AGGRAVATING ENVIRONMENTAL FACTORS IN CHRONIC OBSTRUCTIVE RESPIRATORY DISEASES

Ellen Lanckacker

Promotor: Prof. Dr. Guy Joos
Co-promotor: Dr. Tania Maes

Thesis submitted to fulfil the requirements for the degree of

"Doctor of Philosophy in Medical Sciences"

2013

This work was supported by the Interuniversity Attraction Poles (IUAP) Program, Belgian State, Belgian Science Policy, Project P6/35 and the Concerted Research Action of the University of Ghent (BOF/GOA 01251504).

Cover:
left, above – Jaws of a house dust mite, shown at 1500x magnification
right, down – Electron microscopic photograph Staphylococcus aureus

ISBN: 9789081196246
Department of Respiratory Medicine
Laboratory for Translational Research in Obstructive Pulmonary Diseases
Ghent University Hospital
De Pintelaan 185
9000 Ghent
BELGIUM

List of publications

Robays LJ*, ***Lanckacker EA****, Moerloose KB, Maes T, Bracke KR, Brusselle GG, Joos GF, Vermaelen KY. * *both authors contributed equally*
Concomitant inhalation of cigarette smoke and aerosolized protein activates airway dendritic cells and induces allergic airway inflammation in a TLR-independent way.
J Immunol. 2009 Aug 15 ;183(4) :2758-66.

Lanckacker EA, Tournoy KG, Hammad H, Holtappels G, Lambrecht BN, Joos GF, Maes T.
Short cigarette smoke exposure facilitates sensitization and asthma development in mice.
Eur Respir J. 2012 Aug 16. [Epub ahead of print]

Huvenne W*, ***Lanckacker EA****, Krysko O, Bracke KR, Demoor T, Hellings PW, Brusselle GG, Joos GF, Bachert C, Maes T. * *both authors contributed equally*
Exacerbation of cigarette smoke-induced pulmonary inflammation by *Staphylococcus aureus* enterotoxin B in mice. Respir Res. 2011 May 27;12:69.

Lanckacker EA, Robays LJ, Joos GF, Vermaelen KY.
A new danger in the air : how pulmonary innate immunity copes with man-made airborne xenobiotics. J Innate Immun. 2010;2(2):96-106.

Maes T, Provoost S, ***Lanckacker EA***, Cataldo DD, Vanoirbeek JA, Nemery B, Tournoy KG, Joos GF. Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. Respir Res. 2010 Jan 21;11:7.

Table of contents

Abbreviations

iNK	invariant natural killer
DC	dendritic cell
APC	antigen-presenting cell
TLR	toll-like receptor
PAMP	pathogen-associated molecular pattern
CO	carbon monoxide
NOx	nitrogen oxides
SO2	sulphur dioxide
PAH	polycyclic aromatic hydrocarbon
PM	particulate matter
CS	cigarette smoke
COPD	chronic obstructive pulmonary disease
Ig	immunoglobulin
HDM	house dust mite
Treg	regulatory T-cell
MHCII	major histocompatibility complex II
IL	interleukin
CCR	CC chemokine receptor
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
MBP	major basic protein
ZO	zonula occludens
CCL	CC chemokine ligand
MCP-1	monocyte chemotactic protein-1
MIP-3α	macrophage inflammatory protein-3α
PRR	pathogen recognition receptor
PAR	protease-activated receptor
DAMP	damage-associated molecular pattern
GM-CSF	granulocyte macrophage colony-stimulating factor
TSLP	thymic stromal lymphopoietin

ATP	adenosine triphosphate
UA	uric acid
EMT	epithelial-to-mesenchymal transition
EGF	epithelial growth factor
TGF-β	transforming growth factor-β
ETS	environmental tobacco smoke
LPS	lipopolysaccharide
VEGF	vascular endothelial growth factor
DEP	diesel exhaust particles
OVA	ovalbumin
ROS	reactive oxygen species
CXCL	chemokine CXC ligand
MMP	matrix metalloproteinase
IFN-γ	interferon-γ
$LT\alpha_1\beta_2$	lymphotoxin-$\alpha_1\beta_2$
LTβR	lymphotoxin-β receptor
ICAM-1	intercellular adhesion molecule-1
RSV	respiratory syncytial virus
S. aureus	*Staphylococcus aureus*
TCR	T-cell receptor
SE	*Staphylococcus aureus* enterotoxin
SEB	Staphylococcus aureus enterotoxin B
HNEC	human nasal epithelial cells
ELISA	enzyme-linked immunosorbent assay
RT	reverse transcriptase
PCR	polymerase chain reaction
HMGB-1	high-mobility group box 1
HA	hyaluronic acid or hyaluronan
LMW	low molecular weight
HMW	high molecular weight
WT	wild type
KO	knockout

Summary

Asthma and chronic obstructive pulmonary disease (COPD) are respiratory disorders characterized by shortness of breath, mucus production and cough. Over the last decades, the incidence and severity of both diseases has increased continuously, especially in the industrialized West. Although genetic predisposition is a fundamental factor underlying both disorders, the observed increase in prevalence has occurred too rapidly to be explained by genetic variations alone, implicating a role for environmental factors.

The strongest predictive factor for the development of asthma is the sensitization to house dust mite (HDM) allergens. Additionally, epidemiological studies have demonstrated that cigarette smoke (CS) exposure is a considerable risk factor for the development or progression of allergic asthma. To better understand the impact of CS exposure on immunological responses towards allergens, we took advantage of a previously established mouse model in which CS breaks inhalation tolerance to the "surrogate" allergen ovalbumin. Investigating the impact of CS inhalation on key aspects of airway dendritic cells (DCs), revealed enhanced DC recruitment and maturation in mice concomitantly exposures to CS and ovalbumin for 3 weeks. Prolonged CS inhalation further amplified the DC-mediated transport of inhaled ovalbumin to the draining lymph nodes. In order to unravel the underlying mechanisms leading to a Th2-oriented immune response, we hypothesized a role for the endotoxin-like effects of CS in facilitating allergic sensitization by triggering the endotoxin-recognition receptor TLR4 on epithelial cells or DCs. Surprisingly, neither TLR4 deficient mice, nor the adaptor protein MyD88 knockout mice were protected against CS-induced facilitated Th2 immunity, suggesting that other pathways are involved.

Importantly, the differences in biochemical and immunogenic characteristics of the surrogate allergen ovalbumin and real-life allergens (e.g. HDM) may have a profound impact on the mechanisms behind the elicited allergic response. Accordingly, we established a novel mouse model with improved clinical relevance, using HDM as real-life allergen, together with CS as indoor pollutant. We provided biological proof that CS indeed favours HDM-driven asthma development, as illustrated by increased characteristics of asthma, such as enhanced eosinophilia, elevated production of Th2-related cytokines, increased airway

hyperresponsiveness and HDM-specific serum IgG1. Furthermore, we demonstrated that CS inhalation during the sensitization phase is sufficient to induce asthma development in mice. We found that only a few days of CS inhalation during the initial allergen contact already enhanced DC recruitment, activation and migration to the lymph nodes, supporting the local induction of HDM-specific Th2 immunity. Finally, using IL1RI deficient mice, we demonstrated IL1RI to be necessary to prime local Th2 responses in the lymph nodes.

Chronic CS inhalation is an important risk factor for the development of COPD. In addition, repeated CS exposure may compromise the epithelial barrier function against invading pathogens, making COPD patients more prone to develop acute exacerbations with aggravation of symptoms. The discovery of specific IgE antibodies directed against *Staphylococcus aureus* enterotoxins in patients with COPD, provides indications that these antigens may act as potential aggravating factors of COPD pathophysiology. To focus on the disease-aggravating role of bacterial superantigens, we designed a novel mouse model of concomitant exposure to CS and *Staphylococcus aureus* enterotoxin B. Simultaneous exposure to both stimuli resulted in a significant aggravation of hallmark features of CS-induced pulmonary inflammation, such as a marked increase in $CD8^+$ T lymphocytes and neutrophils, enhanced goblet cell hyperplasia and the formation of dense lymphoid aggregates in the lung.

In conclusion, we demonstrated a role for environmental factors in the induction, progression or aggravation of asthma and COPD, using mouse models of combined exposures to natural and anthropogenic environmental stimuli.

Samenvatting

Astma en chronisch obstructief longlijden (COPD) zijn veel voorkomende respiratoire ziekten, met klachten als kortademigheid, chronisch hoesten en slijm. De laatste jaren is de prevalentie en de ernst van beide ziekten, vooral in de geïndustrialiseerde landen, sterk toegenomen. Niettegenstaande de genetische achtergrond een belangrijke risicofactor kan zijn in het ontstaan van astma en COPD, worden vooral omgevingsfactoren verantwoordelijk geacht voor de snel toegenomen prevalentie.

De belangrijkste predictieve factor voor het ontstaan van astma, is de sensibilisatie ten opzichte van huisstofmijt (HSM) allergenen. Uit epidemiologische studies blijkt dat sigarettenrook (SR) eveneens een belangrijke trigger is in het ontstaan en de progressie van de ziekte. Om inzichten te verwerven in de moleculaire mechanismen verantwoordelijk voor SR-geïnduceerd astma, werd initieel gebruik gemaakt van een reeds bestaand muismodel waar SR de normale inhalatietolerantie ten opzichte van het “surrogaat” allergeen ovalbumine verstoort. Onderzoek naar het effect van SR op het gedrag van pulmonaire dendritische cellen (DCs), de belangrijkste immuunsensors in de long, onthulde een verhoogde rekrutering en maturatie van DCs in muizen die 3 weken werden blootgesteld aan een combinatie van SR en ovalbumine. Langdurige SR inhalatie amplificeerde eveneens het DC-gemedieerde transport van dit antigen naar de drainerende lymfeknopen. Een belangrijk mechanisme, verantwoordelijk voor de DC activatie en de ontwikkeling van Th2 responsen in de long, is de TLR4 – endotoxine pathway. Daarom werden TLR4 deficiënte muizen, alsook adaptor eiwit MyD88 knock-out muizen blootgesteld aan sigarettenrook in combinatie met OVA aerosol. De deficiënte muizen bleken echter op even efficiënte wijze als de wildtypes allergische inflammatie te genereren in de luchtwegen, wat doet vermoeden dat alternatieve mechanismen betrokken zijn in de ontwikkeling van SR-geïnduceerd astma.

Het verschil in biochemisch en immunogeen karakter tussen het surrogaat allergeen ovalbumine en werkelijke allergenen (vb. HSM) kan echter de onderliggende mechanismen van een allergische respons in sterke mate beïnvloeden. Bijgevolg werd een nieuw muismodel ontwikkeld, gebruik makend van HSM als klinisch relevant allergeen, gecombineerd met SR als omgevingspolluent. Gebruik makend van dit model kon biologisch

bewijs geleverd worden dat SR inderdaad de ontwikkeling van HSM-geïnduceerd astma kan beïnvloeden, wat zich vertaalde in een toegenomen astma fenotype, zoals een stijging in eosinofilie, verhoogde productie van Th2-gerelateerde cytokines, toegenomen luchtweg hyperreactiviteit en HSM-specifiek serum IgG1. Verder bleek de blootstelling aan SR tijdens de sensitisatie fase reeds voldoende om astma te ontwikkelen. Meer nog, een kortstondige SR blootstelling van slechts enkele dagen, tijdens de eerste contacten met het allergeen, was reeds voldoende om DCs in verhoogde mate te activeren en te rekruteren naar de long, alsook hun migratie naar de lymfeknopen te stimuleren en zó HSM-specifieke Th2 immuniteit te induceren. Finaal kon, gebruik makend van IL1RI deficiënte muizen, aangetoond worden dat IL1RI noodzakelijk is voor de inductie van deze Th2 responsen in de lymfeknopen.

De chronische blootstelling aan SR is een belangrijke risicofactor voor de ontwikkeling van COPD. Aangezien SR de mucosale weerstand tegen invasieve pathogenen verzwakt, kan dit COPD patiënten gevoeliger maken voor de ontwikkeling van acute exacerbaties met versterkte symptomen tot gevolg. De ontdekking van specifieke immunoglobulines in COPD patiënten, gericht tegen de virulente enterotoxines afkomstig van *Staphylococcus aureus*, doet vermoeden dat deze antigenen mogelijks een rol spelen in de aggravatie van COPD pathofysiologie. Om dit te onderzoeken, ontwikkelden we een nieuw muismodel van gecombineerde blootstelling aan SR en *Staphylococcus aureus* enterotoxine B. Gelijktijdige blootstelling aan beide stimuli resulteerde in een significante aggravatie van typische kenmerken van CS-geïnduceerde pulmonaire inflammatie, zoals een verhoogde toename van $CD8^+$ T lymfocyten en neutrofielen, toegenomen slijmbeker hyperplasie en de vorming van compacte lymfoide aggregaten in de long.

In conclusie, gebruik makend van muismodellen waarin de blootstelling aan natuurlijke en antropogene omgevingsstimuli werden gecombineerd, hebben we aangetoond dat omgevingsfactoren een belangrijke rol kunnen spelen in de inductie, progressie en aggravatie van astma en COPD.

PART I: INTRODUCTION

In the following 3 chapters, we will highlight different aspects of asthma and COPD pathogenesis, which will be addressed in the thesis further on.

CHAPTER 1: ANCIENT MEETS NOVEL: PULMONARY INNATE IMMUNITY AND THE RISE OF ANTHROPOGENIC STIMULI

1

1.1. Pulmonary innate immunity

The innate immune system of the lung is one of the most critical homeostatic systems of the body. Life-threatening damage to the delicate gas-exchange structures can occur either by failure to rapidly detect and clear inhaled airborne pathogens, or as a result of an unbridled inflammatory response. Hence, the integrity of the lung must be protected at all times.

Despite their structural vulnerability, the lungs defend themselves effectively through a combination of mechanical, humoral (adaptive immunity) and cellular mechanisms [1,2]. The airway epithelial lining, composed of ciliated and mucous-secreting cells, provide the first line of defense against invading pathogens [3,4]. Beating of the cilia moves a continuous stream of mucus, trapping and ejecting inhaled pathogens from the lung. Antimicrobial peptides and pulmonary surfactant proteins, synthesized by the epithelium of the deeper alveolar zones, further constitute an additional immediate mechanism of defense [5]. Phagocytic cells, like alveolar macrophages and neutrophils, complete the picture by neutralizing persistent pathogens that broke through. Other important sensors of innate immunity are invariant natural killer (iNK) T cells and pulmonary dendritic cells (DCs).

