Universitat Autònoma de Barcelona

EFFECTS OF DIESEL EXHAUST PARTICLES ON ALLERGIC AND CHEMICAL-INDUCED ASTHMA

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DOCTORAL THESIS

EFFECTS OF DIESEL EXHAUST PARTICLES ON ALLERGIC AND CHEMICAL-INDUCED ASTHMA

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LIST OF ABBREVIATIONS

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16 rRNA	Small-subunit ribosomal rna
ACh	Acetylcholine
AHR	Airway hyperresponsiveness
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
AP	Ammonium persulfate
ARE	Antioxidant-response element
ARNT	Aryl hydrocarbon receptor nuclear translocator
ASM	Airway smooth muscle
AUC	Area under the curve
BABB	2:1 mixture of benzyl alcohol and benzyl benzoate
BAL	Bronchoalveolar lavage
BC	Black carbon
BCR	B cell receptor
brLN	Bronchial lymph node
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLP	Chitinase-like protein

CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CXC	Chemokine
CXCL	Chemokine ligand
DALY	Disability-adjusted life years
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DELFIA	Dissociation-enhanced lanthanide fluorescent immunoassay
DEP	Diesel exhaust particle
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DS	Digestion solution
EAR	Early asthmatic response
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EPX	Eosinophil peroxidase
FcεRI	High-affinity IgE receptor
FEV_x	Forced expired volume in x seconds
FMO	Fluorescence minus one
FV	Flow volume

FVC	Forced vital capacity
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRIDREC	Grid reconstruction algorithm
H	Tissue elastance
HAH	Halogenated aromatic hydrocarbons
HDM	House dust mite
HMW	High molecular weight
HPC	High positive control
Hz	Hertz
ICS	Inhaled corticosteroid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
LAR	Late asthmatic response
LMW	Low molecular weight
LPC	Low positive control
LRT	Lower respiratory tract

LT	Leukotriene
M_3R	Muscarinic 3 receptor
MAMP	Microbe-associated molecular patterns
MBP	Major basic protein
MCP-1	Monocyte chemoattractant protein 1
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MLEM	Maximum-likelihood expectation maximization algorithm
MRI	Magnetic resonance imaging
MMP	Metalloprotease
NC	Negative control
NGF	Nerve growth factor
NIST	National institute of standards and technology
NK	Natural killer cell
NPFE	Negative pressure-driven forced expiration maneuver
Nrf2	Nuclear factor-erythroid 2-related factor 2
OA	Occupational asthma
OCS	Oral corticosteroid
OPT	Optical projection tomography
OVA	Ovalbumin

PAH	Polycyclic aromatic hydrocarbons
PAMP	Pathogen-associated molecular pattern
PC20	Provocative concentration causing a 20% drop in FEVx
PEEP	Positive end expiratory pressure
PG	Prostaglandin
PGHS	Prostaglandin synthase
PM	Particulate matter
Pmax	Maximum inflation pressure
ppm	Parts per million
PRR	Pattern recognition receptor
QP3	Quick-prime 3
REGIII	Regenerating islet-derived III factor
Rn	Central airway resistance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
s.c.	Subcutaneous injection
SCFA	Short-chain fatty acids
SD	Standard deviation
SHE	Soybean hull extract
SRM	Standard reference material

SVOC	Semi-volatile organic compound
T2	Type 2 Endotype
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
Th	Helper T cells
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptors
TLSP	Thymic stromal lymphopoietin
TRAP	Traffic-related air pollution
TRF	Time-resolved fluorometry
TRP	Transient receptor potential channel
URT	Upper respiratory tract
VOC	Volatile organic compound
WHO	World health organization
Zrs	Input impedance

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SUMMARY

Air pollution is known to induce and exacerbate asthma pathophysiology, but the immunological effects of diesel exhaust particles (DEP) are still not well characterized and seem to differ depending on the pre-existing asthma endotype: type 2 (T2), which is subdivided further into Th2 and ILC2-driven; non-T2; and a mixed Th2/Th17 endotype. In the T2 endotype, traffic-related air pollution (TRAP) has been demonstrated to enhance respiratory sensitization to environmental allergens, while it has been suggested that DEPs act as adjuvants in the induction of allergic asthma. In the Th2-driven T2 endotype it seems that DEPs may increase the antigenic capacity of certain aeroallergens; while in the ILC2-driven T2 endotype, they may stimulate lung epithelial cells to produce high levels of interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP), cytokines related to ILC2s and eosinophilic asthma. In the non-T2 endotype, DEPs may aggravate neutrophilic asthma by increasing the production of both IL-17A and IL-17F cytokines. Lastly, our group reported that coexposure to soybean hull extract (SHE) and DEPs may favour a mixed Th17/Th2 response, not only involving a Th2 inflammation with IL-4, IL-5, IL-13 and IL-10, but also increasing levels of chemokines related to the Th17 response such as IL-17A, IL-17F, and CCL20 cytokines.

The present doctoral thesis aims to study the effects of DEP exposure on two different asthma endotypes by assessing respiratory mechanics, airway hyperresponsiveness (AHR), innate and adaptive immune responses, oxidative stress and particle deposition patterns. The first study (*Chapter 1*) aims to study the effects of DEP exposure in a mouse model of chemical-induced asthma due to ammonium persulfate (AP), a non-type 2 endotype. This study demonstrates that sensitization and inhalation of AP induce a T2 low asthma endotype, characterized by asthma-like lung parameters such as higher levels of central airway resistance, tissue elastance, and AHR, and airway inflammation consisting in eosinophilia, and lower levels of tolerogenic dendritic cells

(DC). The DEP-exposed group showed slightly modified lung mechanics, airway inflammation with neutrophilia, higher levels of Th2-related DCs, lower levels of alveolar macrophages, and reduced levels of cytokines such as IL-13 and IFN-γ. The group coexposed to AP and DEPs presented exacerbated signs of asthma consisting principally of asthma-like lung function, airway inflammation with higher levels of oxidative stress-sensitive DCs, lower total macrophages, and a differential pattern of DEPs deposition in the lungs, restricted to large conducting airways.

The second study (*Chapter 2*) aims to study the effects of DEPs exposure in a mouse model exposed to non-asthmagenic doses of SHE, a Type 2 endotype. In this second chapter, the inhalation of 3 $mg \cdot ml^{-1}$ SHE induced a mild airway inflammation consisting in increased levels of eosinophils, B cells, Th2-related DCs, total and resident monocytes, and reductions in levels of NK cells, tolerogenic DCs, total and alveolar macrophages. The inhalation of DEPs alone triggers airway inflammation with neutrophilia. Lastly, the inhalation of SHE and DEPs together induced a mixed Th2/Th17 asthma endotype characterized by asthma-like lung function with higher levels of AHR and central airway resistance (Rn), airway inflammation consisting in higher levels of SHE-specific immunoglobulin (Ig)-E in serum, H_2O_2 in bronchoalveolar lavage (BAL), NK cells, oxidative stress-sensitive DCs, and lower levels of Th1-related DCs.

RESUMEN

Se sabe que la contaminación ambiental induce y exacerba el asma, aunque los mecanismos inmunológicos por los cuales las partículas diesel (DEP) modulan el asma no están del todo descritos y parecen diferir según el endotipo asmático: tipo 2 (T2), subdividido según si la respuesta es dirigida por las células Th2 o ILC2; el no T2; y el endotipo mixto Th2/Th17. En el endotipo T2 se ha demostrado que la contaminación del aire relacionada con el tráfico rodado (TRAP) incrementa la sensibilización a ciertos alérgenos ambientales, mientras que se ha propuesto que las DEPs actúan como adyuvante en la inducción del asma alérgico. En el endotipo T2 mediado por las células Th2, parece ser que las DEPs podrían aumentar la capacidad antigénica de ciertos aeroalérgenos; mientras que en el endotipo mediado por las ILC2, las DEPs podrían estimular el epitelio pulmonar a producir altos niveles de interleucina (IL)-33, IL-25 y la limfopoietina tímica estromal (TLSP), citocinas relacionadas con la vía de las ILC2 y el asma eosinofílica. En el endotipo no T2, las DEPs podrían agravar el asma neutrofílica aumentando la producción de citocinas como la IL-17A e IL-17F. Por último, nuestro grupo ha descrito que la coexposición a alérgeno de cáscara de soja (SHE) y DEPs podría favorecer una respuesta mixta Th2/Th17, involucrando no solo la inflamación Th2 con IL-4, IL-5, IL-13 e IL-10, sino también una respuesta Th17 con niveles crecientes de quimiocinas tales como la IL-17A, IL-17F y CCL20.

