations, the results of the rapid assay presented high agreement with the ELISA results. The visual interpretation of the assay showed high sensitivity and specificity, comparable to those recorded with other rapid assays measuring other aeroallergens[13–15]. Furthermore, the line intensity (analysed by densitometry, an objective measurement) of samples categorized as negative was significantly lower than that of positive and double positive samples.

The rapid strip assay's main advantage is its speed, but this may not yet be a decisive factor in the case of airborne samples because the time needed to obtain the sample is still too long. However, the assay may represent a simpler, less expensive method for soybean aeroallergen environmental monitoring that can be performed in non-laboratory environments. Furthermore, although speed may not be decisive in environmental monitoring, it may constitute a real advantage for industrial hygiene monitoring, as some of the sampling procedures widely used in occupational hygiene take only a short time (for example, surface wipe sampling, dust sampling or bulk sampling).

The rapid immunoassay described is fast, simple, and sensitive and does not require expensive equipment or specific skills. It has considerable potential in the field of environmental monitoring for screening soybean aeroallergens levels in port cities with soybean harbor facilities where allergen levels are not currently measured.

So far, all efforts to prevent new soybean asthma epidemics have focused on reducing the levels of soybean aeroallergens. However, the possible effects of the combination of pollution and soybean allergens have been overlooked. One of the objectives of this thesis was to study how the combination of soybean allergens and diesel exhaust particles can affect the induction or exacerbation of asthma.

In this thesis, a soybean asthma murine model has been developed and the effect of air pollution, in particular DEP exposure, has been assessed. The study shows that the repeated intranasal administration of soybean, in the form of a sufficient concentration of soy hull extract (SHE) leads to a severe asthmatic response with an increased AHR, a robust inflammatory response in BAL, and increased levels of total IgE, Th2 cytokines, and also Th17 related cytokines. The study also demonstrates that DEP can synergize with SHE to produce an asthmatic response even when the concentration of SHE is too low to produce a response on its own (3mg/ml; Soy3). Besides, the immunological mechanism underlying the effect of the combination of low SHE concentration (Soy3) with DEP seems to be not a Th2 mechanism, but a Th17-related response.

To our knowledge, there has been hardly any experimental research into the airway immune-inflammatory responses of asthma due to soybean. The possible interaction between soybean allergens and DEP, and its effects on the onset and development of asthma have never been studied.

Until now, the effects of the combined exposure to aeroallergens and DEP have been studied mainly in animal models based on the administration of ovalbumin (OVA) or house dust mite (HDM). However, OVA is not associated with asthma in humans[22] and therefore is not a physiologically relevant aeroallergen, and HDM is mainly an indoor allergen. Besides, asthma murine models, both acute and chronic, are based on the assumption that the underlying mechanism is an allergen-driven Th2-type inflammation[22,23]. Nevertheless, human asthma is a complex multifactorial disease, and while the Th2 allergen-driven response has proven to be highly relevant especially in the case of allergic asthma, epidemiological studies suggest that in many situations this Th2 allergen-driven concept is not the primary mechanism for the disease development and progression. In fact, in recent years the contribution of other factors to the development, progression and exacerbation of asthma (among them, air pollution and other immunological mechanisms with additional types of T cells such as Th17 cells) is increasingly being recognized[22–26].

The murine model of asthma due to soybean developed in this thesis has shown that repeated exposure to soybean aeroallergens at a sufficient concentration (5mg/ml; Soy5) leads to an asthmatic response with increased AHR, increased eosinophils and neutrophils in BAL, and also higher levels of IgE in serum. However, our results also show that this response is not only based on a Th2 mechanism, with higher levels of IL-4, IL-5 and IL-13, but on a mechanism that involves these Th2-related cytokines and also Th17-related mediators, such as IL-17A, IL-17F and the C-C motif chemokine 20 (CCL20).

IL-17A and IL-17F are two proinflammatory cytokines whose increased levels in the lung of asthmatic patients seem to directly correlate with disease severity (i.e., increased AHR to methacholine)[27] and are known to recruit, activate and regulate the migration of neutrophils[27–29]. Those cytokines are produced mainly by Th17 cells but can also be produced by other immune cells and other types of cell like bronchial epithelial cells[29,30]. Th17 cells are a subset of T helper cells reported to play important roles in host defense mechanisms and in a diverse group of immune-mediated diseases, including asthma[28,29]. As a matter of fact, Th17 cells have already been described as alternative drivers of severe asthma pathophysiology in addition to the Th2 pathway[30,31], and Th17-driven inflammation has been shown to be increased in patients with severe asthma[30]. Animal model studies have also established a causative link between Th17 cells and increased levels of IL-17A and IL-17F in glucocorticoid-insensitive asthma[27,29–31]. CCL20 is a chemokine derived from bronchial epithelial cells in response to several stimuli such as proinflammatory cytokines and ambient particulate matter, which has been implicated in the pathogenesis of asthma[32]. CCL20 is a functional ligand for C-C chemokine receptor type 6 (CCR6),

and via this receptor is able to attract Th17 cells into the site of airway inflammation[28,29,32]. It has also been reported that the CCL20/CCR6 system can play a pivotal role in allergic airway responses such as AHR, airway eosinophilia, and production of IL-5 and IgE[29,32,33].