Pulmonary DCs are a heterogenous population of antigen-presenting cells (APC) with the unique ability to initiate appropriate adaptive immune responses in the lung [6]. They reside in tissues in close contact to the external environment and form an extensive network immediately above and beneath the basement membrane and within the interalveolar septa. Their specialized surface receptors (e.g. C-type lectin receptors, Toll-like receptors (TLRs)) recognize highly conserved pathogen-associated molecular patterns or PAMPs [7]. Inhalation of invading pathogens unveils the innate character of airway DC dynamics, reflected by their fast and massive recruitment into the airways and lungs [8]. Capture of inhaled antigens leads to DC activation and migration to the T cell zones of draining thoracic lymph nodes and depending on the nature of the antigen, DCs will selectively promote Th1, Th2 or Th17 immunity [9].

1.2. The rise of anthropogenic stimuli [#]

Throughout evolution, innate immunity had to co-evolve constantly in order to remain effective. Dating back millions of years ago, the explosive colonization of the land by a myriad of organisms characteristic for that time, must have brought a constant selection pressure on the very first airway innate immune defenses, especially with the development of the first vertebrate lungs 360 million years ago [10,11]. Seen on this time-scale, human evolution is an extremely recent event.

Since prehistoric times, and boosted by the first industrial revolution, human activity has resulted in the ever increasing release of airborne xenobiotic compounds, most of them derived from the combustion of fossil fuels (e.g. diesel, charcoal) [12] or tobacco [13]. Xenobiotics (Greek *xenos* = foreigner, stranger) are chemically synthesized compounds that do not exist naturally and are thus 'foreign to the body' [14]. Carbonaceous particles (e.g. diesel exhaust particles), as well as a broad array of volatile compounds (carbon monoxide (CO), nitrogen oxides (NOx), sulphur dioxides (SO_2)) and the more carcinogenic polycyclic aromatic hydrocarbons (PAHs) are all important classes of pollutants with 'unnatural' effects. Because of their small size (< 10 µm PM_{10}, < 2.5 µm $PM_{2.5}$, < 0.1 µm $PM_{0.1}$), airborne particulates can be easily inhaled and absorbed into the bloodstream or deposited in the deeper zones of the lung, causing adverse respiratory and cardiac effects [15-17].
Furthermore, due to socioeconomic changes, higher levels of combustion products have been reached since the widespread use of tobacco. Emerging evidence suggests a prominent role for cigarette smoke (CS) as indoor risk factor for the development of respiratory diseases [18]. Cigarette smoke is a major and entirely preventable cause of disease in middle- and high-income countries and kills nearly 6 million people each year. Unless urgent action is taken, the annual death toll can rise to more than eight million by 2030 [19].

The question arises how the immune system of the lung deals with these evolutionary recent anthropogenic stimuli. It can be assumed that confrontation of un-evolved airway DCs with these modern agents induces aberrant immune responses in the lung, giving a new view on the pathogenesis of several chronic inflammatory pulmonary diseases, like asthma and chronic obstructive pulmonary disease (COPD).

[#] Based on: 'A new danger in the air: how pulmonary innate immunity copes with man-made airborne xenobiotics.' Lanckacker EA, Robays LJ, Joos GF, Vermaelen KY. J Innate Immun. 2010;2(2):96-106.

CHAPTER 2: ALLERGIC ASTHMA

2

2.1. Introduction

Asthma is a chronic inflammatory disease, characterized by recurrent episodes of wheezing, breathlessness, chest tightness and cough. The symptoms are usually associated with increased airway hyperresponsiveness and variable airflow obstruction, which is often reversible, either spontaneously or after treatment [20].

Extensive work has been done to understand the complex aetiology of the asthmatic disease. Most asthmatic patients are atopic or genetically predisposed to produce immunoglobulin (Ig)E antibodies to common environmental allergens, like house dust mites (HDMs), molds, grass pollen or animal dander. More than 50% of all asthma cases are attributed to atopic (allergic) Th2-high eosinophilic asthma, however an important part is non-Th2 prone [21,22]. Non-Th2-related asthma is often associated with severe asthmatic disease and characterized by a mixed Th1/Th2 phenotype and neutrophilic airway inflammation [23]. The introductory part of this thesis will especially focus on atopic Th2-prone allergic asthma. For more information about different asthma phenotypes, we refer to the review of Wenzel *et al*. [23].

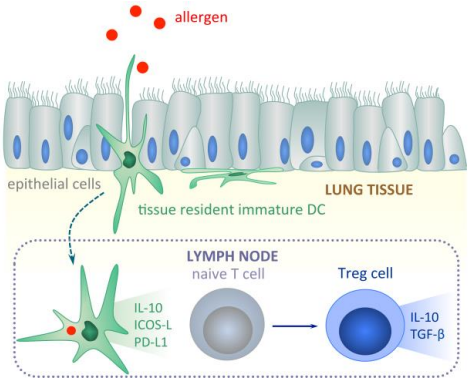
Allergic asthma results from aberrant immune responses towards inhaled aeroallergens and originates from allergic sensitization, followed by repetitive and persistent re-exposure to the same allergen. Typical for allergic asthma is the chronic inflammatory response, accountable for almost all the cardinal features of asthma pathophysiology. The persistence and nature of the inflammation, largely determines the severity of the disease, which can vary from intermittent to mild, moderate or severe persistent asthma or can even be fatal [24].

2.1.1. Inhalation tolerance

DCs play a crucial role in determining the outcome of an immune response upon mucosal antigen encounter. The mucosal environment itself instructs DCs to induce an immune response that is either tolerogenic or immunogenic. In *steady state* or in the absence of any 'danger' signal, tissue resident immature DCs express low levels of co-stimulatory molecules on their cell surface. DCs will take-up harmless environmental compounds and present them

in the lymph nodes, inducing regulatory T cells (Tregs) who will further maintain the homeostasis in the lung (***Figure 1***).

In healthy individuals, immune tolerance is the normal functional outcome upon allergen exposure. Failure of these endogenous tolerance mechanisms are thought to be responsible for allergen sensitization and the development of an aberrant immune response.



Arrow: ----→ migration; ——→ differentiation. Figure by Lanckacker EA

2.1.2. Mucosal allergic sensitization towards aeroallergens

The strongest predictive factor for the development of asthma, is the sensitization to aeroallergens. Allergic sensitization is defined as the phase between the initial encounter of the allergen in the body and the development of increased IgE sensitivity, however without causing any allergic symptoms yet. Sensitization generally begins in early childhood and may last a few days, several months or even years.

The airway DCs are the key regulators of allergic sensitization in the lung. They form an extensive network immediately above and beneath the airway basement membrane and project their dendrites in between the epithelial cells to sample the incoming air without breaking the epithelial integrity [25]. DCs have to sense (exogenous) danger signals to get properly activated. This danger signal can originate directly from the allergen itself or from the local tissue environment and determines the nature of the subsequent immune response. Especially the release of Th2-skewing mediators from the airway epithelium are important (see ***Chapter 2.2***). Once activated, DCs take up the encountered allergens, process them into small peptides and present them, in the context of major histocompatibility complex (MHC) II molecules, to naïve $CD4^+$ T cells in the lymph nodes (***Figure 2***). During their migration to the lymph nodes, DCs differentiate into professional antigen presenting cells, expressing costimulatory molecules (CD80, CD86) that favour the differentiation and clonal expansion of allergen-specific T helper 2 (Th2) effector cells [26]. Moreover, the differentiation

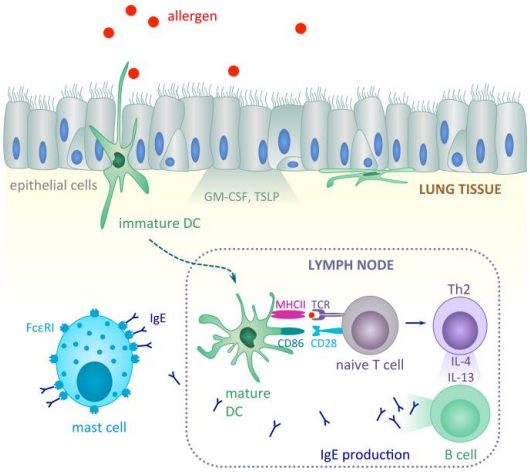


Figure 2: *Mucosal sensitization in atopic patients. Arrow: ----> migration; ——> differentiation.* *Figure by Lanckacker EA, based on Galli et al. Nature. 2008 Jul 24;454(7203):445-54*

towards the Th2 phenotype seems to be dependent on the so-called 'early IL-4', probably released by basophils as primary source [27]. The induction of a Th2 immune response results in the further release of interleukin (IL)-4 and IL-13. These cytokines will stimulate allergen-specific B cells to undergo immunoglobulin class switch recombination from IgM to IgE [28]. Allergen-specific IgE binds the high affinity IgE receptor FcεRI expressed on mast cells and basophils and remains there until re-exposure of the same allergen occurs (***Figure 2***). Once initiated, the IgE response can be further amplified by basophils, mast cells and activated eosinophils. From now on, the subject is sensitized, allergen re-exposure may result in a rapid allergic reaction.

2.1.3. Allergic inflammation and asthma development

In sensitized individuals, re-introduction of the allergen will crosslink adjacent IgE molecules on mast cells and basophils, activating them to release the content of their granules into the surrounding fluids (***Figure 3***). The rapid release of preformed and newly synthesized proinflammatory mediators contributes to the acute signs and symptoms of the allergic inflammatory response. The *early phase* of an allergic reaction typically occurs within minutes or even seconds following allergen exposure. The local release of proinflammatory mediators such as histamine, eicosanoids (e.g. leukotriene (LT) B4, prostaglandin (PG) D2) and proteases (e.g. tryptase) induces vasodilation, bronchoconstriction and excessive mucus production. During the *late phase* reaction, other mast cell mediators such as multifunctional cytokines (e.g. IL-8, CC chemokine ligand (CCL)-2, tumor necrosis factor (TNF)-α) and growth factors (e.g. granulocyte macrophage colony-stimulating factor (GM-CSF)) further induce chemotactic recruitment and activation of eosinophils, neutrophils and Th2 effector cells. The late phase reaction takes place 2 to 6 hours after allergen rechallenge and may even persist a few days. The local recruitment of Th2 cells implies the production of

Th2-cytokines which account for the complex features of asthma. The infiltration of eosinophils in the lungs, is mediated by IL-5 in concert with CC chemokine receptor (CCR)3 chemokines, such as eotaxin. IL-5 is also important for the proliferation, activation and survival of eosinophils. The IgE antibody production requires the release of IL-4 and IL-13. In addition, these cytokines will be responsible for the up-regulation of vascular cell adhesion molecule (VCAM)-1 on endothelial cells, facilitating transendothelial migration of very late antigen (VLA)-4 positive inflammatory cells. Furthermore, IL-13 together with IL-9 can change the excitability of bronchial smooth muscle cells, inducing airway hyperresponsiveness and goblet cell hyperplasia. IL-9 is also identified as potent mast cell growth and differentiation factor (***Figure 3***) [24].

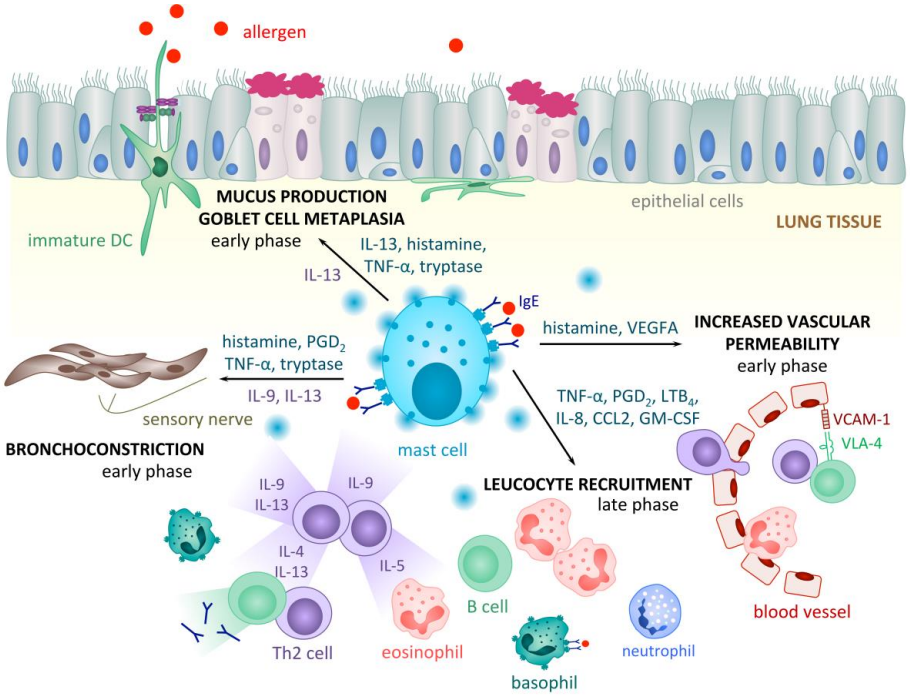


Figure 3: *Early and late phase response after allergen rechallenge. The early phase response is characterized by mast cell activation through cross-linking of allergen-specific IgE and results in bronchoconstriction, increased vascular permeability and mucus production. The late phase reaction is characterized by infiltration of the airway wall with eosinophils and lymphocytes. Arrow: ⟶ functional consequences of mast cell mediator release.*

Figure by Lanckacker EA, based on Galli et al. Nature. 2008 Jul 24;454(7203):445-54.

2.1.4. Chronic allergic inflammation

Continuous or repetitive allergen exposure results in a persistent inflammatory response. In the beginning, the inflammation is largely restricted to the conducting airways. However, as

the disease becomes more chronic, cellular infiltrates (eosinophils, lymphocytes, neutrophils) disperse to the trachea and the smaller airways and finally, take up residence in the tissues. Especially the presence of eosinophils is characteristic for chronic allergic airway inflammation. Eosinophils are thought to be major contributors to tissue damage, as activated eosinophils release potential tissue damaging superoxides, eicosanoids, major basic protein (MBP) and a range of cytokines and chemokines [29]. Interestingly, eosinophilic inflammation and airway remodeling, have been reported in biopsies from young children, suggesting that these changes might be initiated, unexpectedly, early in life [30]. Repetitive tissue damage due to chronic airway inflammation is associated with substantial thickening of the airway wall and may account for severe airflow obstruction and decline in lung function, as observed in established asthma. Airway wall remodeling includes epithelial fragility, goblet cell metaplasia and submucosal gland enlargement, increased deposition of extracellular matrix proteins (tenascin, fibronectin, and type I, III and V collagen) and increased smooth muscle mass index [31]. In addition, the mitotic activity of the airway epithelium, required for restoration of the denuded place, may be suppressed in patients with asthma and may explain the abnormal repair response to injury [32].

2.2. Role of the airway epithelium

Airway epithelial cells make up the first line of defense against various particles and aeroallergens. The physical barrier function of the epithelium depends on the coordinate expression of tight junction (e.g. claudins, occludin, zonula occludens (ZO)-1, 2, 3) and adherens junction proteins (E-cadherin, β-catenin) [33]. Most of the clinically relevant allergens have protease activity, degrading these transmembrane proteins to promote allergen access to the DC network underneath. Loss of E-cadherin for example has been described in biopsies from patients with asthma [34] and may facilitate allergic sensitization by modulating DC biology [35].