La presente tesis doctoral tiene como objetivo estudiar el efecto de la exposición a las DEPs sobre dos endotipos de asma diferentes, mediante la evaluación de la función respiratoria, la hiperreactividad bronquial (AHR), la inflamación pulmonar, el estrés oxidativo y el patrón de deposición de las DEPs en las vías aéreas. El primer estudio (Capítulo 1) evalua los efectos de la exposición a DEPs en un modelo murino de asma químico a sales de amonio de persulfato (AP), un endotipo no T2. Dicho estudio demuestra que la sensibilización y la inhalación de AP induce un endotipo asmático

tipo T2 bajo, caracterizado por función pulmonar de tipo asmática, elevados niveles de resistencia de las vías aéreas centrales, elastancia tisular y AHR; e inflamación de las vías respiratorias con eosinofilia y bajos niveles de células dendríticas (CD) tolerogénicas. El grupo expuesto a las DEPs se caracteriza por tener una función pulmonar ligeramente alterada; inflamación de las vías respiratorias con neutrofilia, niveles superiores de CDs relacionadas con la vía Th2 y niveles inferiores de macrófagos alveolares; y niveles reducidos de citocinas tales como la IL-13 e IFN-γ. El grupo coexpuesto a AP y DEPs presenta signos de asma exacerbado, que consiste principalmente en una función pulmonar tipo asmática; inflamación de las vías respiratorias con niveles superiores de CDs sensibles al estrés oxidativo y cantidades más bajas de macrófagos totales; presentando también una deposición diferencial de las DEPs en los pulmones, hallándose principalmente en las vías superiores.

El segundo estudio (Capítulo 2) analiza los efectos de la exposición a DEPs en un modelo murino expuesto a bajas concentraciones de SHE, un entotipo T2. En este segundo capítulo, la inhalación de 3 mg ml^{-1} de SHE induce una leve inflamación de las vías respiratorias con niveles elevados de eosinófilos, células B, CDs relacionadas con la vía Th2, monocitos totales y residentes, así como niveles reducidos de células NK, CDs tolerogénicas, macrófagos totales y alveolares. La inhalación de las DEPs desencadena neutrofilia en las vías respiratorias. Por último, la inhalación de SHE y DEPs conjuntamente induce un endotipo de asma mixto Th2/Th17, caracterizado por una función pulmonar asmática con niveles elevados de AHR y resistencia de las vías aéreas centrales (Rn); e inflamación de las vías respiratorias con niveles superiores de inmunoglobulina (Ig) de tipo E específica a SHE en suero, H_2O_2 en lavado broncoalveolar (BAL), células NK, CDs sensibles al estrés oxidativo y niveles más bajos de CDs relacionadas con la vía Th1.

1. GENERAL INTRODUCTION

1.1 ASTHMA

Asthma is a heterogeneous disease characterized by chronic airway inflammation and by respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation (1). Chronic inflammation of the airways is associated with airway hyperresponsiveness (AHR), an exaggerated airway-narrowing response often triggered by factors such as allergens, irritants, exercise, viruses, and other agents on the environment (1,2).

Asthma is a global problem, with an estimated 300 million affected individuals worldwide (1). The prevalence ranges from 1 to 18% of the population in different countries (1). In its Global Burden of Disease Study, the World Health Organization (WHO) estimated that asthma causes 13.8 million disability-adjusted life years (DALY) and 346,000 deaths worldwide every year (3).

Case fatality rates depend on the management of the disease. With the exception of refractory asthma, the disease can be managed by both non-pharmacological and pharmacological approaches. On the one hand, cessation of exposure to the causative agent represents the main non-pharmacological approach in occupational asthma (OA) (4–6). On the other, pharmacological treatments have included corticosteroids, β2 agonists, muscarinic receptor antagonists, leukotriene receptor antagonists and theophylline. In addition, novel biological agents focus on the regulation of immunoglobulin (Ig)-E and interleukin (IL)-4, IL-5, and IL-13 pathways, among others (7).

1.1.1 Immunological mechanisms in asthma

Asthma is a complex disorder resulting from the interaction between the environment and the host’s genome. Atopy, the genetic tendency to develop antibodies against common allergens, is associated with asthma and other immunological diseases, such

as allergic rhinitis and atopic dermatitis (eczema). In fact, a history of atopy in early life seems to be one of the key factors increasing an individual's risk of persistent asthma (8).

Due to its heterogeneity, asthma comprises various clinical presentations with different underlying pathophysiological mechanisms. Since the 1990s, asthma has been stratified according to clinical history, physiology, trigger, inflammatory cell profile, severity and control (9,10). However, these classifications do not truly reflect the underlying immunological mechanisms.

Atempts to characterize and classify asthma have led to the concept of asthma phenotypes and endotypes. The term "phenotype" is defined as a recognisable cluster of similar clinical presentations of a certain disease, while "endotype" is a disease subtype with a clearly elucidated pathophysiology (9). Today, depending on airway inflammation and the underlying immune mechanisms, asthma is mainly classified into type 2, non-type 2 and mixed Th2/Th17 endotypes.

1.1.1.1 Type 2 endotype

Type 2 (T2 or T2 high) asthma is the most common and the most widely studied endotype, characterized by the presence of eosinophils and type 2 inflammatory markers. T2 asthma occurs in more than 80% of children sensitized to environmental allergens and in most of their adult peers. These patients tend to have co-morbidities like atopic dermatitis and allergic rhinitis, have a high risk of exacerbations and respond well to systemic corticosteroids and anti-IL-5 treatment (11).

The pathophysiology of T2 asthma begins when allergens such as those from animal dander, dust mites, pollens, fungi, air pollution and viruses damage the airway epithelial barrier. These protease-containing allergens break proteins that mediate tight junctions, such as E-cadherin, claudin-18, and α- and β-catenins, and disrupt barrier structures (12). Airway epithelial cells detect these agents through pattern recognition

receptors (PRRs) and act as sentinels, responding rapidly by releasing thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 (11).

Depending on the response to these alarmins, eosinophilic asthma can occur in an allergic or nonallergic manner. While TSLP acts on type 2 helper T cells (Th2), IL-33 and IL-25 activate type 2 innate lymphoid cells (ILC2s). Thus, on the basis of inflammatory pathway, T2 asthma can be subdivided further into Th2 and ILC2-driven endotypes.

1.1.1.1.1 Type 2 Th2-driven endotype

Thymic stromal lymphopoietin initiates the innate phase of allergic T2 immune responses by promoting immature dendritic cells (DCs) to produce IL-8, eotaxin-2, thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) and by inducing mast cells to produce IL-5, IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-6 (13).

TSLP-activated DCs capture extracellular antigens in the airways, internalize and transport them to drain lymph nodes where they are presented on the type II major histocompatibility complex (MHCII). Antigen presentation occurs with the specific binding of MHCII and T cell receptors (TCRs) of naïve cluster of differentiation (CD)4+ T cells, leading to the expression of GATA3 transcription factor and the subsequent transformation to allergen-specific CD4+ Th2 cells (12,14).

A specific subset of Th2 cells migrate to the B cell follicle to initiate immunoglobulin class switching from IgM to IgE. Another subset of Th2 cells migrates back to the airway mucosa to express IL-4, IL-5, IL-9, IL-13, GM-CSF and TNF-α while inhibiting the expression of Th1-related IFN-γ and IL-12 cytokines (13,15).

Once sensitized, exposure of the airways to the allergen results in a mast cell-driven early asthmatic response (EAR). The allergen is recognized by the circulating allergen-

specific IgE, wich binds to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. These cells release histamine, serotonin, prostaglandin (PGD_2) and leukotriene C_4 (LTC_4), inducing airway smooth muscle cell contraction, mucus secretion and vasodilatation. A late asthmatic response (LAR) is caused by Th2 cytokines and eosinophils. IL-5 cytokine promotes the differentiation and maturation of IL-5Rα+ eosinophil progenitors in the bone marrow, as well as their subsequent mobilization to the airways (16).

Upon stimulation, eosinophils release cytokines like IL-13 and IL-5, eotaxins, granule mediators, leukotrienes, and cytotoxic proteins like major basic protein (MBP), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). With this myriad of inflammatory mediators, eosinophils induce airway remodelling, smooth muscle contractility and mucous hypersecretion (12–16).

1.1.1.1.2 Type 2 ILC2-driven endotype

Innate lymphoid cells play an important role in airway-barrier tissue homeostasis, repair, and modelling. In contrast to both T and B cells, ILC2s lack antigen-specific receptors (TCR and BCR respectively), but are directly regulated by various mediators such as cytokines, lipid mediators, neuropeptides, hormones and nutrients (17).

In nonallergic eosinophilic asthma, allergens, air pollutants and microbes induce epithelial cells to release IL-33 and IL-25. These cytokines are the agents responsible for activating ILC2s in an antigen-independent manner via their respective receptors (IL-17RB and T1/ST2). NF-kβ and MAPK induce the GATA3 transcription factor. In some cases, co-stimulatory cytokines such as TSLP, IL-2, IL-7 and IL-9 are required to fully activate ILC2s via the STAT5 transcription factor. In contrast, cytokines like type 1 IFNs, type 2 IFN, and IL-27 are resported to inhibit ILC2s proliferation and type 2 cytokine production via the STAT1 transcription factor (17).

Activated ILC2s produce high amounts of cytokines, chemokines and peptides including IL-4, IL-5, IL-6, IL-9, IL-13, GM-CSF, amphiregulin, eotaxin and methionine-enkephalin (16,17). In fact, ILC2s produce 10-fold more IL-5 and IL-13 than activated Th2 cells, and are thus crucial in he increase in the T2 response (12).