The combined administration of an aeroallergen and DEP and its effects on asthma and some asthma hallmarks such as AHR and pulmonary inflammation have been described in previous studies using other murine models with different allergens and protocols. Acute asthma models based on OVA administration[34,35] and chronic models that rely on repeated HDM administration[36–38] have demonstrated that exposure to DEP alone cannot produce an asthmatic response and has no effect on AHR. Nonetheless, the effect of DEP on pulmonary inflammation, and more specifically on neutrophilic inflammation, is a controversial subject in the literature. Since DEP alone has been reported to cause an increase in pulmonary neutrophils[38–40], in our study, the absence of significant changes in neutrophil counts in BAL when administering only DEP is a controversial result. However, exposure to DEP without changes in neutrophil counts has also been described in both human[41] and animal studies[37,42,43]. Moreover, and although this may seem contradictory, other studies have suggested that the absence of significant changes in the neutrophil count can be compatible with a significant increase in the levels of well-known activators of the production of IL17 and Th17 cell differentiation[30] such as IL-6 and TGF-β[41,43]. In addition, these controversial results may be the consequence of the different types and compositions of DEP used in different studies, since it has been reported that different types of DEP produce different outcomes at an inflammatory level[44]. In fact, it has recently been reported that, while the administration alone of carbon black particles (the core of DEP), causes an increase in the number of neutrophils, the administration of whole DEP does not modify neutrophilic inflammation, showing that differences in the composition of DEP may have an important impact on the neutrophilic inflammation outcome[45].

When DEP are administered in combination with an allergen, they provoke a marked increase in AHR in comparison to the effects observed when either the allergen[34–38,40] or DEP are administered alone. Several studies have related exposure to a combination of allergen and DEP with increases in pulmonary inflammation[40,46]. In fact, increases in both neutrophil and eosinophil counts in BAL are a common feature in many murine models exposed to a combination of DEP and aeroallergens[36,37,40,46].

Muranaka *et al.* were the first to describe the effect of DEP and its combination with an allergen on the production of IgE[47]. Since then, however, the effect of DEP exposure on the production of IgE has become a controversial issue in the literature; while some authors observed significant increases in IgE levels [34–37,40], others reported no changes[40,46].

In our murine model, the outcome of combined administration of SHE with DEP with regard to the different asthma hallmarks assessed depends on the allergen dose administered. A SHE solution capable of producing an asthmatic response by itself (Soy5) in combination with DEP results in increased AHR, pulmonary inflammation and levels of IgE in comparison with the control group. There are no significant changes in any of these asthma hallmarks in comparison with the administration of the aeroallergen dose alone, although the asthmatic response seems to be stronger when the allergen is given in combination, with higher levels of AHR, pulmonary inflammation, and IgE. Nevertheless, the combined administration of Soy5 and DEP produces a cytokine profile different from the one observed when Soy5 is administered alone. Soy5+DEP promotes increased levels of IL-4, IL-5 and all the Th17-related cytokines analysed and also decreased levels of interferon-γ (IFN-γ) and IL-31, but IL-13 and IL-10 present no significant variations compared to controls. The reductions in both IFN-γ, a Th1 signature cytokine that can also act as an inhibitor of Th17 differentiation[27,30,48], and IL-31, a Th2-related cytokine whose production is induced by IL-4[49], together with the absence of IL-13 and increased levels compared to controls of IL-17A, IL-17F and CCL20, suggest a mixed Th2/Th17 cytokine response.

The results of the Soy5+DEP cytokine profile, concurrently with the increased levels of IL-17F and CCL20 promoted by exposure to DEP alone, support the already described activation of a Th17-mediated response related to DEP exposure[36,38,50–53]. This Soy5+DEP cytokine profile also suggests that DEP exposure may have an effect on the Th2 response and produce a partial downregulation of this Th2 response which may be mediated by the reciprocal negative regulation described between the Th2 and Th17 pathways[31].

The combination of DEP and Soy3 (a SHE concentration incapable by itself of producing an asthmatic response) produces significant increases in AHR and pulmonary inflammation not only compared to the control group, but also (and more importantly) compared with the Soy3 group. As stated above, the literature suggests that the immune mechanism underlying the effects of soybean aeroallergens on the onset and exacerbation and even the asthma outbreaks is an allergic asthma response, based on allergen-driven Th2 inflammation, in which interleukins IL-4, IL-5 and IL-13 are the main mediators[54–56]. However, Soy3+DEP promotes AHR and eosinophilic and neutrophilic pulmonary inflammation without increasing the levels of IgE, and

more importantly without the involvement of IL-4 and IL-13. The absence of the Th2 mediators most widely described as hallmarks of allergic asthma, combined with significant increases in Th17- related cytokines, suggests that a Th17-mediated mechanism might be the mechanism underlying these increases in AHR and neutrophilic and even eosinophilic pulmonary inflammation with a combination of soy at low concentrations and DEP.