The airway epithelium is pivotal in the development of immune responses in the lung (***Figure 4A***). The unique interplay between epithelial cells and DCs is crucial for the induction of inflammatory disorders, such as asthma [36]. Epithelial cells and DCs interact through the release of cytokines and other soluble compounds. CCL2 and CCL20 for example are produced by airway epithelial cells following environmental exposures and are known to

attract monocytes and immature DCs to the lung [37-39]. In addition, airway epithelial cells express an array of pattern recognition receptors (PRRs) (TLRs, NOD-like Receptors, C-type lectins, Protease-activated receptors (PARs)), used to rapidly detect and respond to pathogens or their PAMPs; and to damage-associated molecular patterns (DAMPs), released upon cell damage or stress. Signalling through PRRs leads to NF-κB activation and the subsequent release of pro-inflammatory cytokines by airway epithelial cells. Hammad *et al.* demonstrated that epithelial activation of TLR4 in response to HDM or LPS drives the production of GM-CSF, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 [40]. These pleiotropic innate cytokines share the capacity to instruct DCs to mount Th2-mediated cell responses in the lung. TSLP and GM-CSF for example are key initiators of allergic airway inflammation. Neutralization of GM-CSF during sensitization, strongly reduced all salient features of HDM-induced asthma [41,42] and overexpression of both cytokines in the murine airway compartment induces Th2 sensitization to otherwise innocuous antigens [43,44]. In addition, naive CD4+ T cells primed by TSLP-stimulated DCs produce the typical proallergic cytokines IL-4, IL-5 and IL-13 [45]. IL-25, also known as IL-17E, has been shown to regulate type 2 immunity through its direct effect on TSLP-DC activated Th2 memory cells and directly stimulates activated memory cells to further differentiate into Th2 effector cells [46,47]. Furthermore, IL-33, a member of the IL-1 family, interacts with the orphan receptor ST2, preferentially expressed on DCs, Th2 cells and mast cells. IL-33-activated DCs are crucial for T-cell proliferation and Th2 polarization in the draining lymph nodes [48]. Also other members of the IL-1 family, such as IL-1α and IL-1β, are released by bronchial epithelial cells upon environmental exposures. Willart *et al.* showed that IL-1α, and to a lesser extent IL-1β, is sufficient to promote mucosal sensitization to inhaled protein allergens [42].

In addition to the production of cytokines, DAMPs such as, uric acid (UA) and extracellular adenosine triphosphate (ATP) are increased in the airways of mice and asthmatic patients following persistent allergen exposure [49-51]. Kool *et al*. demonstrated an important role for UA in mediating HDM-induced Th2 cell immunity through the production of innate cytokines (GM-CSF, TSLP and IL-25) and the recruitment of inflammatory DCs [49]. Furthermore, Idzko *et al.* identified a crucial role for ATP in the process of Th2 sensitization. Administration of exogenous ATP to the murine lung induced a break of inhalation tolerance and the enhanced recruitment and activation of lung DCs [50].

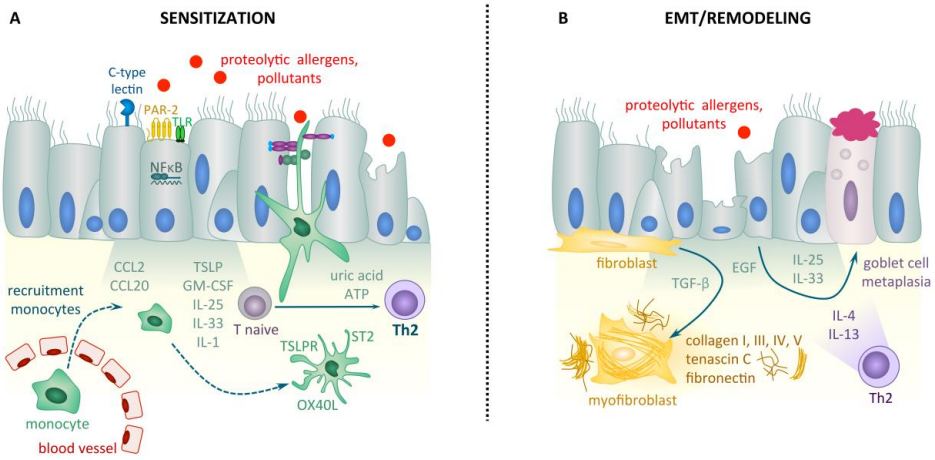


Figure 4: *Role of the airway epithelium during sensitization and airway wall remodeling.*
Arrows: ----→ migration; ——→ differentiation.
Figure 4A by Lanckacker EA, based on Hammad et al. Nat Rev Immunol. 2008 Mar;8(3):193-204.

The airway epithelium is not only crucial during mucosal sensitization. The dynamic changes of the epithelium due to persistent environmental insults, may alter epithelial-mesenchymal communication and may play an important role in airway wall remodeling [52] (***Figure 4B***). One of the early events in the so-called epithelial-to-mesenchymal transition (EMT) is the disassembly of tight junction proteins and the epithelial release of repair markers such as epithelial growth factor (EGF) and transforming growth factor (TGF)-β. EGF is a key cytokine in promoting goblet cell metaplasia [53], however other cytokines (IL-13, IL-25, IL-33) secreted from cells of the innate and adaptive immune system are also involved. TGF-β controls the proliferation of fibroblasts and their transdifferentiation into myofibroblasts, which promote the synthesis of extracellular matrix components, like collagen or fibronectin [54].

2.3. Risk factors for allergic sensitization

Endogenous as well as exogenous factors may affect the chance of being sensitized to common allergens. Host factors (atopy, gender, age, race) combined with environmental influences (environmental allergens, pollutants, infection history, birth order, diet, drugs, obesity) will determine the progression of asthma as a disease.

Genetic studies identified distinct regions within the human genome, closely associated with asthma, atopy and bronchial hyperresponsiveness. Gene variants on chromosome *17q21* associated with the expression of *ORMDL3*, are linked with asthma susceptibility and early-onset asthma, especially in the presence of environmental tobacco smoke (ETS) [55,56]. Furthermore, variations within the *TSLP* and *IL33* genes and within the *IL1RL1* gene, encoding the IL-33 receptor, have shown associations with many atopic phenotypes [57,58]. However, the relationship between allergy and atopy is rather complicated. Not everyone with atopy develops clinical manifestations of allergy and not everybody with clinical symptoms is atopic.

Besides genetic factors, personal aspects such as **age** or **gender**, are known risk factors for asthma. Boys suffer more often from asthma than girls, whereas in adults the prevalence is reversed [59]. Importantly, early childhood represents the 'prime time' for initial sensitization. Many reports already demonstrated the presence of allergen-reactive T-cells in cord blood, giving evidence for transplacental priming [60]. Moreover, infant T cells are predominantly Th2-prone and neonatal APCs represent a relatively immature phenotype, explaining the increased risk for Th2 sensitization early in life [61].

According to the hygiene hypothesis, a lack of early childhood exposure to infectious agents increases the risk for allergic disease [62]. Exposure to microbes or their biological products (e.g. endotoxin/lipopolysaccharaide (LPS)) can protect against the development of atopy by skewing the Th1/Th2 balance away from the allergy-promoting Th2-cells. However, the hygiene hypothesis has been abandoned by most researchers as being too simplistic and failure of endogenous tolerance mechanisms, rather than a Th1/Th2 imbalance may be pivotal in the development of allergic disease [63].

Enhanced susceptibility to allergic sensitization can be further attributed to **environmental factors**, such as the exposure to indoor or outdoor **allergens** (e.g. HDM, cockroach, cat, dog, mould *vs.* grass, weed, or birch pollens). Especially the role of HDM will be discussed extensively in ***Chapter 2.4***.

The risk for childhood asthma strongly depends on the level of allergen exposure. Reducing the indoor *allergen dose* or the *frequency* of allergen contact will lower the sensitization risk. However, there is no linear relationship between early allergen exposure and asthma

incidence. As illustrated in ***Figure 5*** [64], the sensitization risk increases with the allergen dose, until a plateau is reached. Beyond this level, a further increase in allergen concentration, rather reduces the potential sensitization risk.

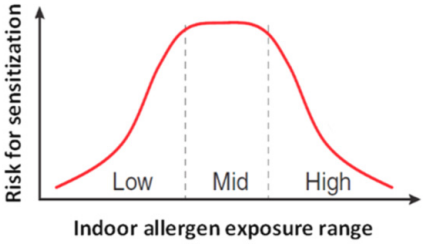


Figure 5*: Risk for sensitization in relation to allergen concentration.*
Adapted from Holt et al. Nat Immunol. 2005 Oct;6(10):957-60.

The sensitizing potential of numerous allergens not only depends on the quantity of the inhaled substance but also on the *intrinsic properties* of the allergen itself. Many of the common aeroallergens responsible for asthma development are proteases, including major HDM allergen *Der p 1* or cat allergen *Fel d 1*. In addition, allergens with protease activity are able to mediate sensitization of non-protease proteins (e.g. pollen allergens) when simultaneously inhaled [65,66]. Some allergens that lack protease activity can induce epithelial barrier dysfunction indirectly through the induction of the angiogenic cytokine vascular endothelial growth factor (VEGF) [67]. Furthermore, *anthropogenic climate changes*, such as increases in CO_2 concentration or surface temperature, may alter allergen production, distribution and allergenicity [68]. Plants may vary their pollination calendar, advancing and prolonging their pollination period, with subsequent effects on the severity and prevalence of allergic disease [69,70].

Other factors contributing to the onset or aggravation of allergic diseases are indoor or outdoor **environmental pollutants** (e.g. tobacco smoke, ozone, diesel exhaust). Particularly *tobacco smoke* has been shown to act synergistically with allergen exposure to induce or enhance immune regulated lung diseases. Hence, the role of CS in asthmatic disease will be fully discussed in ***Chapter 2.5.***

Furthermore, the causal relationship between proximity to roadways and asthma [71] is correlated with *ozone* [72] and/or *diesel exhaust* [73,74]. Both the organic matter adsorbed to diesel exhaust particles (DEP) and the non-extractable carbon core are thought to be

responsible for the adjuvant effect [75]. DEP can absorb aeroallergens, released by pollen grains, which may increase the allergen load in nose and lung [76,77]. Moreover, several murine sensitization models using ovalbumin (OVA) or HDM (*Der f*) in the presence of DEP reveal an increase in antigen-specific IgG1 or enhanced pulmonary inflammation and goblet cell metaplasia [78,79].

Both tobacco smoke as well as diesel exhaust particles are complex vectors of reactive oxygen species (ROS), polycyclic aromatic hydrocarbons (PAHs) and a myriad of other noxious xenobiotic compounds. The potential effects of PAHs and oxidative stress on pulmonary DCs and their impact on subsequent immune responses in the lung are extensively reviewed in ***Chapter 7.***

Finally, respiratory tract **viruses** or **bacterial infections** have emerged as the most frequent triggers for acute exacerbations in both children and adults. The role of viruses during asthma exacerbations is nicely reviewed by Jackson *et al*. [80,81].

2.4. House dust mite allergens

50 to 85 % of asthmatics are HDM allergic [82]. In addition to asthma, other common allergic disorders such as rhinitis, rhinoconjunctivitis and atopic dermatitis are associated with HDM allergy.

2.4.1. Our intimate associates

The perennial indoor HDM *Dermatophagoides pteronyssinus* belongs to the taxonomical class of the Arachnida, subclass Acari and is more closely related to spiders, scorpions and horseshoe crabs than to insects. Dust mites are ubiquitous throughout humid areas of the world and are 8-legged as all acari are. Their size can vary between 0.2 to 0.4 mm which is barely visible to the naked eye. During their life cycle, they transform from eggs to larva, protonymphs, tritonymphs and adults, with an optimal growth temperature around 25°C. Common HDMs feed on human skin flakes, with our bedroom as the focus of infestation [83-85].

Mite bodies and mite feces are the predominant source of many allergens. Fecal pellets are composed of food, debris and proteolytic enzymes, bound together by mucus and covered in a chitinous peritrophic membrane. The average intact mite dropping is 10 – 40 µm in diameter which can be deposited in the conducting airways after inhalation [86]. Allergens associated with mite fecal matter are enzymes that originate from the mite's digestive tract. Other sources of allergens may be components of the mite saliva, or soluble proteins in their body fluids [84].

2.4.2. House dust mite allergenicity

Allergenicity is the property of being an allergen or being able to induce allergic sensitization. Moreover, the ability of an allergen to induce IgE is a measure of the allergic

Allergen	Biological Action	Mol Weight kDa	Allergenicity Reference	Functional Consequence
Der p 1	Cysteine protease (papain-like)	24	97	Disruption of tight junctions. Cytokine, chemokine & growth factor production. Eosinophil and mast cell degranulation. Fibroblast maturation & collagen production.
				Smooth muscle proliferation.
Der p 2	MD-2 related lipid recognition domain	15	98	Molecular mimicry of MD-2.
				Presents LPS to TLR4 resulting in activation of inflammatory genes
Der p 3	Trypsin (serine protease).	31	99	Disruption of tight junctions.
				Cytokine, chemokine & growth factor production. Eosinophil and mast cell degranulation. Fibroblast maturation & collagen production.
				Smooth muscle proliferation.
Der p 4	Alpha amylase	60	100	
Der p 5	alpha-helical protein of unknown function found exclusively in mites moderately cross reactive with Der p 21	14		Thought to bind hydrophobic ligands resulting in stimulation of the innate immune system
Der p 6	Chymotrypsin (serine protease)	25	101	Disruption of tight junctions. Cytokine, chemokine & growth factor production. Eosinophil and mast cell degranulation. Fibroblast maturation & collagen production. Smooth muscle proliferation.
Der p 7	Binds lipopeptide polymyxin B Strucurally homologous to lipid binding proteins	26, 30 and 31	102	Does not specifically bind LPS but can be a ligand for other bacterial lipids. Structurally similar to LPS binding protein. Interaction with innate immune system.
Der p 8	Glutathione S-transferase	27	103	
Der p 9	Collagenolytic serine protease	29	104	Disruption of tight junctions. Cytokine, chemokine & growth factor production. Eosinophil and mast cell degranulation. Fibroblast maturation & collagen production. Smooth muscle proliferation.
Der p 10	Tropomyosin	36	105	
Der p 11	Paramyosin	103	106	
Der p 12	Chitinase (lacks a catalytic domain)			
Der p 13	Lipocalin Lipid transporter			
Der p 14	Apolipophorin High molecular weight allergen found in lipid bodies and transport particles	177	49	IL-4 and IL-13 release from peripheral blood mononuclear cells of allergic donors
Der p 15	Chitinase			
Der p 18	Chitinase			
Der p 20	Arginine kinase		107	
Der p 21	alpha-helical protein of unknown function found exclusively in mites moderately cross reactive with Der p 5			
Der p 23	Unknown function, homology to peritrophin-A domain (PF01607)	14		

***Table 1**: Characterized allergens of the HDM species D. pteronyssinus.*
Adapted from Gregory et al. Trends Immunol. 2011 Sep;32(9):402-11.

state. HDM fecal pellets contain many allergens with either proteolytic activity or enhanced TLR stimulatory capacity. *D. pteronyssinus* produces more than 20 different allergen groups, classified according to their sequence homology and biological function (***Table 1***) [87,88]. Of the 19 denominated allergens, the major IgE binding capacity has been reported for the group 1, 2, 3, 7, 9, 11, 14 and 15 allergens.