Cytokine IL-5 promotes eosinophil maturation, while IL-13 is known to impair the epithelial barrier of human bronchial epithelial cells (18). Thus, ILC2s account for the severe eosinophilic inflammation in the absence of the classical Th2-mediated allergic response.

Interestingly, it has been reported that in some allergic conditions IL-33-induced activation of ILC2 is resistant to glucocorticoid treatment (19). Furthermore, late-onset eosinophilic asthma, characterized by prominent blood and sputum eosinophilia refractory to corticosteroid treatment, has been associated with ILC2-driven production of IL-5 and IL-13 (12). Thus, the IL-33-ILC2-IL5-eosinophil axis is associated with a severe phenotype of steroid-resistant eosinophilic asthma.

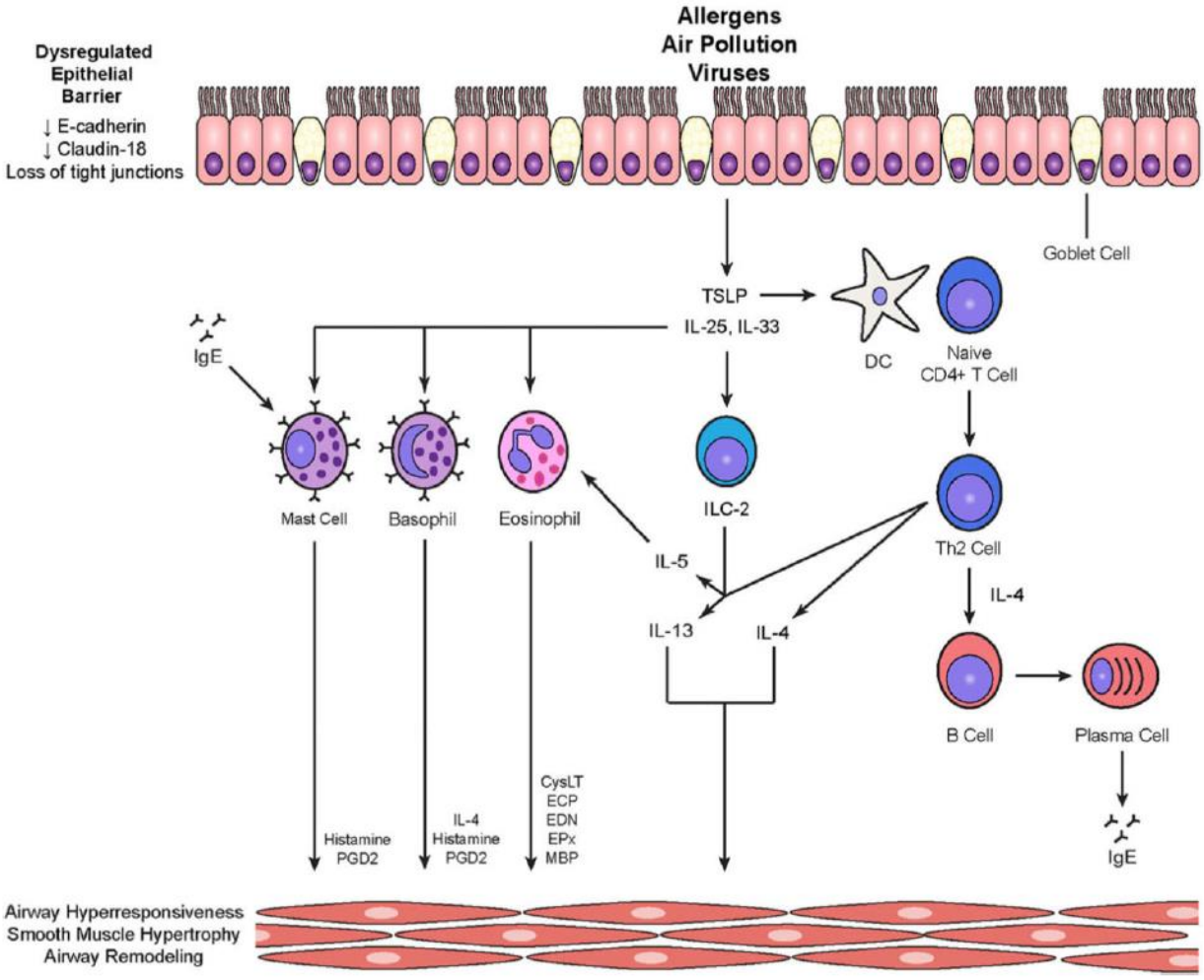

Figure 1. T2 asthma pathways. Damage to the airway epithelial barrier enables the penetration of environmental factors, inducing the release of alarmins such as TSLP, IL-25, and IL-33. TSLP primes dendritic cells to induce the differentiation of naïve T cells into Th2 cells. IL-25 and IL-33 induce ILC2s, mast cells, eosinophils, and basophils. Activated ILC2s and Th2 cells, produce IL-5 and IL-13. IL-5 promotes eosinophil differentiation and survival. IL-13, IL-4 and other inflammatory mediators from mast cells, basophils, and eosinophils affect airway hyperresponsiveness, smooth muscle hypertrophy, and airway remodelling. Figure from Kuruvilla *et al.* (12).

1.1.1.2 Non-type 2 endotype

Non-type 2 (non-T2 or T2 low) asthma is relatively under-studied, and is usually defined with the absence of eosinophils and type 2 inflammatory markers. These patients tend to have late-onset asthma with a high risk of exacerbations and a poor response to inhaled and oral corticosteroids (ICS and OCS respectively) (12).

Non-T2 asthma can be further subdivided into neutrophilic and paucigranulocytic endotypes depending on the cellular findings in sputum specimens.

1.1.1.2.1 Neutrophilic asthma

Neutrophilic airway inflammation is a term used to describe a subtype of asthma associated with high (40 - 70%) sputum neutrophils. It is the least know asthma phenotype and is usually related to tract irritation and lung injury caused by infections, ageing, intensive exercise, cold air, cigarette smoke, air pollution and gastroesophageal reflux (20).

Pathogens and injured cells release specific pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). Both PAMPs and DAMPS are recognised by toll-like receptors (TLR), a specific type of pattern recognition receptor (PRR) present in epithelial and immune cells (21). TLR2 and TLR4 activation induces epithelial cells to secrete IL-6, IL-8 and leukotriene B4 (LTB_4), Th1 cells to secrete CXCL1, IL-8, IL-1β, IFN-γ and TNF-α and macrophages to secrete IL-8 and NF-kβ (22). Chemokines like IL-6, TGF-β, IL-1β and IL-23 differentiate naïve CD4+ T cells into Th17 cells. Th17 cells are known to secrete IL-17A, IL-17F and IL-22. Chemokines like IL-6, IL-8, IL-17F, IL-22, LTB_4, IFN-γ and TNF-α represent the main factors for neutrophil recruitment (23,24). However, C5a, CXCL1, CXCL5, matrix metalloprotease-9 (MMP-9), elastase and alpha-1 antitrypsin are also known to contribute (24). In fact, neutrophil-produced elastase increases IL-8 levels and inactivates tissue inhibitor of metalloproteinase-1 (TIMP-1), thus promoting a feed forward loop in neutrophil recruitment and neutrophil production of MMP-9, respectively (25).

A variety of neutrophil subpopulations have been found which differ in terms of their maturity and activity levels (26). In asthmatic patients, mature neutrophils induce angiogenesis, airway remodeling, mucus hypersecretion, airway smooth muscle

hyperresponsiveness and lung function impairment (24). Although ICS suppress airway inflammation, corticosteroids have been reported to inhibit neutrophil apoptosis (27). Other mechanisms that may also enhance neutrophilic inflammation are abnormal airway epithelial cell activity, impaired macrophage efferocytosis and altered airway microbiome (24).

1.1.1.2.2 Paucigranulocytic asthma

Paucigranulocytic asthma is a term used to describe a subtype of asthma associated with normal sputum levels of both eosinophils and neutrophils with persistent airway obstruction. The mechanisms underlying this endotype may be dysfunctions in the nerves, smooth muscle cells and vascular tissue in the airways (12,22,24).

Acetylcholine (ACh), the primary transmitter of parasympathetic nerve fibres in the airways, is also synthesized by airway surface epithelial cells in response to environmental factors like air pollutants, cigarette smoke, allergens and contractile agonists (28,29). It is traditionally associated with the induction of airway smooth muscle (ASM) contraction and mucus secretion, being recognized by muscarinic 3 receptor (M_3R). In fact, an exaggerated release of neuronal ACh increases cytosolic Ca^{+2} concentrations and the subsequent activation of the contractile apparatus, resulting in a noninflammatory endotype of asthma in which there is evidence of inflammation-independent AHR (29).

Acetylcholine not only acts on ASM, but also has effects at cellular level. It induces epithelial cells to proliferate and release GM-CSF, LTB_4, TGF-β and IL-8, goblet cells to express MUC5AC and produce mucous, fibroblasts to proliferate and secrete collagen, IL-6, IL-8 and MMP-2, macrophages to secrete monocyte chemoattractant protein 1 (MCP-1) and TGF-β, mast cells to infiltrate, and cytolytic T cells to proliferate (24). Chemokines like IL-6, IL-8, MCP-1 and TGF-β modulate leukocyte trafficking and induce basement membrane thickening due to the deposition of extracellular matrix.