These results may seem at odds with the general mechanistic paradigm of allergic asthma with eosinophilia and Th2 cytokines, but although Th17 cells (and their related cytokines IL-17A and IL-17F) are usually associated with neutrophilic inflammation[27,29,30], they may also be involved in many other aspects of asthma. In fact, levels of IL-17 and IL-17F have been reported to correlate with AHR severity in human asthma[28,30] and have also been implicated in the asthma-related remodeling process of the airway[28,29,31]. Furthermore, IL-17F may have multiple biological effects on bronchial epithelial cells[29,30,32,57], airway smooth muscle cells, fibroblasts, endothelial cells[29,30] and even a direct effect on eosinophils[29,30,58].

While eosinophilia of the airway is a phenomenon usually associated with Th2 allergen-driven inflammation and with the presence of IL-5 produced by Th2 cells, these cells are not the only ones able to produce this cytokine. Eosinophils themselves are actually among the main producers of IL-5[58–60]. Th17 cells and their *milieu* of cytokines may also play a role in eosinophil production, maturation, survival, and cytolytic activity. In fact, the combination of IL-5 and IL-17A or IL-17F enhances the activation of eosinophils but, while IL-5 is crucial to increasing eosinophil survival, Th17 cytokines enhance the cytolytic function of these cells, promoting the degranulation process[31,58]. Some authors propose that there is a synergistic effect between IL-5 and Th17 cytokines, and have also suggested the presence of a mechanism of eosinophil activation and maturation directly mediated by Th17 via the production of GM-CSF and related to DEP exposure which may support Th17-driven eosinophilia independently of Th2[61,62]; this may explain the immunologic mechanism underlying the effect of the combination of Soy3+DEP.

While IL-31 seems to be involved in allergic asthma, the studies conducted so far have produced contradictory results. The results from both our Soy5+DEP and our Soy3+DEP groups reveal reduced levels of this cytokine. Initial studies using asthma murine models found an increase in IL-31 receptor mRNA levels in lung tissue and lung cell infiltrates, suggesting that IL-31 may be relevant for disease development[63]. However, recent studies of the IL-31 receptor propose that IL-31 signaling may be required to negatively regulate Th2 type responses rather than

exacerbate them[64,65]. We think that the decreased level of IL-31 in these groups may be related to the Th17 differentiation process and to TGF-β, which appears to be able to suppress IL-31 expression at mRNA and protein level[66]. Despite these contradictory and as yet unexplained results regarding the effect of IL-31 on the Th2 response and asthma, in all studies published so far relatively high concentrations of IL-31 (50–500 ng/ml) have been required to stimulate signal transduction on the cytokine receptor[67]. These results indicate that the affinity of IL-31 to the receptor may be low, and provide a possible explanation for the high level of IL-31 in the BAL in our control group.

To our knowledge, this is the first study to standardize a murine model of asthma due to soybean and to assess the combined effect of soybean aeroallergens and DEP. These results show that the continuous administration of soybean allergens at a certain concentration is able to trigger an asthmatic response. They also show that coexposure to soybean allergens and DEP results in a stronger asthmatic response, enhancing airway hyperresponsiveness and pulmonary inflammation even when the concentration of soybean allergen is unable to promote an inflammatory response by itself. The model also provides evidence that the mechanism underlying soybean asthma is a mixed Th2/Th17 response, and that DEP are able to enhance the allergenic effect of soybean through a Th17-mediated mechanism. These findings suggest that particulate matter monitoring, as a surrogate of DEP exposure, may be a useful addition to allergen monitoring in the attempts to prevent new asthma outbreaks.

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CONCLUSIONS

Conclusions

- The rapid test developed is a simple, fast and sensitive way to monitor soybean aeroallergens in environmental samples without needing expensive equipment or specific skill personnel.

- This rapid immunoassay has considerable potential for the environmental monitoring screening of soybean aeroallergens levels in port cities with soybean harbor facilities.

- The continuous administration of soybean allergens at a certain concentration is capable of triggering an asthmatic response in a murine model.

- The underlying immunological mechanism of asthma due to soybean in this model is a mixed Th2/Th17 response.

- The combined exposure to soybean allergens and DEP produces stronger asthmatic responses, increasing airway hyperresponsiveness and pulmonary inflammation.

- DEP is capable of enhancing the allergenic effect of soybean allergens even when the levels are too low to produce an asthmatic response by itself.

- The mechanism by which DEP enhance the effect of soybean allergens over asthma response is mediated by a Th17 response.

- Particulate matter monitoring, as a surrogate of DEP exposure, may be a useful addition to the allergen monitoring in the attempts to prevent new asthma outbreaks.