The first major mite allergen identified is ***Der p 1***, a member of the group 1 allergens. Group 1 allergens are cysteine proteases that share sequence identity with the catalytic site of the plant enzyme papain [89]. *Der p 1* cleaves intracellular tight junctions proteins, with putative proteolysis sites identified in occludin and claudin-1 [90]. *Der p 1* can promote the PAR-2-mediated release of proinflammatory cytokines, such as IL-6 and IL-8 [91]. *Der p 1* allergens are ubiquitous in house dust. Levels starting from 100 ng/g carpet dust are associated with increased sensitization risk [92].

The second major HDM allergen ***Der p 2*** does not possess intrinsic proteolytic activity, although recently found to be very allergenic. Trompette *et al.* demonstrated that *Der p 2* has structural and functional homology with MD-2, the chaperone protein necessary for TLR4 signalling. *Der p 2* reconstituted the LPS-driven TLR4 activation in the absence of MD-2, suggesting the auto-adjuvant activity of *Der p 2* [93]. This is of special importance for the lung, as airway epithelial cells express TLR4, but little or no MD-2 [94].

Der p 3, 6 and ***9*** represent the three HDM serine proteases which display tryptic, chymptryptic and collagenolytic activities, respectively. Together with group 1 allergens, they account for almost 79% of the proteolytic activity found in house dust [95]. Similar to mite cysteine proteases, they induce increased epithelial permeability through the cleavage of occludin and ZO-1 [96].

Der p 7 and ***Der p 14*** are lipid-binding proteins. They elicit strong IgE and T-cell responses in patients with mite allergy. Recently, *Der p 7* is found to be similar to the LPS-binding protein which interacts with TLRs after binding LPS and other bacterial-derived lipid ligands [97]. The discovery that group 7, as well as group 2 allergens, are structurally similar to different proteins in the TLR pathway, further strengthens the connection between dust mite allergens, innate immunity and allergy [87].

In addition to the diverse array of protein allergens, we should not underestimate the role of the non-protein compounds present in dust mites. HDM extracts are often contaminated with low **endotoxin** levels. *Bartonella* or other Gram-negative species are thought to be the source of this LPS content [98]. Phipps *et al.* found that mice deficient for MyD88 or the LPS receptor TLR4, do not develop common features of allergic asthma [99]. Recently, Hammad *et al.* elegantly demonstrated that TLR4, present on epithelial cells, activates Th2 cell immunity to HDM allergens, through the release of pro-innate cytokines [40]. Together, these observations suggest an important role for the low LPS content present in dust mite extracts. The glucose-derived **β-glucan** moieties within HDM extract were shown to participate in the early events of allergic airway disease. β-glucan motifs stimulate epithelial cells to release CCL20, which is a major chemokine for the attraction of lung DCs [37].
Also other contaminating products might regulate HDM-induced allergic disease. **Chitin**, a glucosamine-based polymer which is part of the mite exoskeleton, induces the accumulation of IL-4 expressing eosinophils and basophils in the murine lung [100,101].

2.4.3. The use of HDM extract in experimental research: benefits and pitfalls

The identification and isolation of mites from house dust, and the ability to grow them in culture, have led to the production of HDM extracts currently used in research. HDM extracts are made from an aqueous extraction of a variable mixture of whole mites, nymphs, fecal pellets, eggs and spent culture medium. Based on the number of proteins they contain, and based on the production methods they use, extracts are **difficult to standardize**. Since allergenicity originates from mites and their fecal pellets, only extracts containing high concentrations of both body and fecal allergens should be used in clinical testing and therapy. It is however possible that some allergens are highly abundant in the environment but poorly represented in the extracts. This could be due to a poor extraction process (e.g. due to hydrophobicity) or may be the result of protein lability. *Der p 3, 7* and *14* for example are shown to be unstable in aqueous solutions [102]. Furthermore, the **complexity** of HDM extracts makes it difficult to understand the contribution of the different HDM elements. Although it would be very interesting to clarify the function of each individual allergen, humans are not exposed to one single protein, but to a whole range of compounds. To

capture the full complexity of HDM allergens, we need to design preclinical mouse models, using HDM extracts in their entirety.

The use of HDM to model asthma has some interesting benefits. HDM is one of the most important **real-life allergens** associated with allergic disease, and is clinically far more relevant than the previously used model innocuous antigen ovalbumin (OVA) [103]. Some HDM proteins posses **intrinsic Th2-promoting adjuvant activity** and have the capacity to induce natural sensitization through the respiratory tract [104]. Whereas prolonged OVA exposure induces inhalation tolerance rather than allergic inflammation [105], HDM can mimic the **chronicity** of human asthmatic disease. Johnson *et al*. described a murine model of intranasal HDM delivery for up to 7 weeks, showing sustained eosinophilic airway inflammation along with severe structural changes of the airway wall (goblet cells, collagen deposition, increased smooth muscle content) [106].

2.5. Cigarette smoke exposure

Smoking is one of the most common addictions of modern times and a risk factor for respiratory diseases like asthma and COPD. Although the devastating health impact of CS is well known, over 1 billion people continue to smoke.

2.5.1. Introduction

CS is an aerosol of liquid droplets (particulate phase) suspended within a mixture of gases and semi-volatile compound (gaseous phase). More than 4000 chemicals have been identified within CS, including carcinogens (PAHs, tobacco specific nitrosamines, acrolein), noxious gases (CO, NO_x) or toxic compounds (nicotine, acetone) [107,108].

Two different emissions can be distinguished, both with different composition and properties. Mainstream cigarette smoke is the smoke actually drawn into the mouth during puffs (active smoking), whereas sidestream smoke is released from the smoldering end of the cigarette. Additionally, the term environmental tobacco smoke (ETS) is used as a mixture of sidestream smoke and exhaled mainstream smoke after dilution and aging. Inhalation of ETS is called 'unvoluntary' or passive smoking [109].

2.5.2. Epidemiology of cigarette smoking and asthma [¥]

CS is a major risk factor for the promotion or aggravation of asthmatic disease. However, due to differences in composition, the impact of ETS and active smoking on allergic sensitization and ensuing asthma development, may differ to some extent.

A large body of evidence establishes a link between **ETS** exposure and the increased risk for asthma development [110,111]. Both *in utero* maternal smoke exposure [112] as well as early postnatal exposure [113] or any parental smoking at home significantly increases the risk of developing asthma later in life [112,114]. The risk for childhood asthma, wheeze or chronic cough even increases with the number of parents who smoke [115].
The relationship between allergic sensitization and ETS exposure is however, less evident [116,117]. ETS exposure promotes the induction of Th2 cytokines in the nasal fluid of allergic patients [118], and increases the sensitization risk in children where both parents are allergic [119]. Furthermore, ETS exposure is dose-dependently correlated with greater asthma severity, diminished lung function and poorer asthma control, both in children [120,121] and adults [122-124].

Unlike ETS, the impact of **active smoking** on asthma development is more controversial. Active smoking increases asthma prevalence [125,126] and the risk of new onset asthma [127], especially among patients with allergic rhinitis [128,129] and those adults exposed to maternal smoke *in utero* [130]. Current smoking is associated with increased disease severity [131], higher severity scores [132] and less controlled asthma [133-135]. Moreover, smoking asthmatics have an accelerated decline in lung function, which may improve after smoke cessation [136]. Finally, current smokers have an increased risk for the sensitization to HDM allergens, but not for grass pollen or cat allergens [137].

The clinical manifestations and inflammatory responses between smoking asthmatics and patients with COPD may, in some individuals, overlap. In addition, a long duration of asthma and a high pack year smoking history may ultimately result in the development of COPD.

[¥] Based on 'Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation.' Maes T, Provoost S, Lanckacker EA, Cataldo DD, Vanoirbeek JA, Nemery B, Tournoy KG, Joos GF. Respiratory Research. 2010;11:7.

2.5.3. Animal models of cigarette smoking and asthma

In contrast to the clinical relevance, most animal models of asthma used to rely on OVA as "surrogate" allergen. OVA is no naturally occurring allergen and is not associated with any form of asthma in real life, however most of the insights regarding the mechanisms underlying asthmatic disease, result from mouse models of OVA-induced experimental asthma.

Murine models, investigating the role of CS (ETS *versus* active smoking) on asthma pathogenesis, discriminate between the impact on sensitization and asthma development or aggravation. Results from animal models with [138,139] or without [140-143] prior sensitization to OVA, all agree that ETS exposure may facilitate allergic **sensitization**, as demonstrated by increased serum IgE, eosinophil counts, Th2 cytokines, airway hyperresponsiveness and/or the development of a memory response [140]. However, the role of active smoking as an adjuvant for allergic sensitization in mice is less univocal. Some studies indicate that active smoking may break inhalation tolerance to OVA [144-146], whereas others do not [146,147]. In addition, different animal studies have demonstrated a role for ETS in the **development** [138,139] or **aggravation** of "established" asthmatic disease [148]. *In utero* maternal ETS exposure aggravated subsequent adult responses to OVA [143]. In contrast to the reports on ETS, the results of active smoking on the development and aggravation of asthma are again controversial [147,149-154], probably because of the dose-dependent effect of CS as adjuvant or anti-inflammatory agent [146,151].

As we learned from ***Chapter 2.4***, real-life allergens such as HDM, are far more complex than OVA, which is a single chicken egg protein. The difference in biochemical and immunogenic character between OVA and common aeroallergens may have an impact on the nature of the elicited allergic response and may even impact the relevance of the murine asthma model. Recently, a few animal studies already used HDM in combination with CS. Botelho *et al.* demonstrated an attenuated effect of CS on "established" HDM-induced asthma [155], whereas Blacquière *et al.* showed that maternal smoking during pregnancy induces airway remodeling in HDM-exposed adult mice offspring [156].

CHAPTER 3: CHRONIC OBSTRUCTIVE PULMONARY DISEASE

3

3.1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of illness and death worldwide. The disease affects about 10% of the general population and is predicted to be the third leading cause of death by 2030 [19].

COPD is a chronic inflammatory condition, characterized by progressive and poorly reversible obstruction of the smaller airways of the lung. Patients suffering from COPD experience abnormal sputum production, chronic cough, wheezing and shortness of breath. Cigarette smoking is by far the most important risk factor for the development of the disease, however only 20 to 30% of heavy smokers develops COPD, suggesting that genetic factors might be important during the disease process [157].

Despite similarities of some clinical features between asthma and COPD, there is a marked difference in the location and pattern of inflammation, linked to differences in the immunological mechanisms underlying the disease, and differences in therapy.

Typical hallmark features of COPD pathophysiology include chronic inflammation, lymphoid follicle formation, emphysema and airway wall remodeling (***Figure 6***).

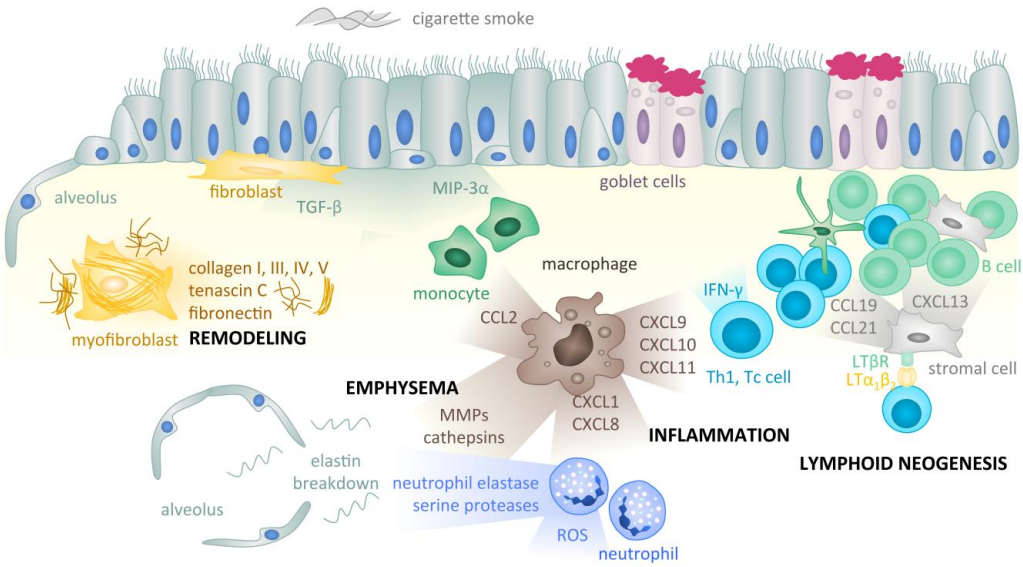


Figure 6: *Immunological aspects of chronic obstructive pulmonary disease.* *Figure by Lanckacker EA.*

3.1.1. Pulmonary inflammation in COPD

The inflammatory disease pattern observed in smokers and patients with COPD is diverse in many respects. The accumulation of inflammatory components appears to contribute to lung injury and serves as a self-perpetuating stimulus for further immune activation. The cellular composition of the infiltrates varies among patients, but there is typically an accumulation of macrophages, neutrophils, CD8+ cytotoxic T cells, and to a lesser extent CD4+ Th1 lymphocytes [158].

Macrophages orchestrate the inflammation in COPD through the release of chemokines, attracting neutrophils (chemokine CXC ligand (CXCL)-1, CXCL-8), monocytes (CCL2) and T lymphocytes (CXCL9, CXCL10, CXCL11) to the airways and lungs (***Figure 6***). Moreover, macrophages release a whole repertory of proteolytic enzymes (matrix metalloproteinase (MMP)9, MMP12, cathepsins) suggesting a potential role during tissue damage and emphysema [159]. Increased numbers of activated neutrophils are found in sputum from smokers and patients with COPD. Neutrophils are a potent source of inflammatory mediators, including ROS and tissue destructive proteases, such as neutrophil elastase, MMPs or cathepsin G. Neutrophil proteases may contribute to alveolar destruction and are potent stimuli of mucin production and secretion [160,161]. Upon the release of CXCL-9, -10 and -11 by macrophages, T lymphocytes (which are predominantly CD8+ cytotoxic T-cells (Tc)) accumulate in the airways and lungs. Lung CD8+ T cells may contribute to the progression of COPD through the release of cytolytic enzymes (perforin, granzyme B) responsible for apoptosis or necrosis of structural cells (e.g. epithelial and endothelial cells) [162,163]. Although less abundant, CD4+ Th1 lymphocytes are also found in smokers with COPD, and at least two subtypes have been found. CD4+ Th1 cells are largely responsible for the production of high levels of the Th1-typical cytokine interferon (IFN)-γ, whereas CD4+ Th17 cells regulate tissue inflammation by producing IL-17A and IL-17F.