Indeed, collagen is deposited not only in the subepithelial space but also in the deeper submucosal layer (28,29).

To summarise, altered neuronal production of ACh induces AHR, airway wall remodelling, ASM hypertrophy and hyperplasia, extended fibrosis and mucous production in the absence of a clearly defined airway inflammation.

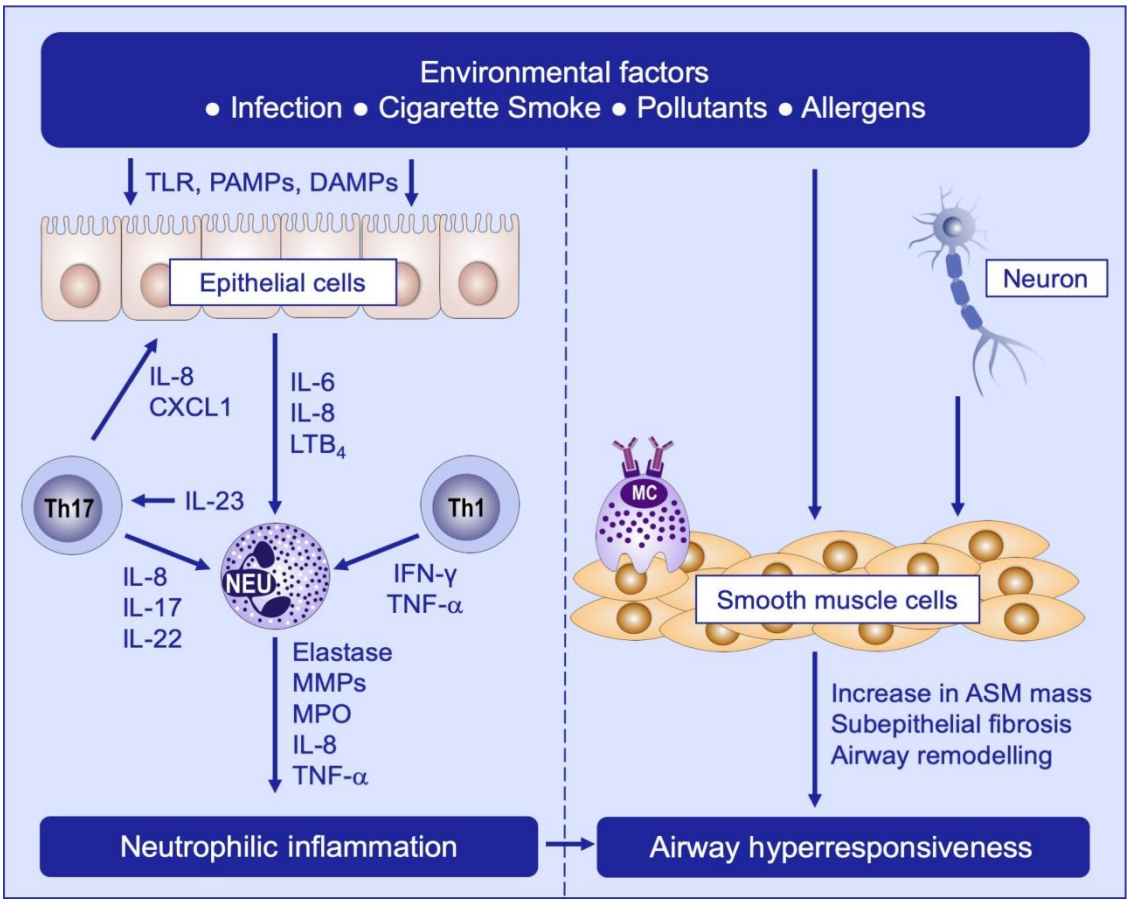

Figure 2. Non-T2 asthma pathways. Environmental factors induce formation of PAMPs and DAMPS, which are recognised by TLRs in airway epithelial cells and induce neutrophilic inflammation. Altered neuronal production of ACh induces AHR through inducing ASM hyperreaction. Figure from Sze *et al.* (24).

1.1.1.3 Mixed Th2/Th17 endotype

The assumption that asthma is an exclusive Th2 or Th17 disorder is probably an oversimplification, as in many cases there is a considerable overlap in the types of cytokines involved. A mixed Th2/Th17 endotype was first described by Cosmi *et al.*,

who detected circulating memory CD4+ T cells that produced both IL-17A and IL-4 cytokines (30). These dual-positive Th2/Th17 cells have been associated with glucocorticoid resistance, airway obstruction, and bronchial hyperreactivity (31).

The relationship between type 17 and type 2 immune responses is highly complex and not well understood. It has been reported that the injured epithelium, through chitinases and chitinase-like proteins (CLPs), induces innate γδT and Th17 cells to secrete neutrophil-activating cytokines such as IL-1b, IL-6, IL-17 and IFNγ (32). In contrast, these cytokines have also been reported to induce eosinophilia, via GM-CSF and IL-5 signalling, and to suppress neutrophilic inflammation by inducing both ILC2 and Th2 cells to secrete IL-4 and IL-13 cytokines (31). It has also been suggested that IL-4 and IL-13 amplify Th17 responses by up-regulating CD209a expression on dendritic cells (33).

Other authors have reported that Th2/Th17 dual lymphocytes may derive from Th17 cells exposed to IL-4 and IL-13 or Th2 lymphocytes in presence of IL-1b, IL-6 and IL-21 cytokines (32). Previously, our group has shown that coexposure to air pollution in an allergic asthma murine model shifts the immune response from a type 2 to a mixed Th2/Th17, inducing the allergic response and highlighting the fact that air pollutants trigger the asthmatic response when the allergen concentration is too low to cause a response by itself (34).

1.2 AIR POLLUTION

Air pollution is defined as a mixture of natural and man-made airborne substances that may harm humans, animals, vegetation or materials (35). The manufacturing, transportation and combustion of fossil fuels for the generation of energy are the main sources of anthropogenic air pollutants (36). These side-products can be classified into gaseous pollutants, organic compounds, heavy metals and particulate matter (PM); gaseous pollutants and PM are the most important.

The most common gaseous pollutants are sulphur, nitrogen and carbon oxides (SO_x, NO_x and CO_x respectively), ozone (O_3) and reactive hydrocarbons, which are also known as semi-volatile organic compounds (SVOCs). Primary pollutants are those released directly into the atmosphere, such as SO_x, NO_x, CO_x and SVOCs. Secondary pollutants are the ones generated in the atmosphere: ozone, sulphuric acid and ammonium nitrate (35).

Particulate matter is defined as a complex mixture of airborne particles, dust, pollen, smoke, and liquid droplets, usually classified by their aerodynamic diameter. Particles with a diameter <10µm are known as PM_{10}, those with a diameter <2.5µm as $PM_{2.5}$ and those with a diameter <0.1µm as $PM_{0.1}$. The PM_{10} fraction includes $PM_{2.5}$ and $PM_{0.1}$, $PM_{2.5}$ includes $PM_{0.1}$, and the term PM_{coarse} refers to particulate matter between 2.5 and 10µm. Black carbon (BC) and diesel exhaust particles (DEP) are part of this PM. BC is defined as a mixture of particles and oil droplets emitted in both anthropogenic and naturally-occurring soot, while DEPs are side-products of the incomplete combustion of diesel-fuel engines (37,38).

Each pollutant has specific physicochemical properties that define its capability to reach, accumulate and affect tissues. SO_2 is known to affect the upper airways, NO_2 and O_3 reach and affect the lower airways, and CO diffuses directly into the bloodstream causing hypoxia (35). The size of the PM determines the depth they reach

along the respiratory tract, and the chemicals on the surface determine the potential toxicity. PM_{10} penetrate the nose, throat and larynx; $PM_{2.5}$ reach into the trachea, bronchi and bronchioli, and $PM_{0.1}$ enter the alveolar region, passing through the alveolar-capillary membrane, diffusing into the blood circulation and inducing systemic toxicity (39–42).

Air pollution has been associated with disability and mortality (43,44), affecting the central nervous system (CNS) (45) and fertility (46) and causing lung (47–51), cardiovascular (52,53) and immune-related (54,55) diseases. Among lung diseases, the exposure to air pollutants increases the risk of certain respiratory infections (56) and chronic respiratory diseases (57). Recent studies relate the exposure to $PM_{2.5}$, PM_{coarse}, PM_{10}, O_3, SO_2 and NO_2 with cough, phlegm, bronchial hyperresponsiveness and a lower lung function (57–60). Furthermore, PM are associated with lung cancer (61) and chronic laryngitis (62), O_3, $PM_{2.5}$ and NO_x with emphysema (58), $PM_{2.5}$, PM_{10} and NO_2 with chronic obstructive pulmonary disease (COPD) (59), and DEPs, SO_2, NO_2, $PM_{2.5}$ and BC with asthma (60,63–65).

1.2.1 Diesel exhaust particles

Diesel exhaust particles are the most common PM. Generated during the incomplete combustion of diesel-fuel engines, these particles form aggregates measuring from >30μm to <0.1μm in diameter. These particles consist of an elemental carbon core surrounded by toxic compounds such as sulphuric acid, redox-active quinines, metal ions like Cu, Fe, Ca, Mg, Na, environmental allergens and SVOCs like halogenated and polycyclic aromatic hydrocarbons (HAH and PAH, respectively) (Figure 3) (66–69).