3.1.2. Lymphoid follicles in COPD

Lymphoid neogenesis refers to the development of organized lymphoid structures which act as secondary lymphoid organs during sustained chronic inflammation. Lymphoid follicles are

associated with severe COPD and are known to participate in adaptive humoral and T cell mediated responses to antigen [164,165].

Lymphoid tissue formation is the result of a highly coordinated interplay between inflammatory cells, resident stromal cells, adhesion molecules, chemokines and cytokines (***Figure 6***). Upon chronic inflammation, activated lymphocytes express lymphotoxin-$\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) on their surface membrane. Binding of $LT\alpha_1\beta_2$ to the respective lymphotoxin-β receptor (LTβR) on neighbouring stromal cells (probably fibroblasts or fibroblast precursors), induces the expression of adhesion molecules (intercellular adhesion molecule (ICAM)-1, VCAM-1) and chemokines [166,167]. Homeostatic lymphoid chemokines orchestrate lymphocyte homing and compartimentalization of the lymphoid organ. CCL19 and CCL21 attract CCR7+ T lymphocytes and mature DCs to the T cell zone of the follicle, whereas CXCL13 attracts CXCR5+ B cells and is involved in germinal centre formation. Persistent chronic inflammation will stimulate lymphoid aggregates to become highly organized lymphoid follicles, with follicular DCs, Ig-producing plasma cells, and high endothelial venules, which allow the additional supply of naïve lymphocytes [168].

It is however not clear yet, whether lymphoid follicles are beneficial or not. Lymphoid follicles may develop as a local back up mechanism to protect against infectious agents or may be a source of auto-antibodies, maintaining the ongoing inflammatory response [165].

3.1.3. Airway wall remodeling and emphysema in COPD

Whereas in asthma, remodeling is mainly located in the larger airways of the lung, it predominantly affects the smaller airways and the alveolar wall in COPD patients. Especially peripheral airway wall fibrosis, goblet cell metaplasia and emphysema can be distinguished.
Emphysema is the abnormal enlargement of air spaces in the lung and results from destruction of tissues supporting the alveolar wall (elastin breakdown) [169]. Destruction of lung parenchyma may be the result of an imbalance between proteases and antiproteases. As a clinical outcome, emphysematous lung destruction reduces maximal expiratory airflow by decreasing the natural elastic recoil force that drives the air out of the lungs [170].

3.2. Exacerbations

The natural history of COPD is typically interrupted by acute exacerbations or acute aggravation of symptoms. Exacerbations accelerate the decline in lung function, resulting in reduced physical activity, poorer quality of life and an increased risk of death.

Despite the importance of exacerbations, we know relatively little about their incidence, their determinants and their immunological effects in COPD.

Exacerbations are usually triggered by either bacterial or viral respiratory tract infections. The most commonly isolated bacterial species are nontypable *Haemophilus influenzae*, *Streptococcus pneumonia* and *Moraxella catarrhalis*. The major respiratory viruses associated with COPD exacerbations are rhinovirus, influenza or respiratory syncytial virus (RSV) (***Table 2***) [171].

Microbial Pathogens in COPD		
Microbe	**Role in Exacerbations**	**Role in Stable Disease**
Bacteria		
Haemophilus influenzae	20-30% of exacerbations	Major role
Streptococcus pneumoniae	10-15% of exacerbations	Miror role
Moraxella catarrhalis	10-15% of exacerbations	Minor role
Pseudomonas aeruginosa	5-10% of exacerbations, prevalent in advanced disease	Probably important in advanced disease
Enterobacteriaceae	Isolated in advanced disease, pathogenic significance undefined	Undefined
H. haemolyticus	Isolated frequently, unlikely cause	Unlikely
H. parainfluenzae	Isolated frequently, unlikely cause	Unlikely
Staphylococcus aureus	Isolated infrequently, unlikely cause	Unlikely
Viruses		
Rhinovirus	20-25% of exacerbations	Unlikely
Parainfluenza virus	5-10% of exacerbations	Unlikely
Influenza virus	5-10% of exacerbations	Unlikely
Respiratory syncytial virus	5-10% of exacerbations	Controversial
Coronavirus	5-10% of exacerbations	Unlikely
Adenovirus	3-5% of exacerbations	Latent infection seen, pathogenic significance undefined
Human metapneumovirus	3-5% of exacerbations	Unlikely
Atypical bacteria		
Chlamydophila pneumoniae	3-5% of exacerbations	Commonly detected, pathogenic significance undefined
Mycoplasma pneumonia	1-2% of exacerbations	Unlikely
Fungi		
Pneumocystis jiroveci	Undefined	Commonly detected, pathogenic significance undefined

Table 2: *Microbial pathogens in COPD.*
Adapted from Sethi et al. N Engl J Med. 2008 Nov 27;359(22):2355-65.

Approximately 25% of patients with stable COPD show bacterial colonization of the lower respiratory tract, and even higher bacterial counts (50%) are observed during acute exacerbations [172].

The reason for the increased incidence of pulmonary bacterial colonization in patients with COPD may be failure of their macrophages to clear pathogens due to reduced phagocytic capacity [173,174]. Moreover, inflammatory mucus exudates present in the airways of patients with COPD, are the ideal soil for bacterial colonization and growth.

COPD exacerbations are associated with increased pulmonary and systemic inflammation. Almost all COPD exacerbations are marked by increased sputum neutrophilia, and, or eosinophils during viral infections. The presence of microbial PAMPs due to invading pathogens, increases the expression of PRRs, which may lead to a subsequent increase of neutrophils. Furthermore, a specific antibody response will be generated, characterized by increased production of serum IgG and tissue-local production of protective mucosal IgA [158].

3.3. Disease modifying role of Staphylococcus aureus enterotoxins

Although bacterial *Staphylococcus aureus* (*S. aureus*) has only infrequently been isolated from the airways of patients with COPD exacerbations (***Table 2***), significantly increased levels of IgE antibodies against *S. aureus* enterotoxins have been identified in serum [175]. Therefore, the role of these superantigens, as potential aggravating factor of COPD pathogenesis, might be underestimated.

S. aureus is an opportunistic pathogen, which is part of the normal microflora of the human skin and upper respiratory tract. About 25% of healthy persons may be persistently or transiently colonized. Although nasal carriage is a predictor for staphylococcal infections, colonization does not ordinarily cause disease [176]. Especially impaired mucosal barrier function, due to injury of environmental insults, creates the “opportunity“ for the pathogen to infect [177]. Once they are infiltrated, *S. aureus* strains secrete a number of pyrogenic toxins, known as superantigens because of their potent immunostimulatory effects. Unlike conventional antigens, they bind to MHCII molecules outside the antigen-binding groove and are presented as unprocessed proteins to certain T cells expressing specific T-cell receptor

(TCR) Vβ genes (***Figure 7***). As a consequence, superantigens stimulate up to 20% of T cells while only 1 in 10^5-10^6 cells are activated upon normal antigen presentation. Among the 19 different exotoxins synthesized and released by *S. aureus*, toxic shock syndrome toxin-1 (TSST-1) and enterotoxins A, B, C, D and E are the best known [178,179].

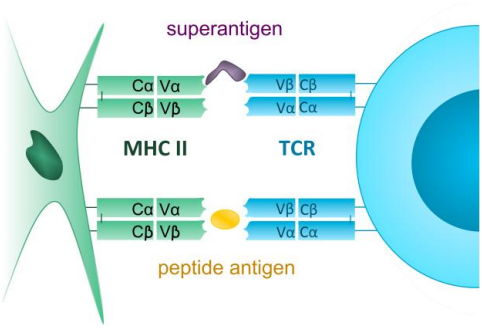


Figure 7: *Presentation of conventional antigens versus superantigens.*
Figure by Lanckacker EA, based on Papageorgiou et al. Structure 1997 Aug 15;5(8):991-6.

Clinical evidence for a role of *S. aureus* enterotoxins (SEs) in skin and airway allergies have been well documented. Atopic dermatitis patients are frequently colonized with *S. aureus* and the presence of enterotoxin specific antibodies is positively correlated with disease severity [180,181].

Also in patients with chronic rhinosinusitis, an increased colonization rate of *S. aureus* has been found, especially in patients with nasal polyps [182]. Remarkably, 40% of nasal polyposis patients develops lower respiratory tract conditions later in life, mainly severe asthma. Recently, the group of Bachert *et al.* hypothesized a possible causal role for *S. aureus* enterotoxin IgE, instead of allergenic IgE, as a risk factor for the development of severe asthmatic disease in nasal polyposis patients [183].

Experimental data have suggested *S. aureus* enterotoxins as disease modifying agents. As shown by *in vitro* studies, SEB exerts a direct pro-inflammatory effect on human nasal epithelial cells (HNEC). SEB stimulation of freshly isolated HNECs, induces chemokine (e.g. CCL-2) and growth factor release (e.g. GM-CSF), resulting in the recruitment and prolonged survival of granulocytic cells [184]. Furthermore, SEB drives the maturation of human monocyte-derived DCs and promotes Th2 cell polarization, probably through TLR2 signaling [185,186].

Using *in vivo* animal studies, *S. aureus* enterotoxins were shown to aggravate allergic airway inflammation in mice [186,187]. Moreover, SEB is able to facilitate allergic sensitization to the innocuous antigen OVA. Concomitant inhalation of SEB and OVA augmented DC migration and maturation as well as enhanced allergen-specific T lymphocyte proliferation [188]. However, the disease modifying role of *S. aureus* enterotoxins in CS-induced pulmonary diseases has not been investigated yet.

Reference List

1. Mizgerd JP. Acute lower respiratory tract infection. *N. Engl. J. Med.* 2008; 358: 716-27.
2. Parker D, Prince A. Innate immunity in the respiratory epithelium. *Am. J. Respir. Cell Mol. Biol.* 2011; 45: 189-201.
3. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest* 2002; 109: 571-7.
4. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N. Engl. J. Med.* 2010; 363: 2233-47.
5. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat. Rev. Immunol.* 2005; 5: 58-68.
6. GeurtsvanKessel CH, Lambrecht BN. Division of labor between dendritic cell subsets of the lung. *Mucosal. Immunol.* 2008; 1: 442-50.
7. Willart MA, Lambrecht BN. The danger within: endogenous danger signals, atopy and asthma. *Clin. Exp. Allergy* 2009; 39: 12-9.
8. McWilliam AS, Nelson D, Thomas JA, Holt PG. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* 1994; 179: 1331-6.
9. Vermaelen K, Pauwels R. Pulmonary dendritic cells. *Am. J Respir Crit Care Med.* 2005; 172: 530-51.
10. Kimbrell DA, Beutler B. The evolution and genetics of innate immunity. *Nat. Rev. Genet.* 2001; 2: 256-67.
11. Cooper MD, Herrin BR. How did our complex immune system evolve? *Nat. Rev. Immunol.* 2010; 10: 2-3.
12. Ristovski ZD, Miljevic B, Surawski NC, Morawska L, Fong KM, Goh F, Yang IA. Respiratory health effects of diesel particulate matter. *Respirology.* 2012; 17: 201-12.
13. Invernizzi G, Ruprecht A, Mazza R, Rossetti E, Sasco A, Nardini S, Boffi R. Particulate matter from tobacco versus diesel car exhaust: an educational perspective. *Tob. Control* 2004; 13: 219-21.
14. *www. medical-dictionary. thefreedictionary. com/xenobiotic* 2012.
15. Sun Q, Hong X, Wold LE. Cardiovascular effects of ambient particulate air pollution exposure. *Circulation* 2010; 121: 2755-65.
16. Gauderman WJ, Avol E, Gilliland F, Vora H, Thomas D, Berhane K, McConnell R, Kuenzli N, Lurmann F, Rappaport E, Margolis H, Bates D, Peters J. The effect of air pollution on lung development from 10 to 18 years of age. *N. Engl. J. Med.* 2004; 351: 1057-67.
17. Pope CA, III, Burnett RT, Thun MJ, Calle EE, Krewski D, Ito K, Thurston GD. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *JAMA* 2002; 287: 1132-41.
18. Stampfli MR, Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat. Rev. Immunol.* 2009; 9: 377-84.
19. *World Health Organization: www. who. int/topics/smoking/en* 2012.
20. *Global Initiative for Asthma: www. ginasthma. org* 2012.
21. Platts-Mills TA. The role of immunoglobulin E in allergy and asthma. *Am. J. Respir. Crit Care Med.* 2001; 164: S1-S5.

22. Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? *Thorax* 1999; 54: 268-72.
23. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat. Med.* 2012; 18: 716-25.
24. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature* 2008; 454: 445-54.
25. Sung SS, Fu SM, Rose CE, Jr., Gaskin F, Ju ST, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J. Immunol.* 2006; 176: 2161-72.
26. van Rijt LS, Vos N, Willart M, Kleinjan A, Coyle AJ, Hoogsteden HC, Lambrecht BN. Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of TH2 effector responses in a mouse model of asthma. *J. Allergy Clin. Immunol.* 2004; 114: 166-73.
27. Yoshimoto T. Basophils as T(h)2-inducing antigen-presenting cells. *Int Immunol.* 2010; 22: 543-50.
28. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat. Rev. Immunol.* 2003; 3: 721-32.
29. Kariyawasam HH, Robinson DS. The eosinophil: the cell and its weapons, the cytokines, its locations. *Semin. Respir. Crit Care Med.* 2006; 27: 117-27.
30. Pohunek P, Warner JO, Turzikova J, Kudrmann J, Roche WR. Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma. *Pediatr. Allergy Immunol.* 2005; 16: 43-51.
31. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am. J. Respir. Crit Care Med.* 2001; 164: S28-S38.
32. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J.* 2000; 14: 1362-74.
33. Anderson JM, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am. J. Physiol* 1995; 269: G467-G475.
34. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Can. J. Physiol Pharmacol.* 2008; 86: 105-12.
35. Jiang A, Bloom O, Ono S, Cui W, Unternaehrer J, Jiang S, Whitney JA, Connolly J, Banchereau J, Mellman I. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity.* 2007; 27: 610-24.
36. Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat. Med.* 2012; 18: 684-92.
37. Nathan AT, Peterson EA, Chakir J, Wills-Karp M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *J. Allergy Clin. Immunol.* 2009; 123: 612-8.
38. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, Brusselle GG. Cigarette Smoke-Induced Pulmonary Inflammation and Emphysema Are Attenuated in CCR6-Deficient Mice. *J Immunol.* 2006; 177: 4350-9.
39. Provoost S, Maes T, Joos GF, Tournoy KG. Monocyte-derived dendritic cell recruitment and allergic T(H)2 responses after exposure to diesel particles are CCR2 dependent. *J. Allergy Clin. Immunol.* 2012; 129: 483-91.

40. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.
41. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J. Immunol.* 2004; 173: 6384-92.
42. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* 2012; 209: 1505-17.
43. Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei XF, Snider DP, Xing Z, Jordana M. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest* 1998; 102: 1704-14.
44. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, Gyarmati D, Aye T, Campbell DJ, Ziegler SF. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat. Immunol.* 2005; 6: 1047-53.
45. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt RR, Bazan F, Kastelein RA, Liu YJ. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 2002; 3: 673-80.
46. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, Liu YJ, Zhu Z, Dong C. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J. Exp. Med.* 2007; 204: 1509-17.
47. Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, Hippe A, Corrigan CJ, Dong C, Homey B, Yao Z, Ying S, Huston DP, Liu YJ. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J. Exp. Med.* 2007; 204: 1837-47.
48. Besnard AG, Togbe D, Guillou N, Erard F, Quesniaux V, Ryffel B. IL-33-activated dendritic cells are critical for allergic airway inflammation. *Eur. J. Immunol.* 2011; 41: 1675-86.
49. Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa, Hammad H, Lambrecht BN. An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity.* 2011; 34: 527-40.
50. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC, Jr., Lambrecht BN. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat. Med.* 2007; 13: 913-9.
51. Muller T, Vieira RP, Grimm M, Durk T, Cicko S, Zeiser R, Jakob T, Martin SF, Blumenthal B, Sorichter S, Ferrari D, Di Virgillio F, Idzko M. A potential role for P2X7R in allergic airway inflammation in mice and humans. *Am. J. Respir. Cell Mol. Biol.* 2011; 44: 456-64.
52. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr. Opin. Allergy Clin. Immunol.* 2012; 12: 53-9.
53. Enomoto Y, Orihara K, Takamasu T, Matsuda A, Gon Y, Saito H, Ra C, Okayama Y. Tissue remodeling induced by hypersecreted epidermal growth factor and

amphiregulin in the airway after an acute asthma attack. *J. Allergy Clin. Immunol.* 2009; 124: 913-20.
54. Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur. Respir. J.* 2006; 27: 208-29.
55. Bouzigon E, Corda E, Aschard H, Dizier MH, Boland A, Bousquet J, Chateigner N, Gormand F, Just J, Le Moual N, Scheinmann P, Siroux V, Vervloet D, Zelenika D, Pin I, Kauffmann F, Lathrop M, Demenais F. Effect of 17q21 variants and smoking exposure in early-onset asthma. *N. Engl. J. Med.* 2008; 359: 1985-94.
56. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, Depner M, von Berg A, Bufe A, Rietschel E, Heinzmann A, Simma B, Frischer T, Willis-Owen SA, Wong KC, Illig T, Vogelberg C, Weiland SK, von Mutius E, Abecasis GR, Farrall M, Gut IG, Lathrop GM, Cookson WO. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* 2007; 448: 470-3.
57. Harada M, Hirota T, Jodo AI, Hitomi Y, Sakashita M, Tsunoda T, Miyagawa T, Doi S, Kameda M, Fujita K, Miyatake A, Enomoto T, Noguchi E, Masuko H, Sakamoto T, Hizawa N, Suzuki Y, Yoshihara S, Adachi M, Ebisawa M, Saito H, Matsumoto K, Nakajima T, Mathias RA, Rafaels N, Barnes KC, Himes BE, Duan QL, Tantisira KG, Weiss ST, Nakamura Y, Ziegler SF, Tamari M. Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 2011; 44: 787-93.
58. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, von Mutius E, Farrall M, Lathrop M, Cookson WO. A large-scale, consortium-based genomewide association study of asthma. *N. Engl. J. Med.* 2010; 363: 1211-21.
59. de Marco R, Locatelli F, Sunyer J, Burney P. Differences in incidence of reported asthma related to age in men and women. A retrospective analysis of the data of the European Respiratory Health Survey. *Am. J. Respir. Crit Care Med.* 2000; 162: 68-74.
60. Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, Holt PG. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol.* 1998; 160: 4730-7.
61. Holt PG, Upham JW, Sly PD. Contemporaneous maturation of immunologic and respiratory functions during early childhood: implications for development of asthma prevention strategies. *J Allergy Clin. Immunol.* 2005; 116: 16-24.
62. Bloomfield SF, Stanwell-Smith R, Crevel RW, Pickup J. Too clean, or not too clean: the hygiene hypothesis and home hygiene. *Clin. Exp. Allergy* 2006; 36: 402-25.
63. Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 2002; 3: 715-20.
64. Holt PG, Thomas WR. Sensitization to airborne environmental allergens: unresolved issues. *Nat. Immunol.* 2005; 6: 957-60.
65. Fattouh R, Pouladi MA, Alvarez D, Johnson JR, Walker TD, Goncharova S, Inman MD, Jordana M. House Dust Mite Facilitates Ovalbumin-specific Allergic Sensitization and Airway Inflammation. *Am. J Respir Crit Care Med.* 2005; 172: 314-21.
66. Porter P, Susarla SC, Polikepahad S, Qian Y, Hampton J, Kiss A, Vaidya S, Sur S, Ongeri V, Yang T, Delclos GL, Abramson S, Kheradmand F, Corry DB. Link between allergic asthma and airway mucosal infection suggested by proteinase-secreting household fungi. *Mucosal. Immunol.* 2009; 2: 504-17.

67. Antony AB, Tepper RS, Mohammed KA. Cockroach extract antigen increases bronchial airway epithelial permeability. *J. Allergy Clin. Immunol.* 2002; 110: 589-95.
68. Ziska LH, Beggs PJ. Anthropogenic climate change and allergen exposure: The role of plant biology. *J. Allergy Clin. Immunol.* 2012; 129: 27-32.
69. Fitter AH, Fitter RS. Rapid changes in flowering time in British plants. *Science* 2002; 296: 1689-91.
70. Ziska LH, Gebhard DE, Frenz DA, Faulkner S, Singer BD, Straka JG. Cities as harbingers of climate change: common ragweed, urbanization, and public health. *J. Allergy Clin. Immunol.* 2003; 111: 290-5.
71. Kramer U, Koch T, Ranft U, Ring J, Behrendt H. Traffic-related air pollution is associated with atopy in children living in urban areas. *Epidemiology* 2000; 11: 64-70.
72. Diaz-Sanchez D, Proietti L, Polosa R. Diesel fumes and the rising prevalence of atopy: an urban legend? *Curr. Allergy Asthma Rep.* 2003; 3: 146-52.
73. Morgenstern V, Zutavern A, Cyrys J, Brockow I, Koletzko S, Kramer U, Behrendt H, Herbarth O, von Berg A, Bauer CP, Wichmann HE, Heinrich J. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am. J. Respir. Crit Care Med.* 2008; 177: 1331-7.
74. Nordling E, Berglind N, Melen E, Emenius G, Hallberg J, Nyberg F, Pershagen G, Svartengren M, Wickman M, Bellander T. Traffic-related air pollution and childhood respiratory symptoms, function and allergies. *Epidemiology* 2008; 19: 401-8.
75. Nilsen A, Hagemann R, Eide I. The adjuvant activity of diesel exhaust particles and carbon black on systemic IgE production to ovalbumin in mice after intranasal instillation. *Toxicology* 1997; 124: 225-32.
76. Knox RB, Suphioglu C, Taylor P, Desai R, Watson HC, Peng JL, Bursill LA. Major grass pollen allergen Lol p 1 binds to diesel exhaust particles: implications for asthma and air pollution. *Clin. Exp. Allergy* 1997; 27: 246-51.
77. Ormstad H, Johansen BV, Gaarder PI. Airborne house dust particles and diesel exhaust particles as allergen carriers. *Clin. Exp. Allergy* 1998; 28: 702-8.
78. Ichinose T, Takano H, Miyabara Y, Sagai M. Long-term exposure to diesel exhaust enhances antigen-induced eosinophilic inflammation and epithelial damage in the murine airway. *Toxicol. Sci.* 1998; 44: 70-9.
79. Ichinose T, Takano H, Sadakane K, Yanagisawa R, Yoshikawa T, Sagai M, Shibamoto T. Mouse strain differences in eosinophilic airway inflammation caused by intratracheal instillation of mite allergen and diesel exhaust particles. *J. Appl. Toxicol.* 2004; 24: 69-76.
80. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. *J. Allergy Clin. Immunol.* 2010; 125: 1178-87.
81. Jackson DJ, Sykes A, Mallia P, Johnston SL. Asthma exacerbations: origin, effect, and prevention. *J. Allergy Clin. Immunol.* 2011; 128: 1165-74.
82. Nelson RP, Jr., DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. *J. Allergy Clin. Immunol.* 1996; 98: 258-63.
83. Nadchatram M. House dust mites, our intimate associates. *Trop. Biomed.* 2005; 22: 23-37.
84. Arlian LG, Platts-Mills TA. The biology of dust mites and the remediation of mite allergens in allergic disease. *J. Allergy Clin. Immunol.* 2001; 107: S406-S413.

85. Hart BJ. Life cycle and reproduction of house-dust mites: environmental factors influencing mite populations. *Allergy* 1998; 53: 13-7.
86. Tovey ER, Chapman MD, Platts-Mills TA. Mite faeces are a major source of house dust allergens. *Nature* 1981; 289: 592-3.
87. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 2011; 32: 402-11.
88. Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int. Arch. Allergy Immunol.* 2002; 129: 1-18.
89. Furmonaviciene R, Shakib F. The molecular basis of allergenicity: comparative analysis of the three dimensional structures of diverse allergens reveals a common structural motif. *Mol. Pathol.* 2001; 54: 155-9.
90. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J. Clin. Invest* 1999; 104: 123-33.
91. Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, Stewart GA. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J. Immunol.* 2002; 169: 4572-8.
92. Wahn U, Lau S, Bergmann R, Kulig M, Forster J, Bergmann K, Bauer CP, Guggenmoos-Holzmann I. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. *J. Allergy Clin. Immunol.* 1997; 99: 763-9.
93. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, Karp CL. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009; 457: 585-8.
94. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB, Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am. J. Physiol Lung Cell Mol. Physiol* 2004; 287: L428-L437.
95. Stewart GA, Boyd SM, Bird CH, Krska KD, Kollinger MR, Thompson PJ. Immunobiology of the serine protease allergens from house dust mites. *Am. J Ind. Med.* 1994; 25: 105-7.
96. Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, Cannell MB, Stewart GA, Garrod DR, Robinson C. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. *Clin. Exp. Allergy* 2001; 31: 279-94.
97. Mueller GA, Edwards LL, Aloor JJ, Fessler MB, Glesner J, Pomes A, Chapman MD, London RE, Pedersen LC. The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins. *J Allergy Clin. Immunol.* 2010; 125: 909-17.
98. Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16S ribosomal DNA in house dust mite cultures. *J Allergy Clin. Immunol.* 2005; 116: 1296-300.
99. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, Mattes J, Barry J, Davidson S, Oreo K, Smith L, Mansell A, Matthaei KI, Foster PS. Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 responses. *Am. J. Respir. Crit Care Med.* 2009; 179: 883-93.
100. Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 2007; 447: 92-6.

101. Lee CG, Da Silva CA, Lee JY, Hartl D, Elias JA. Chitin regulation of immune responses: an old molecule with new roles. *Curr. Opin. Immunol.* 2008; 20: 684-9.
102. Thomas WR, Smith WA, Hales BJ. The allergenic specificities of the house dust mite. *Chang Gung. Med. J.* 2004; 27: 563-9.
103. Epstein MM. Do mouse models of allergic asthma mimic clinical disease? *Int. Arch. Allergy Immunol.* 2004; 133: 84-100.
104. Ishii A, Takaoka M, Matsui Y. Production of guinea pig reaginic antibody against the house dust mite extract, Dermatophagoides pteronyssinus, without adjuvant. *Jpn. J. Exp. Med.* 1977; 47: 377-83.
105. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged Inhaled Allergen Exposure Can Induce Persistent Tolerance. *Am. J Respir Cell Mol. Biol.* 2007; 36: 573-84.
106. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am. J. Respir. Crit Care Med.* 2004; 169: 378-85.
107. Stedman RL. The chemical composition of tobacco and tobacco smoke. *Chem. Rev.* 1968; 68: 153-207.
108. Thielen A, Klus H, Muller L. Tobacco smoke: unraveling a controversial subject. *Exp. Toxicol. Pathol.* 2008; 60: 141-56.
109. Maes T, Provoost S, Lanckacker EA, Cataldo DD, Vanoirbeek JA, Nemery B, Tournoy KG, Joos GF. Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. *Respir. Res.* 2010; 11: 7.
110. Bousquet J, Vignola AM. Exposure to environmental tobacco smoke and adult asthma. *Allergy* 2001; 56: 466-9.
111. Jaakkola MS, Piipari R, Jaakkola N, Jaakkola JJ. Environmental tobacco smoke and adult-onset asthma: a population-based incident case-control study. *Am. J Public Health* 2003; 93: 2055-60.
112. Skorge TD, Eagan TM, Eide GE, Gulsvik A, Bakke PS. The adult incidence of asthma and respiratory symptoms by passive smoking in uterus or in childhood. *Am. J Respir Crit Care Med.* 2005; 172: 61-6.
113. Wang C, Salam MT, Islam T, Wenten M, Gauderman WJ, Gilliland FD. Effects of in utero and childhood tobacco smoke exposure and beta2-adrenergic receptor genotype on childhood asthma and wheezing. *Pediatrics* 2008; 122: e107-e114.
114. von Mutius E. Environmental factors influencing the development and progression of pediatric asthma. *J Allergy Clin. Immunol.* 2002; 109: S525-S532.
115. Cook DG, Strachan DP. Health effects of passive smoking. 3. Parental smoking and prevalence of respiratory symptoms and asthma in school age children. *Thorax* 1997; 52: 1081-94.
116. Strachan DP, Cook DG. Health effects of passive smoking .5. Parental smoking and allergic sensitisation in children. *Thorax* 1998; 53: 117-23.
117. Lannero E, Wickman M, van Hage M, Bergstrom A, Pershagen G, Nordvall L. Exposure to environmental tobacco smoke and sensitisation in children. *Thorax* 2008; 63: 172-6.
118. Diaz-Sanchez D, Rumold R, Gong H, Jr. Challenge with environmental tobacco smoke exacerbates allergic airway disease in human beings. *J Allergy Clin. Immunol.* 2006; 118: 441-6.