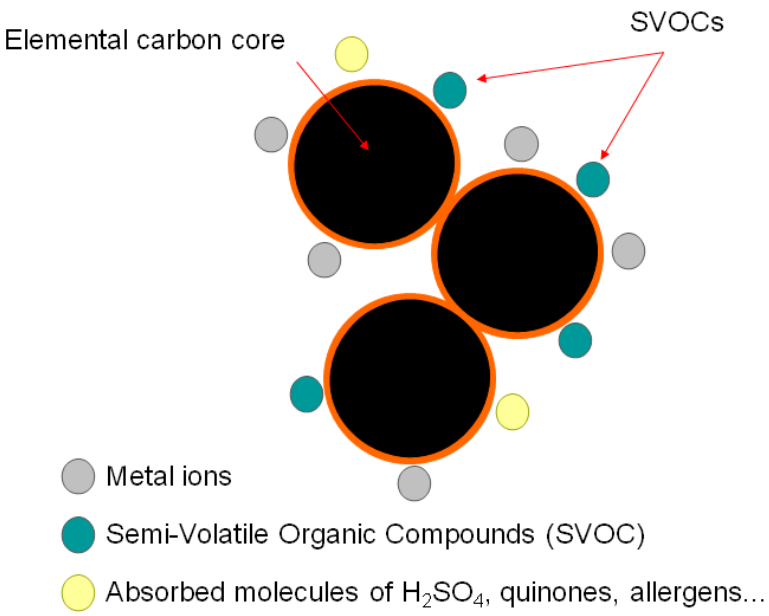

Figure 3. DEP composition. DEP comprise an elemental carbon core surrounded by a variety of toxic compounds. Figure modified from Muñoz *et al.* (63).

1.2.1.1 Interaction between diesel exhaust particles and the immune system

DEPs are known to produce both oxidative and nitrosative stress with the formation of reactive oxygen and nitrogen species (ROS/RNS) in the airways (63). Bleck *et al.* reported that human bronchial epithelial cells exposed to DEPs induced immune disorders by increasing the production of TSLP and ROS in a sustained manner (70). Further research demonstrated that TSLP acts on Jagged-1 and OX40L expression in myeloid dendritic cells, driving mucosal immunity towards a type 2 immune response (71).

Aromatic hydrocarbons like HAH and PAH surrounding DEPs are recognised by the aryl hydrocarbon receptor (AhR), present in a wide range of immune and non-immune cells located along the airways (72). As regards immune cells, AhR is highly expressed in Th17 and γδT cells (73). After binding to its ligands, AhR translocates into the nucleus acting as a transcription factor for T cell differentiation genes such as Foxp3 and GATA3, inflammatory proteins such as IL-1β, IL-2, IL-6, IL-8, IL-17, IL-22, GM-

CSF, MMP-1, TNF-α and NF-kβ, other transcription factors like MAPK, STAT1 and STAT5, specific genes such as prostaglandin synthase (PGHS), inducible nitric oxide synthase (iNOS), detoxification enzymes such as CYP1A1 and CYP1B1, and other xenobiotic metabolizing enzymes (73,74). The expression of proinflammatory cytokines stimulates iNOS, contributing to the generation of RNS and thus to nitrosative stress (75). ROS are released with the activation of neutrophils; under the effect of Nox1, activated by IL-4 and TNF-α, as an effect of transition metals, and as a result of the metabolization of PAHs by CYP1A1/2 and CYP1B1, among other biological processes (76). Low levels of oxidative stress induce the nuclear factor-erythroid 2-related factor 2 (Nrf2)/antioxidant-response element (ARE) pathway, leading to the expression of antioxidant and phase II genes (77). In case of an excessive imbalance, high levels of ROS induce AP-1, NF-kβ and STAT3 expression, promoting inflammatory gene expression, growth and proliferation, and cytotoxic effects like apoptosis and necrosis (78).

DEPs are also recognised by TLRs in airway macrophages and epithelial cells, inducing the expression of IL-1, IL-6, IL-8, IL-25, IL-33, TNF-α, TSLP and GM-CSF, and promoting a type-2 immune response via Th2 or ILC2 (79,80).

1.2.1.2 Effects of diesel exhaust particles on asthma incidence

It is widely accepted that air pollution exacerbates asthma pathogenesis by causing oxidative stress, altering the immune homeostasis and modifying the genetic regulation of biological processes such as inflammation, physiology and susceptibility (35). What is more controversial is whether air pollution can induce asthma pathogenesis by itself.

Asthma varies considerably across the life course, and so does the effect of air pollutants on asthma pathogenesis. In adults, the association between DEPs exposure and asthma induction is still being debated. Some clinical studies report that DEPs alone induce asthma symptoms and bronchoconstriction in healthy adults (81) and

increase airway resistance in both healthy and asthmatic individuals (82), while other authors conclude that the current evidence is insufficient to indicate any causal role between DEPs and asthma during adulthood (83). The precise mechanisms linking DEPs exposure and adult asthma are still unknown, but some authors propose that it may be caused by the induction of oxidative stress by volatile organic compounds (VOCs) on the surface, the induction of a prolonged airway inflammation, immune activation of Th2, Th17 and ILC2 pathways, the exacerbation of allergic symptoms, or the induction of epigenetic changes and modifications of the host's microbiome (63).

In the paediatric population, the association between DEPs exposure and asthma incidence is much more evident. Epidemiological studies have associated DEPs with respiratory symptoms like cough, wheeze, and shortness of breath (84). Furthermore, other pollutants such as BC, NO_2, $PM_{2.5}$ and PM_{10}, have been associated with the development of transient and persistent asthma (85). Two studies have shown that truck-traffic and concentrations of black smoke and soot measured in schools are significantly associated with chronic respiratory symptoms and lower pulmonary function, which are more pronounced in girls than boys (86,87). In line with these results, sigificant associations have been found between traffic-related pollution at home and at school and childhood asthma incidence (88). Furthermore, exposure to $PM_{2.5}$ prenatally and during the first year of life has been related with increases in the absolute risk increase of persistent asthma at the age of five of 4.4% and 4.5% respectively (89). Using the PIAMA prospective birth cohort, Brauer *et al.* demonstrated that certain levels of NO_2 and $PM_{2,5}$ were associated with asthma during the first four years of life (90), while Gehring *et al.* reported that elements attached on the surface of diesel exhaust particles were responsible for asthma development (91).

1.2.1.3 Diesel exhaust particles and the modulation of the pre-existing asthma endotypes

As regards the effect of exposure to air pollution in patients with asthma, the mechanisms of action through which these particles can aggravate the pre-existing pathology are not clear. Nor is it known whether exposure to these particles affects all asthmatics in the same way. Some authors report that distinct immunologic pathways are altered, depending on the pre-existing endotype (63). In the type 2 Th2-driven endotype, traffic-related air pollution (TRAP) has been shown to enhance respiratory sensitization to environmental allergens (92), while it has been suggested that DEPs act as adjuvants in the induction of allergic asthma (34,93,94). In fact, Muranaka *et al.* first reported this effect on IgE production using a murine model of asthma to ovalbumin (OVA) (95). The immunological mechanisms have not been well characterised to date. Some studies hypothesize that DEPs carry allergens on their surface, cause oxidative stress (thus impairing airway epithelial surfaces and enhancing allergen diffusion), induce allergen sensitization by triggering the immune system, or modify the chemical structure of proteins, thus increasing allergen antigenicity (49,50,74). In support of these hypotheses, our group has recently reported that concomitant exposure to DEPs and soybean hull allergen increases the allergenic power of this protein (34). Besides, in the type 2 ILC2-driven endotype, some studies seem to indicate that DEPs interact with the lung epithelium, producing a chronic stimulation of airway epithelial cells and generating high levels of alarmins such as IL-33, IL-25, and TLSP (96,97). These cytokines are a direct stimulus of ILC2 cells involved in the nonallergic eosinophilic immune response (98,99). DEPs have also been reported to exacerbate HDM-asthma by acting on both Th2 and ILC2 pathways, increasing IgG_1 and AHR (100). Indeed, Estrella *et al.* observed that air pollution induces ILC2 cells to produce high amounts of IL-5 and IL-13 while causing AHR (101).

In non-type 2 asthma, DEPs are known to act on transient receptor potential (TRP) channels. These signal receptors are widely distributed throughout the body, expressed on neuronal and non-neuronal cells, such as macrophages and mast cells (102). On vagus nerve termini on the airways, these receptors act as environmental sensors initiating responses to exogenous and endogenous stimuli (69,102–104). DEPs have been reported to activate TRP ankyrin-1 (TRPA1) on murine dorsal root ganglion cells (69) and TRP vanilloid-4 (TRPV4) in bronchial epithelia (105). $PM_{2.5}$ also trigger TRPA1 and TRP vanilloid-1 (TRPV1) receptors in mice (102). Robinson *et al.* demonstrated that PAHs surrounding DEP trigger AhR and mitochondrial production of ROS, enhancing TRPA1 signalling on nociceptive C-fibers (104). Other effectors of TRPA1 and TRPV1 are IL-13, neurogenic inflammation mediator substance P, PGD_2 and nerve growth factor (NGF) (102). As previously described, these factors are also present during asthma events and mediate bronchial smooth muscle contraction, leading to the notion that DEPs may act on airway obstruction.