119. Keil T, Lau S, Roll S, Gruber C, Nickel R, Niggemann B, Wahn U, Willich SN, Kulig M. Maternal smoking increases risk of allergic sensitization and wheezing only in children with allergic predisposition: longitudinal analysis from birth to 10 years. *Allergy* 2009; 64: 445-51.
120. Joad JP. Smoking and pediatric respiratory health. *Clin. Chest Med.* 2000; 21: 37-viii.
121. Mannino DM, Homa DM, Redd SC. Involuntary smoking and asthma severity in children: data from the Third National Health and Nutrition Examination Survey. *Chest* 2002; 122: 409-15.
122. Coultas DB. Health effects of passive smoking. 8. Passive smoking and risk of adult asthma and COPD: an update. *Thorax* 1998; 53: 381-7.
123. Eisner MD, Yelin EH, Katz PP, Earnest G, Blanc PD. Exposure to indoor combustion and adult asthma outcomes: environmental tobacco smoke, gas stoves, and woodsmoke. *Thorax* 2002; 57: 973-8.
124. Tatum AJ, Shapiro GG. The effects of outdoor air pollution and tobacco smoke on asthma. *Immunol. Allergy Clin. North Am.* 2005; 25: 15-30.
125. Avila L, Soto-Martinez ME, Soto-Quiros ME, Celedon JC. Asthma, current wheezing, and tobacco use among adolescents and young adults in Costa Rica. *J Asthma* 2005; 42: 543-7.
126. Kim YK, Kim SH, Tak YJ, Jee YK, Lee BJ, Kim SH, Park HW, Jung JW, Bahn JW, Chang YS, Choi DC, Chang SI, Min KU, Kim YY, Cho SH. High prevalence of current asthma and active smoking effect among the elderly. *Clin. Exp. Allergy* 2002; 32: 1706-12.
127. Genuneit J, Weinmayr G, Radon K, Dressel H, Windstetter D, Rzehak P, Vogelberg C, Leupold W, Nowak D, von Mutius E, Weiland SK. Smoking and the incidence of asthma during adolescence: results of a large cohort study in Germany. *Thorax* 2006; 61: 572-8.
128. Polosa R, Knoke JD, Russo C, Piccillo G, Caponnetto P, Sarva M, Proietti L, Al-Delaimy WK. Cigarette smoking is associated with a greater risk of incident asthma in allergic rhinitis. *J. Allergy Clin. Immunol.* 2008; 121: 1428-34.
129. Baena-Cagnani CE, Gomez RM, Baena-Cagnani R, Canonica GW. Impact of environmental tobacco smoke and active tobacco smoking on the development and outcomes of asthma and rhinitis. *Curr. Opin. Allergy Clin. Immunol.* 2009; 9: 136-40.
130. Gilliland FD, Islam T, Berhane K, Gauderman WJ, McConnell R, Avol E, Peters JM. Regular Smoking and Asthma Incidence in Adolescents. *Am. J Respir Crit Care Med.* 2006; 174: 1094-100.
131. Thomson NC, Chaudhuri R, Livingston E. Active cigarette smoking and asthma. *Clin. Exp. Allergy* 2003; 33: 1471-5.
132. Siroux V, Pin I, Oryszczyn MP, Le Moual N, Kauffmann F. Relationships of active smoking to asthma and asthma severity in the EGEA study. Epidemiological study on the Genetics and Environment of Asthma. *Eur. Respir. J.* 2000; 15: 470-7.
133. Polosa R, Russo C, Caponnetto P, Bertino G, Sarva M, Antic T, Mancuso S, Al-Delaimy WK. Greater severity of new onset asthma in allergic subjects who smoke: a 10-year longitudinal study. *Respir. Res.* 2011; 12: 16.
134. Chaudhuri R, McSharry C, McCoard A, Livingston E, Hothersall E, Spears M, Lafferty J, Thomson NC. Role of symptoms and lung function in determining asthma control in smokers with asthma. *Allergy* 2008; 63: 132-5.

135. Chaudhuri R, Livingston E, McMahon AD, Thomson L, Borland W, Thomson NC. Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma. *Am. J. Respir. Crit Care Med.* 2003; 168: 1308-11.
136. Chaudhuri R, Livingston E, McMahon AD, Lafferty J, Fraser I, Spears M, McSharry CP, Thomson NC. Effects of smoking cessation on lung function and airway inflammation in smokers with asthma. *Am. J Respir Crit Care Med.* 2006; 174: 127-33.
137. Jarvis D, Chinn S, Luczynska C, Burney P. The association of smoking with sensitization to common environmental allergens: results from the European Community Respiratory Health Survey. *J. Allergy Clin. Immunol.* 1999; 104: 934-40.
138. Seymour BW, Pinkerton KE, Friebertshauser KE, Coffman RL, Gershwin LJ. Second-hand smoke is an adjuvant for T helper-2 responses in a murine model of allergy. *J. Immunol.* 1997; 159: 6169-75.
139. Seymour BW, Friebertshauser KE, Peake JL, Pinkerton KE, Coffman RL, Gershwin LJ. Gender differences in the allergic response of mice neonatally exposed to environmental tobacco smoke. *Dev. Immunol.* 2002; 9: 47-54.
140. Rumold R, Jyrala M, Diaz-Sanchez D. Secondhand smoke induces allergic sensitization in mice. *J. Immunol.* 2001; 167: 4765-70.
141. Barrett EG, Wilder JA, March TH, Espindola T, Bice DE. Cigarette smoke-induced airway hyperresponsiveness is not dependent on elevated immunoglobulin and eosinophilic inflammation in a mouse model of allergic airway disease. *Am. J. Respir. Crit Care Med.* 2002; 165: 1410-8.
142. Bowles K, Horohov D, Paulsen D, Leblanc C, Littlefield-Chabaud M, Ahlert T, Ahlert K, Pourciau S, Penn A. Exposure of adult mice to environmental tobacco smoke fails to enhance the immune response to inhaled antigen. *Inhal. Toxicol.* 2005; 17: 43-51.
143. Penn AL, Rouse RL, Horohov DW, Kearney MT, Paulsen DB, Lomax L. In utero exposure to environmental tobacco smoke potentiates adult responses to allergen in BALB/c mice. *Environ. Health Perspect.* 2007; 115: 548-55.
144. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res.* 2006; 7: 49.
145. Robays LJ, Lanckacker EA, Moerloose KB, Maes T, Bracke KR, Brusselle GG, Joos GF, Vermaelen KY. Concomitant inhalation of cigarette smoke and aerosolized protein activates airway dendritic cells and induces allergic airway inflammation in a TLR-independent way. *J. Immunol.* 2009; 183: 2758-66.
146. Trimble NJ, Botelho FM, Bauer CM, Fattouh R, Stampfli MR. Adjuvant and anti-inflammatory properties of cigarette smoke in murine allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 2009; 40: 38-46.
147. Robbins CS, Pouladi MA, Fattouh R, Dawe DE, Vujicic N, Richards CD, Jordana M, Inman MD, Stampfli MR. Mainstream cigarette smoke exposure attenuates airway immune inflammatory responses to surrogate and common environmental allergens in mice, despite evidence of increased systemic sensitization. *J Immunol.* 2005; 175: 2834-42.
148. Min MG, Song DJ, Miller M, Cho JY, McElwain S, Ferguson P, Broide DH. Coexposure to environmental tobacco smoke increases levels of allergen-induced airway remodeling in mice. *J Immunol.* 2007; 178: 5321-8.
149. Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, Van Der Strate BW, Kerstjens HA, Timens W, Hylkema MN. Short-term smoke exposure attenuates

ovalbumin-induced airway inflammation in allergic mice. *Am. J. Respir. Cell Mol. Biol.* 2004; 30: 880-5.
150. Melgert BN, Timens W, Kerstjens HA, Geerlings M, Luinge MA, Schouten JP, Postma DS, Hylkema MN. Effects of 4 months of smoking in mice with ovalbumin-induced airway inflammation. *Clin. Exp. Allergy* 2007; 37: 1798-808.
151. Thatcher TH, Benson RP, Phipps RP, Sime PJ. High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function. *Am. J. Physiol Lung Cell Mol. Physiol* 2008; 295: L412-L421.
152. Moerloose KB, Pauwels RA, Joos GF. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. *Am. J. Respir. Crit Care Med.* 2005; 172: 168-72.
153. Van Hove CL, Moerloose K, Maes T, Joos GF, Tournoy KG. Cigarette smoke enhances Th-2 driven airway inflammation and delays inhalational tolerance. *Respir. Res.* 2008; 9: 42.
154. Kim DY, Kwon EY, Hong GU, Lee YS, Lee SH, Ro JY. Cigarette smoke exacerbates mouse allergic asthma through Smad proteins expressed in mast cells. *Respir. Res.* 2011; 12: 49.
155. Botelho FM, Llop-Guevara A, Trimble NJ, Nikota JK, Bauer CM, Lambert KN, Kianpour S, Jordana M, Stampfli MR. Cigarette smoke differentially affects eosinophilia and remodeling in a model of house dust mite asthma. *Am. J Respir. Cell Mol. Biol.* 2011; 45: 753-60.
156. Blacquiere MJ, Timens W, Melgert BN, Geerlings M, Postma DS, Hylkema MN. Maternal smoking during pregnancy induces airway remodelling in mice offspring. *Eur. Respir. J.* 2009; 33: 1133-40.
157. Wan ES, Silverman EK. Genetics of COPD and emphysema. *Chest* 2009; 136: 859-66.
158. Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 2011; 378: 1015-26.
159. Barnes PJ. Alveolar macrophages as orchestrators of COPD. *COPD.* 2004; 1: 59-70.
160. Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, Belaaouaj A. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am. J. Pathol.* 2003; 163: 2329-35.
161. Voynow JA, Fischer BM, Malarkey DE, Burch LH, Wong T, Longphre M, Ho SB, Foster WM. Neutrophil elastase induces mucus cell metaplasia in mouse lung. *Am. J. Physiol Lung Cell Mol. Physiol* 2004.
162. Freeman CM, Han MK, Martinez FJ, Murray S, Liu LX, Chensue SW, Polak TJ, Sonstein J, Todt JC, Ames TM, Arenberg DA, Meldrum CA, Getty C, McCloskey L, Curtis JL. Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. *J. Immunol.* 2010; 184: 6504-13.
163. Cosio MG, Saetta M, Agusti A. Immunologic aspects of chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2009; 360: 2445-54.
164. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2004; 350: 2645-53.
165. Brusselle GG, Demoor T, Bracke KR, Brandsma CA, Timens W. Lymphoid follicles in (very) severe COPD: beneficial or harmful? *Eur. Respir. J.* 2009; 34: 219-30.

166. Demoor T, Bracke KR, Maes T, Vandooren B, Elewaut D, Pilette C, Joos GF, Brusselle GG. Role of lymphotoxin-alpha in cigarette smoke-induced inflammation and lymphoid neogenesis. *Eur. Respir. J.* 2009; 34: 405-16.
167. Aloisi F, Pujol-Borrell R. Lymphoid neogenesis in chronic inflammatory diseases. *Nat. Rev. Immunol.* 2006; 6: 205-17.
168. Gommerman JL, Browning JL. Lymphotoxin/light, lymphoid microenvironments and autoimmune disease. *Nat. Rev. Immunol.* 2003; 3: 642-55.
169. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet* 2004; 364: 709-21.
170. Grumelli S, Corry DB, Song LZ, Song L, Green L, Huh J, Hacken J, Espada R, Bag R, Lewis DE, Kheradmand F. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS. Med.* 2004; 1: e8.
171. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2008; 359: 2355-65.
172. Wilson R. Bacteria and airway inflammation in chronic obstructive pulmonary disease: more evidence. *Am. J. Respir. Crit Care Med.* 2005; 172: 147-8.
173. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha JA, Barnes PJ, Donnelly LE. Defective macrophage phagocytosis of bacteria in COPD. *Eur. Respir. J.* 2010; 35: 1039-47.
174. Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L, Claeys S, Hammad H, Brusselle GG, Vandenabeele P, Krysko DV, Bachert C. Alternatively activated macrophages and impaired phagocytosis of S. aureus in chronic rhinosinusitis. *Allergy* 2011; 66: 396-403.
175. Rohde G, Gevaert P, Holtappels G, Borg I, Wiethege A, Arinir U, Schultze-Werninghaus G, Bachert C. Increased IgE-antibodies to Staphylococcus aureus enterotoxins in patients with COPD. *Respir. Med.* 2004; 98: 858-64.
176. Bachert C, Gevaert P, Zhang N, van Zele T, Perez-Novo C. Role of staphylococcal superantigens in airway disease. *Chem. Immunol. Allergy* 2007; 93: 214-36.
177. Moskowitz SM, Wiener-Kronish JP. Mechanisms of bacterial virulence in pulmonary infections. *Curr. Opin. Crit Care* 2010; 16: 8-12.
178. Fraser JD, Proft T. The bacterial superantigen and superantigen-like proteins. *Immunol. Rev.* 2008; 225: 226-43.
179. Thomas D, Chou S, Dauwalder O, Lina G. Diversity in Staphylococcus aureus enterotoxins. *Chem. Immunol. Allergy* 2007; 93: 24-41.
180. Zollner TM, Wichelhaus TA, Hartung A, Von Mallinckrodt C, Wagner TO, Brade V, Kaufmann R. Colonization with superantigen-producing Staphylococcus aureus is associated with increased severity of atopic dermatitis. *Clin. Exp. Allergy* 2000; 30: 994-1000.
181. Bunikowski R, Mielke M, Skarabis H, Herz U, Bergmann RL, Wahn U, Renz H. Prevalence and role of serum IgE antibodies to the Staphylococcus aureus-derived superantigens SEA and SEB in children with atopic dermatitis. *J. Allergy Clin. Immunol.* 1999; 103: 119-24.
182. van Zele T, Gevaert P, Watelet JB, Claeys G, Holtappels G, Claeys C, van Cauwenberge P, Bachert C. Staphylococcus aureus colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. *J. Allergy Clin. Immunol.* 2004; 114: 981-3.