Regarding the Th2/Th17 mixed endotype, DEPs have been reported to enhance both Th2 and Th17 pathways (54,106). In line with these results, we observed that the co-exposure of soybean hull extract and DEPs triggers an asthmatic response when the aeroallergen concentration is too low to cause asthma by itself, increasing Th2-related cytokines such as IL-4, IL-5, IL-13 and IL-10, and Th17-related cytokines like IL-17A, IL-17F and CCL20 (34).

For all these reasons, and in order to determine which treatments may be of most benefit in each patient, it is essential to broaden our understanding of the immunological mechanisms underlying the interaction between air pollutants and asthma. To achieve this goal, several considerations need to be borne in mind: 1) there is a growing relationship between morbidity and mortality in asthmatic patients when co-exposed to environmental pollution; 2) there is evidence that this interaction may differ depending on the pre-existing asthma endotype; 3) the trend towards

personalised medicine is growing; and 4) biological drugs aiming to regulate immunological pathways involved are undergoing substantial development.

Therefore, the purpose of this doctoral thesis is to study the effects of DEP exposure over two different asthma endotypes by assessing respiratory mechanics, airway hyperresponsiveness, innate and adaptive immune responses, oxidative stress and particle deposition patterns. To do so, two different mouse models have been developed, in which the role of the immune system has been analysed after joint exposure to asthma-inducing agents and DEPs.

2 HYPOTHESIS

HYPOTHESIS

Exposure to diesel exhaust particles in asthmatic individuals worsens bronchial hyperresponsiveness and lung function and increases bronchial inflammation by modifying both the innate and adaptive immunity responses. These modifications differ according to the asthma endotype.

3 OBJECTIVES

3.1 MAIN OBJECTIVE

To study the effects of exposure to diesel exhaust particles on two different asthma endotypes by assessing respiratory mechanics, airway hyperresponsiveness, innate and adaptive immune responses, oxidative stress and particle deposition patterns.

3.2 SECONDARY OBJECTIVES

1) To study the effects of diesel exhaust particles exposure in a mouse model of chemical-induced asthma, a non-type 2 endotype.
2) To study the effects of diesel exhaust particles exposure in a mouse model exposed to non-asthmagenic doses of soybean hull extract, a Type 2 endotype.

4 COMPENDIUM OF PUBLICATIONS

4.1 ARTICLE 1. “THE IMMUNOMODULATORY EFFECTS OF DIESEL EXHAUST PARTICLES IN ASTHMA”

de Homdedeu M, Mj Cruz, S. Sanchez-Díez, Ojanguren I, C. Romero-Mesones, Vanoirbeek J, Vande Velde G, Muñoz X. The immunomodulatory effects of diesel exhaust particles in asthma. Environmental Pollution, Volume 263, Part A, 2020, 114600, ISSN 0269-7491. https://doi.org/10.1016/j.envpol.2020.114600. (https://www.sciencedirect.com/science/article/pii/S0269749120308897)

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DOI: 10.1016/j.envpol.2020.114600

4.2 ARTICLE 2. "ROLE OF DIESEL EXHAUST PARTICLES IN THE INDUCTION OF ALLERGIC ASTHMA TO LOW DOSES OF SOYBEAN"

M. de Homdedeu, M.J. Cruz, S. Sánchez-Díez, S. Gómez-Ollés, I. Ojanguren, D. Ma, X. Muñoz. Role of diesel exhaust particles in the induction of allergic asthma to low doses of soybean. Environmental Research, 2020, 110337, ISSN 0013-9351. https://doi.org/10.1016/j.envres.2020.110337.
(https://www.sciencedirect.com/science/article/pii/S0013935120312342)

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5 OVERALL SUMMARY OF RESULTS

OVERALL SUMMARY OF RESULTS

This doctoral thesis consisted of two studies. Briefly, the first study demonstrated that sensitization and inhalation of AP induced a T2 low asthma endotype, characterized by asthma-like lung parameters, such as higher levels of Rn, H, and AHR; and airway inflammation consisting of eosinophilia, and lower levels of CD11b-Ly6C- DCs. The DEP-exposed group showed slightly modified lung mechanics; airway inflammation with neutrophilia, higher levels of CD11b+Ly6C- DCs, and lower levels of alveolar macrophages; and reduced levels of cytokines, such as IL-13 and IFN-γ. The group coexposed to AP and DEPs presented exacerbated signs of asthma, in the form of asthma-like lung function; airway inflammation with higher levels of CD11b-Ly6C+ DCs, and lower total macrophages; and a differential deposition pattern of DEPs in the lungs, which was restricted to large conducting airways.

As regards the second study, the inhalation of 3 $mg \cdot ml^{-1}$ of SHE induced mild airway inflammation consisting in increased levels of eosinophils, B cells, CD11b+Ly6C- DCs, total and resident monocytes, together with reductions in NK cells, CD11b-Ly6C- DCs, and total and alveolar macrophages. The inhalation of 150µg of DEPs alone triggered airway inflammation with neutrophilia. Finally, the inhalation of SHE and DEPs together induced a mixed Th2/Th17 asthma endotype, characterized by asthma-like lung function with higher levels of AHR and Rn; and airway inflammation consisting in higher levels of SHE-specific IgE in serum, H_2O_2 in BAL, NK cells, CD11b-Ly6C+ DCs, and lower levels of CD11b+Ly6C+ DCs.

Table 1. DEP immunomodulatory effects on non-T2 and T2 asthma endotypes.

		Non-type2 Endotype	Type2 Endotype
Central airway resistance		↑	↑
Tissue elastance		↑	NS
PC20		↓	↓
Specific IgE		-	↑
H_2O_2		NS	↑
Eosinophils		↑	↑↑
Neutrophils		↑	NS
B Cells		NS	↑
NK Cells		↑↑	↑
Monocytes	Total	↓	NS
	Inflammatory	NS	↓
	Resident	NS	↑
Macrophages	Total	↓	↓↓
	Interstitial	↑	↑↑
	Alveolar	↓	↓↓
Dendritic Cells	Total	NS	↑
	CD11b-Ly6C-	↓	↓
	CD11b+Ly6C-	↑	↑
	CD11b+Ly6C+	NS	↓
	CD11b-Ly6C+	↑	↑↑

↑, increases; ↓, decreases; NS, no statistically significant differences; -, not analysed.

6 OVERALL SUMMARY OF DISCUSSION

OVERALL SUMMARY OF DISCUSSION

The results of this doctoral thesis show that exposure to DEPs in a non-type 2 asthma endotype increases Th2-related DCs. Moreover, in the type 2 endotype, coexposure to a non-asthmagenic dose of allergen and DEPs induce asthma features, such as higher levels of specific IgE, H_2O_2, oxidative stress-sensitive DCs, lower levels of Th1-related DCs and total and alveolar macrophage populations.

Although it has long been accepted that air pollution exacerbates and induces asthma pathophysiology (50,63,107,108), the immunological effects of DEPs are still not well characterized and seem to differ depending on the pre-existing asthma endotype (63). For example, in the T2 high endotype, TRAP enhances respiratory sensitization to environmental allergens (92), while it has been suggested that DEPs act as adjuvants in the induction of allergic asthma (34,93,94). In the Th2-driven T2 endotype it seems that DEPs may increase the antigenic capacity of certain aeroallergens (34,109), while in the ILC2-driven T2 endotype they may stimulate lung epithelial cells to produce high levels of IL-33, IL-25, and TLSP, cytokines related with ILC2s and eosinophilic asthma (17,101). In the non-T2 endotype, DEPs may aggravate neutrophilic asthma by increasing the production of both IL-17A and IL-17F cytokines (48). Lastly, our group reported that coexposure to SHE and DEPs could favour a mixed Th2/Th17 response, involving not only a Th2 inflammation with IL-4, IL-5, IL-13 and IL-10, but also increasing levels of chemokines related to the Th17 response such as IL-17A, IL-17F, and CCL20 cytokines (34).

The inhalation of DEPs has been related with oxidative stress; neutrophilia; inflammatory cytokines such as IL-1,IL-5, IL-6, IL-8, IL-13, IL-17A, IL-17F, IL-25, IL-33, TNF-α, TSLP, CCL20 and GM-CSF; AHR and altered lung parameters; lower levels of macrophage populations; and involvement of both Th2 and Th17 immune pathways (63,71,79,96,101,110,111). In the first study, three nasal instillations of 150µg of DEPs

increased CD11b+Ly6C- DCs, which are known to be Th2-related (112). Previous studies have shown that DEPs increase total DCs in both BAL and total lung leukocytes (113); but to our knowledge this is the first description of their ability to promote the type 2 immune response by priming this specific DCs subpopulation, previously reported to be the only DC subset to start the asthmatic response against HDM (114,115).

Dendritic cells are professional antigen-presenting cells endowed with the ability to stimulate naïve T cells. Depending on the agent presented, DCs orchestrate the nature of downstream T cell responses (116). They are believed to be the link between innate and adaptive immune systems, inducing either tolerance or immunity against foreign agents. They can be classified into cDC, pDC, and moDC; cDCs are divided further into cDC1 and cDC2 (116). In mice, lung cDC1 are CD103+, CD11b-, CD207+, XCR1+ and DNGRI+; while lung cDC2 are CD11b+, SIRPα+, and CX_3CR1^{mid}. Lung moDC are CD64+, CD11b+, SIRPα+, MAR-1+, CX_3CR1^{mid} and Ly6C+; and lung pDC are $CD11c^{mid}$, CD11b-, B220+, PDCA-1+, Ly6C+ and SIGLEC-H+ (112). While CD11b- DCs have been reported to be lymphoid-derived, CD11b+ DCs, both moDC and cDC2, are myeloid-derived. moDC are prone to retain antigens and reactivate Th1 cells (117) and cDC2 are decisive in triggering Th2 cell-mediated immunity by producing chemoattractants toward Th2 cells and eosinophils (115).

The inhalation of DEPs alone in the second study did not increase levels of CD11b+Ly6C- DCs. Comparing the two models, DEP exposures varied considerably, as the first group received three inhalations of 150µg of DEPs on days 15, 18 and 21; while the second received nasal instillations of saline five days per week for three weeks, including 150µg of DEPs three days per week. The first group, with three inhalations, exhibited a type 2-related immune response, while the second one, with nine, exhibited a type 17-related immune response. This comparison bears witness to the potential of DEP for activating different pathways in the immune system in a dose-

dependent manner. As observed by Muñoz *et al.*, low exposures to DEPs activate a non-inflammatory antioxidant response, intermediate levels induce a Th2 inflammatory response, and high levels induce an acute inflammatory response with predominance of Th17 and cytotoxic effects (63).

Mice exposed to SHE at 3 mg protein·ml^{-1} during five consecutive days over three weeks also presented higher levels of Th2-related CD11b+Ly6C- DCs. In fact, this treatment produced a T2-like immune response with B cells, eosinophils, resident monocytes, and lower levels of CD11b-Ly6C- DCs. This last subset of dendritic cells, also referred to as cDC1, have been reported to induce tolerance to inhaled antigens and ingest dying epithelial cells, cross-presenting them on MHC class I to CD8+ effector T cells in the lung-draining bronchial lymph node (brLN) (118). These results, together with the absence of any induction of AHR by SHE 3 mg·ml^{-1}, highlight the allergenicity of soybean hull dust and the clear dissociation between airway hyperresponsiveness and inflammation.

Inhalation of AP reduced levels of cDC1, as both AP and AP+DEP groups had significantly lower levels of CD11b-Ly6C- DCs, which are reported to be tolerogenic (112). In addition, AP-sensitized and challenged mice experienced asthma-like lung parameters and eosinophilia. These results show that AP, an irritant, reduces airway tolerance and causes significant lung epithelial damage characterized by AHR and airway inflammation. Furthermore, the AP+DEP group showed increased levels of CD11b-Ly6C+ DCs. Also known as pDCs, these cells are usually found in blood and lymphoid organs in the steady state (119); upon activation, they migrate to non-lymphoid tissues, where they produce large amounts of type I and type III IFNs, expressing MHC class II molecules and stimulating naïve T cells (120). Furthermore, pDCs have been associated with reduced levels of IFN-α in asthma (120) and have been reported to be oxidative stress-sensitive (112), although no statistically significant differences in H_2O_2 were observed in our study.

The ability of air pollutants to induce asthma pathogenesis is controversial, since it varies according to the population reported. In children, epidemiological studies have found DEPs to be associated with respiratory symptoms like cough, wheeze, and shortness of breath (84), while other pollutants such as BC, NO_2, $PM_{2.5}$ and PM_{10} have been associated with the development of transient and persistent asthma (85). Various studies have associated asthma with truck-traffic and black smoke (86,87), traffic-related pollution both at home and at school (88), exposure to $PM_{2.5}$ prenatally and during the first year of life (89), high levels of NO_2 and $PM_{2,5}$ during the first four years of life (90), and elements attached on the surface of diesel exhaust particles (91). In the adult population, some authors have associated asthma with DEP exposure (81,82), though others conclude that the evidence is insufficient (83). Basic research in mouse models of asthma has reported that DEP exposure enhances the allergic response to OVA (121), HDM (100) and SHE (34), among other allergens. Álvarez-Simón *et al.* reported that coexposure to DEPs triggers an asthmatic response when the SHE concentration is too low to cause a response by itself (34). Here, coexposure to 3 mg protein·ml^{-1} SHE and DEPs induced asthma, with increased levels of AHR and Rn, SHE-specific IgE and H_2O_2 in BAL, CD11b-Ly6C+ DCs, and reductions in CD11b+Ly6C+ DCs.

The higher levels of CD11b-Ly6C+ DCs after SHE and DEPs inhalation, as with the coexposure to AP and DEPs, demonstrate the ability of DEP to increase oxidative stress when administered together with other allergens and irritants. Oxidative stress is defined as an imbalance between reactive oxygen and nitrogen species and antioxidants, and results in biological damage (122). While asthma pathogenesis and air pollutants increase the production of reactive oxygen and nitrogen species in the airways (123,124), these species seem to induce asthma disease (125,126). In the second study, the SHE+DEP group experienced increased levels of CD11b-Ly6C+ DCs, lung pDCs sensitive to oxidative stress (112). As regards H_2O_2 in BAL, the

inhalation of SHE, DEPs or both agents together increased levels when compared with the control group, suggesting that the repetitive inhalation induces notable levels of oxidative stress. Although the SHE+DEP group had higher levels than SHE and DEP groups, the differences were not statistically significant. Taken together, these results suggest that pDC are enhanced when an asthmatic individual is exposed to an oxidative stress-causing environment.

The lower levels of CD11b+Ly6C+ DCs after SHE and DEPs inhalation demonstrate the downregulation of Th1 inflammation during the upregulation of the Th2 response. This reduction in moDCs is also associated with the activity of NK cells, a type of innate immune cells which are regarded as the host's first line of defence against tumours and viral infections and are involved in various lung diseases such as asthma (127,128). In asthmatic children, these cells have been reported to expand during acute exacerbation (129). In the adult population, severe asthmatics expressed higher levels of NK activation markers such as CD69, resulting in a positive correlation between eosinophilia and NK levels (130). Using animal models, researchers have found that NK cells contribute to asthma initiation, maintainance and resolution (128). They have also been reported to induce monocytes to differentiate into moDCs by producing GM-CSF, TNF-α and IFN-γ (131). In our studies, the inhalation of DEPs and AP increased NK levels, while SHE induced a reduction. This response of NK cells with the inhalation of SHE is in agreement with the lower levels of moDCs in SHE+DEP group compared with the DEP group, and shows that the inhalation of SHE triggers a type 2 inflammation by lowering the Th1 pathway, thus reducing NK cells and CD11b+Ly6C+ DCs.

As regards SHE-specific IgE, Muranaka *et al.* reported that DEPs enhanced the production of IgE in a mouse model of asthma to OVA (95). In soybean hull dust asthma, Álvarez-Simón *et al.* did not find increases in total IgE levels when mice were coexposed to 3 mg protein·ml^{-1} of SHE and DEPs. In the second chapter, using a

custom DELFIA®, we determined that the coexposure to both agents increased SHE-specific IgE levels. These results indicate the adjuvant activity of DEPs for the production of IgE antibody and are in agreement with the reduced levels of CD11b+Ly6C+ DCs, lung moDCs reported to enhance a type 1 immune response (112).

The effect of DEPs on neutrophilia is controversial; some authors report a causal relationship (34,132), but others do not (34,50,133). In both studies presented here, neutrophil levels in mice increased after the inhalation of DEPs. DEP exposure also reduced alveolar macrophages. In macrophages, the alveolar subpopulation develops from fetal monocytes, phagocytosing foreign agents without inducing an inflammatory response (134) and thus maintaining an immunosuppressive environment (135). In our studies, after engulfing DEPs, these cells seem to undergo apoptosis, as previously reported by Hiraiwa *et al.* (136). In order to restore the alveolar macrophage population, adult monocytes may be recruited to the lungs and differentiated into interstitial macrophages, thus reducing the total monocyte population as well (36) and increasing the proinflammatory response, as previously reported (135). Hussain *et al.* reported that if alveolar macrophages are unable to phagocytose all foreign agents, the activated alveolar macrophages recruit neutrophils into the lungs via MIP-2 and other neutrophil chemoattractants (137), thus inducing a proinflammatory state.

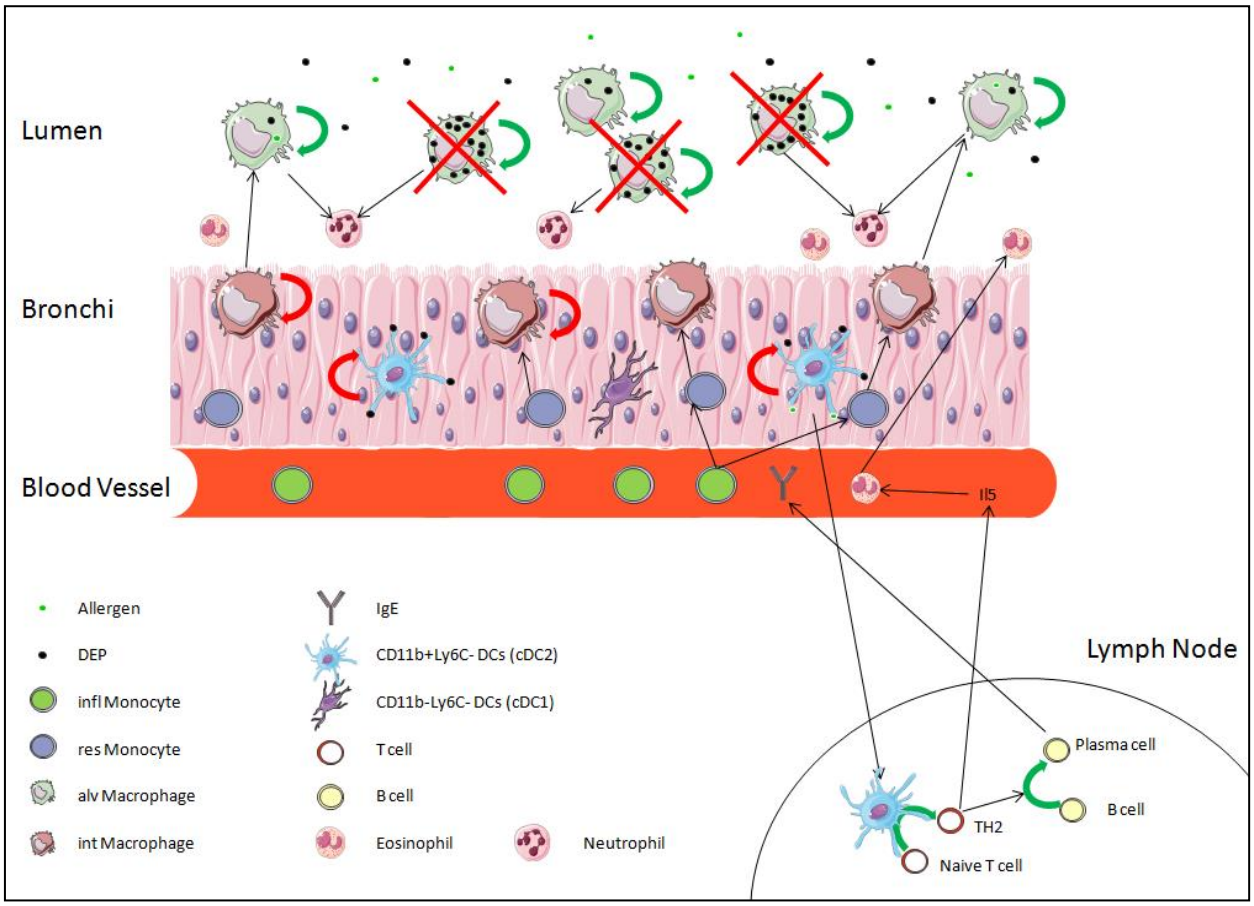

Figure 4. Immunomodulatory effects of DEPs on DCs and macrophages. Diagram showing the proposed mechanisms via which DEPs may act on DCs and alveolar macrophages, altering immune homeostasis in the airways.

Furthermore, coexposure to AP and DEPs reduced levels of total macrophages in total lung leukocytes, suggesting higher macrophage phagocytic activity, apoptosis and renewal. In this situation, optical projection tomography identified a differential deposition of DEPs in lungs. Optical projection tomography is an approach for 3D imaging of small biological specimens, filling the gap between magnetic resonance imaging and confocal microscopy (138). The scanner maximizes its depth-of-focus, producing optical images with a view right through the whole fluorescently-labelled specimen (139). With this technique, we observed that DEPs reached small peripheral airways in non-asthmatic mice, while in asthmatic mice particle deposition was

restricted to the large conducting airways. These differences may be caused by an irritant-induced nasal response to bronchoconstriction or to an increased inflammation of the asthmatic animal's airways, which may mechanically hamper the deposition in the most distal parts of the lung. As a consequence of this differential deposition, mice coexposed to AP and DEPs experienced lower levels of DEP-loaded macrophages in BAL, probably because only the macrophages in the large conducting airways engulfed these particles.

To sum up, the studies included in the present doctoral thesis demonstrate that DEPs modulate the immune system, and that different pathways drive the immune reaction depending on the pre-existing asthma endotype. To the best of our knowledge, the study in *Chapter 1* is the first to assess the combined effect of chemicals and DEPs, showing that DEPs and coexposure to AP and DEPs activate the innate immune response and exacerbate the immune hallmarks of asthma. It also provides the first evidence of the capacity of DEPs to increase the levels of CD11b+Ly6C- DCs, and shows a differential deposition pattern of DEPs in mouse lungs according to asthma status. The study in *Chapter 2* corroborates our previous results and shows that DEP coexposure induces asthma associated with SHE when the dose is too low to generate a response by itself, and does so by raising SHE-specific IgE and H_2O_2 levels and by altering lung leukocyte homeostasis.

7 CONCLUSIONS

CONCLUSIONS

Overall, the studies included in this doctoral thesis propose that the inhalation of diesel exhaust particles modulates the immune system and that the pathways that drive the immune reaction differ depending on the pre-existing asthma endotype.

The main conclusions obtained are:

1. The exposure to diesel exhaust particles activates the innate immune response and exacerbates chemical-induced asthma, a non-type 2 endotype.
2. The inhalation of diesel exhaust particles increases the levels of Th2-related dendritic cells (CD11b+Ly6C-).
3. The deposition pattern of diesel exhaust particles in mouse lungs differs according to asthma status.
4. Diesel exhaust particles coexposure induces asthma to allergens when the dose is too low to generate a response by itself. It does so by inducing airway hyperresponsiveness and altering lung leukocyte homeostasis.
5. The coexposure to allergens and diesel exhaust particles increases allergen-specific IgE and oxidative stress-sensitive dendritic cells (CD11b-Ly6C+) and decreases levels of Th1-related dendritic cells (CD11b+Ly6C+) and alveolar macrophage populations.

8 FUTURE LINES OF RESEARCH

FUTURE LINES OF RESEARCH

The studies that comprise this doctoral thesis, based on experimental animal models, show that the inhalation of DEPs modulates the immune system, and that the pathways that drive its response depend on the pre-existing asthma endotype.

In the first chapter, inhalation of DEPs induced higher levels of CD11b+Ly6C- DCs, although there was no resulting increase in eosinophils or AHR. Further studies analysing the induction of these CD11b+Ly6C- DCs should provide useful information about the interaction between air pollutants, dendritic cells and the type 2 inflammatory response.

In both studies, DEP exposure decreased alveolar macrophages. Within macrophages, the alveolar subpopulation develops from fetal monocytes, phagocytosing foreign agents without inducing an inflammatory response (134), and thus maintaining an immunosuppressive environment (135). Future studies should aim to characterize how macrophages engulf DEPs and the mechanisms of macrophage renewal in order to broaden our understanding of this interaction.

In this doctoral thesis, we have developed a specific flow cytometry panel to discriminate and quantify the relative amounts of eosinophils, neutrophils, T and B cells, NK cells, total, CD11b+Ly6C+, CD11b+Ly6C-, CD11b-Ly6C+ and CD11b-Ly6C- DCs, total, alveolar and interstitial macrophages, and total, inflammatory and resident monocytes from lung tissue immune cells. However, the absence of ILCs limits our understanding of the underlying cellular mechanisms and our ability to fully characterize the role of DEP in the existing endotypes. Further studies should focus on ILCs in order to reach a better understanding of the mechanisms involved.

As previously described, pulmonary sensory receptors detect changes on the airways and elicit reflex events (140). Moreover, TRP channels seem to play a role in asthma, as they have been described as environmental sensors acting on bronchoconstriction

(69,102–105). In the first chapter of this doctoral thesis, coexposure to AP and DEPs caused a differential deposition pattern of DEPs in the lungs, while the inhalation of SHE and DEPs did not. These differences seem to indicate that AP-sensitization and challenge induces a nasobronchial reaction and raise new questions about the role of these responses in asthma. Future studies should analyse the nervous system, focusing on the asthmatic endotype, the inducing agent, and the exposure method.

The role of IgE in asthma has been extensively studied (141–143). In the second chapter, we developed a custom DELFIA® to determine SHE-specific IgE levels and found that they were increased by coexposure to SHE and DEPs. As regards the findings of the first chapter, no immunoassays exist at present to identify AP-specific IgE, since no antibodies can bind ammonium persulfate. The development of an accurate persulfate antigen is an area of active research. Thus, new methods to identify specific IgE to AP and other agents should be created to better characterize asthma and allergy against these agents and the effect of air pollutants.

Taken together, all these proposals should contribute to unravel the mechanisms involved in the modulation of the immune system by DEPs and are likely to improve the treatment of asthma and the quality life of exposed patients.

9 BIBLIOGRAPHY

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