183. Bachert C, van Steen K, Zhang N, Holtappels G, Cattaert T, Maus B, Buhl R, Taube C, Korn S, Kowalski M, Bousquet J, Howarth P. Specific IgE against Staphylococcus aureus enterotoxins: an independent risk factor for asthma. *J. Allergy Clin. Immunol.* 2012; 130: 376-81.
184. Huvenne W, Callebaut I, Reekmans K, Hens G, Bobic S, Jorissen M, Bullens DM, Ceuppens JL, Bachert C, Hellings PW. Staphylococcus aureus enterotoxin B augments granulocyte migration and survival via airway epithelial cell activation. *Allergy* 2010; 65: 1013-20.
185. Mandron M, Aries MF, Brehm RD, Tranter HS, Acharya KR, Charveron M, Davrinche C. Human dendritic cells conditioned with Staphylococcus aureus enterotoxin B promote TH2 cell polarization. *J. Allergy Clin. Immunol.* 2006; 117: 1141-7.
186. Herz U, Ruckert R, Wollenhaupt K, Tschernig T, Neuhaus-Steinmetz U, Pabst R, Renz H. Airway exposure to bacterial superantigen (SEB) induces lymphocyte-dependent airway inflammation associated with increased airway responsiveness--a model for non-allergic asthma. *Eur. J. Immunol.* 1999; 29: 1021-31.
187. Hellings PW, Hens G, Meyts I, Bullens D, Vanoirbeek J, Gevaert P, Jorissen M, Ceuppens JL, Bachert C. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to Staphylococcus aureus enterotoxin B. *Clin. Exp. Allergy* 2006; 36: 1063-71.
188. Huvenne W, Callebaut I, Plantinga M, Vanoirbeek JA, Krysko O, Bullens DM, Gevaert P, van CP, Lambrecht BN, Ceuppens JL, Bachert C, Hellings PW. Staphylococcus aureus enterotoxin B facilitates allergic sensitization in experimental asthma. *Clin. Exp. Allergy* 2010; 40: 1079-90.

PART II: RESEARCH WORK

CHAPTER 4: AIMS OF THE THESIS

4

In the past, animal models have been useful to provide valuable insights regarding the mechanisms of a disease or to predict the safety and efficacy of potential therapeutic drugs. To be clinically relevant, animal models need to mimic the pathogenesis, as well as the heterogeneity of the underlying disease. Phenotypic heterogeneity mainly results from the complex environment we are exposed to, with the emphasis on bacteria, viruses and ambient air pollution. However, most of the current animal models, mimicking asthma or COPD, do not account for this complexity/do not reflect what happens during natural exposure when atmospheric pollution mixtures are inhaled. It is thus a challenge to improve the clinical relevance of existing animal models, using combined exposures. Such combination models will be valuable tools to unravel potential synergistic effects between different environmental compounds and to study how these compounds may impact the underlying disease (heterogeneity).

The aims of this thesis were four-folded:

i. ***The role of cigarette smoke in the development of ovalbumin-induced asthma in mice:*** In our lab, Moerloose *et al.* already established a murine model, in which cigarette smoke (CS) exposure facilitates primary allergic sensitization to an otherwise innocuous antigen, ovalbumin (OVA). We wanted to elucidate the impact of CS on innate immunity and focused on the key innate immune sensors of the lung, the airway DC. Furthermore, we **hypothesized a role for the endotoxin-like effects of CS as an adjuvant to facilitate airway sensitization in mice** and explored a possible role for the TLR4 – MyD88 pathway in our model.

ii. ***The role of cigarette smoke in the development of house dust mite induced-allergic asthma – development of a mouse model:*** Epidemiological studies designate cigarette smoke (CS) as a major risk factor for the development of asthma in children and adults. ***Hence, we hypothesized that CS may facilitate the allergic sensitization to common real-life aeroallergens.*** In a first step, we aimed to design a clinically relevant mouse model, using low doses of HDM as common indoor allergen, combined with CS as major indoor pollutant. Such a combination model may be very useful to investigate how CS adversely affects allergic immune responses in the lung

and how it may impact asthma therapy. In a second step, we tried to elucidate the relative contribution of CS during both sensitization and ensuing asthma development. Thirdly, we investigated whether a very short smoke exposure is sufficient to induce sensitization towards HDM allergens. And finally, we aimed to unravel the impact of short CS exposure on the airway dendritic cell (DC).

iii. ***The role of the respiratory epithelium during cigarette smoke-induced facilitated sensitization and asthma development in mice – preliminary report***: Epidemiological studies, as well as mouse models of asthma, already revealed a crucial role for the airway epithelium in asthmatic disease. Because CS acts as a predisposing factor for asthma, **we hypothesized a role for the airway epithelium during CS-induced facilitated sensitization and disease progression**. In a first step, we wanted to investigate changes in epithelial barrier function after short CS inhalation during primary allergen contact. In a second step, we aimed to elucidate the role of the IL1RI signaling pathway during enhanced early sensitization, whereas finally, we aimed to unravel the role for danger-associated molecular pattern molecules during CS-induced facilitated asthma development.

iv. ***The disease modifying role of bacterial superantigens in CS-induced pulmonary inflammation – development of a mouse model:*** Although *Staphylococcus aureus* (*S. aureus*) is only infrequently isolated in COPD patients, Rohde *et al.* identified significantly increased levels of IgE antibodies against *S. aureus* enterotoxins in serum of patients with COPD. ***Hence, we hypothesized a role for S. aureus enterotoxin B (SEB) as potential aggravating factor of COPD pathogenesis.*** We aimed to design a novel murine model of concomitant exposure to CS and SEB and to investigate the impact of SEB on CS-induced pulmonary and systemic inflammation as well as lymphoid follicle formation and the induction of goblet cells. Such aggravation models may be very useful to elucidate immunological changes during acute exacerbations of COPD.

CHAPTER 5: MODELS AND METHODS USED IN THE THESIS

5

5.1. The development of combined exposure models

5.1.1. Murine model of CS-induced inflammation

Our research group developed a murine CS-exposure model, closely mimicking the important hallmark features of COPD (***Chapter 3***). All combination models used in the thesis are based on this CS exposure protocol [1-3]. For the detailed description, see ***Chapter 5.2.1***.

5.1.2. Murine model of concomitant OVA and CS exposure

In our lab, Moerloose *et al.* already established a mouse model in which simultaneous inhalation of aerosolized OVA (grade III, Sigma-Aldrich, Bornem, Belgium) together with CS, induces features of allergic airway inflammation. To answer the research questions described in the aims of the thesis (***Chapter 4.i***), experiments were based on this combination model. For detailed information about the design of the protocol, we refer to the paper of Moerloose *et al* [4].

5.1.3. Murine model of simultaneous HDM and CS exposure

To unravel the impact of CS on the different stages of asthma pathogenesis, we designed a clinically relevant combination model of HDM and CS, based on current protocols.
The majority of the HDM-driven asthma models used today, are based on work published by Jordana *et al*. The intranasal administration of HDM extract (25 µg protein/10 µl saline) once a day, 5 days/week for up to several weeks, mimics asthma-like lung inflammation [5] as well as the chronicity of the disease [6]. However, the daily application of HDM makes it difficult to distinguish between the sensitization or allergen challenge phase. More recently, Hammad *et al.* described an acute HDM exposure model (100 µg protein/40 µl saline) with clear contrast between the different phases of asthma development (HDM sensitization on day 0, allergen challenge on days 7 and 14) [7]. Hence, we based our combination model on the protocol of Hammad *et al.* but opted to instill 4 times less HDM (Greer Laboratories, Lenoir, NC,USA) in order to evaluate potential aggravating effects upon concomitant CS inhalation. Th2-prone Balb/c mice were used to set up the combination model. Because Balb/c mice are

considered to be more sensitive to CS than C57Bl/6 mice, we decided to lower the intensity of our classic CS exposure protocol (***Chapter 5.2.1***) (3 instead of 4 times, 5 cigarettes/day). Depending on the research aims (***Chapter 4ii, 4iii***), the duration of the exposure period varied from 3 to 17 days.

5.1.4. Murine model of combined SEB and CS exposure

To investigate the role of SEB on CS-induced inflammation, we based our model on the publication from Hellings *et al*, studying the effect of SEB on allergic airway inflammation [8].
The dose of SEB, chosen for our protocol, was in line with dosing experiment by Hellings *et al*. Pilot studies, carried out with nasal or bronchial applications of 5, 50 and 500 ng SEB, demonstrated that 500 ng SEB potently altered bronchial inflammation without clinical signs of wasting disease. Hence, 500 ng SEB (10 µg/ml) (Sigma-Aldrich, Bornem) was chosen to set up this model. Starting from week 2 of the classic CS exposure protocol (***Chapter 5.2.1.***), SEB was administered intranasally (see ***Chapter 5.2.2.***) in C57Bl/6 mice on alternate days.

5.2. Methodology

5.2.1. CS-exposure protocol

Practically, 8 to 10 mice are placed in a plexiglass chamber (7500 cm^3) and are subjected to whole body mainstream CS of 5 simultaneously lit Kentucky Reference cigarettes (2R4F or 3R4F without filters, University of Kentucky, Lexington,KY). This procedure is repeated 4 times a day and is interrupted by a 30 minute smoke free interval to mimic the typically interrupted smoking pattern, observed in human smokers. Because mice do not tolerate undiluted CS, an optimal smoke:air ratio of 1:6 is obtained during the whole protocol. The CS-exposure model is classically performed in non-atopic C57Bl/6 mice, regarded as a Th1 dominant mouse strain with moderate sensitivity to CS.
The serum carboxyhemoglobin levels of CS exposed mice (8.35 ± 0.46% vs. 0.65 ± 0.25% in air exposed mice)[9] are comparable to those measured in human smokers [10] or young children exposed to secondhand CS [11]. Furthermore, 4 weeks of CS exposure are sufficient to mimic CS-induced pulmonary inflammation, as observed in human smokers. However, the

typical characteristics of COPD pathogenesis, including emphysema, lymphoid follicle formation and airway wall remodeling, are obtained after a more chronic exposure period of 24 weeks.

5.2.2. Intranasal instillation

The intranasal application of HDM or SEB is performed in isoflurane (Forene, Abbott, Wavre, Belgium) anesthetized mice, using a continuous flow vaporizer (Harvard Apparatus, Edenbridge, UK) (***Figure 8A***). The mice are manually restrained with the tail anchored between the small finger and the palm and are held in a supine position with the head elevated (***Figure 8B***). The end of the micropipette is placed at the external murine nares and the solution is poured in slowly, causing a rapid increase of the breathing rate. A delivery volume of 35 à 40 µl is known to give a maximum distribution of fluid in the lungs, with increasing volumes resulting in greater relative dosing to the lungs [12]. As illustrated in ***Figure 8C***, 40 µl of cardio green (Sigma-Aldrich, Bornem) nicely deposited in the different lobes of the lungs.

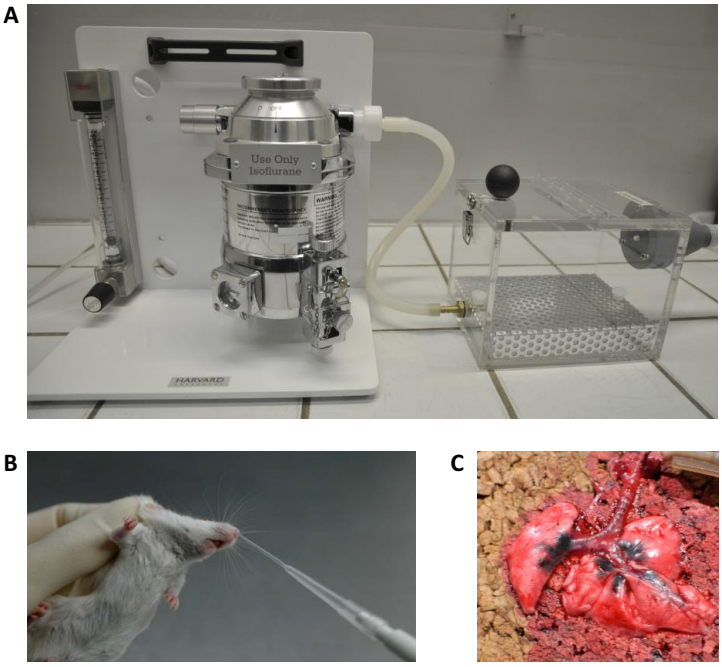


Figure 8: *Intranasal instillation of isoflurane anesthetized mice.*
A. *Isoflurane flow vaporizer;* ***B.*** *Intranasal instillation;* ***C.*** *Cardio green deposition in the lungs.*

5.2.3. Experimental techniques

After completion of the exposure protocol, murine blood and tissue samples are collected and processed to study pulmonary and systemic manifestations. An overview of the collected tissues and their applications is illustrated in ***Figure 9***, followed by a short introduction of the applied techniques. More detailed information about the following techniques can be found in the ***Methods section*** of the original research work (***Chapter 6***).

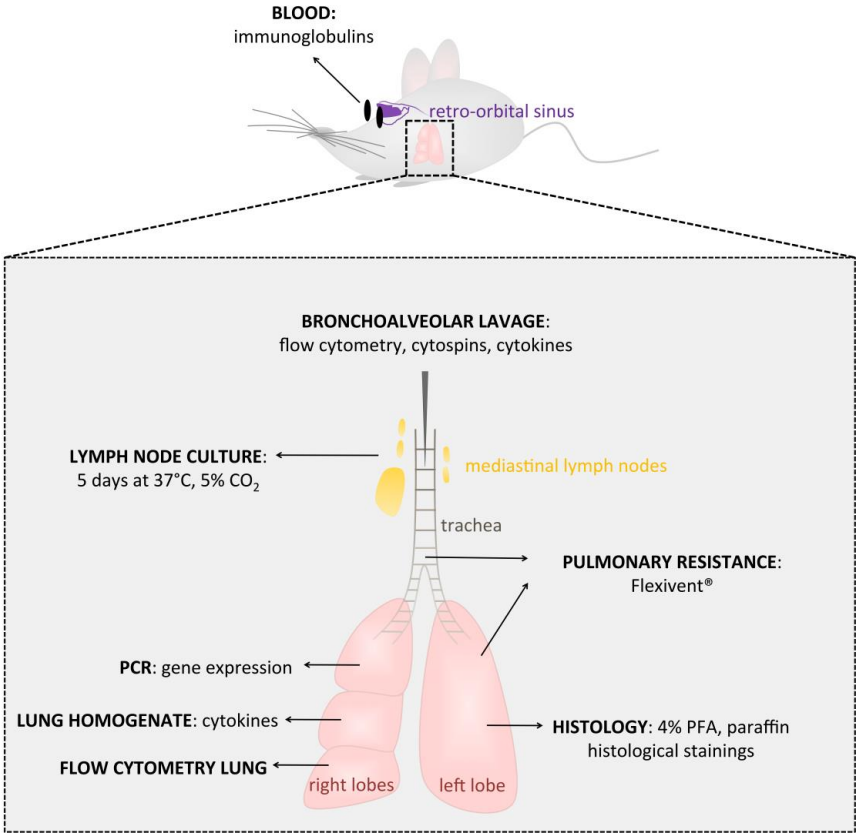


Figure 9*: Overview of the murine samples collected during the endpoint.*
Figure by Lanckacker EA

5.2.3.1. Blood

Blood is collected from the retro-orbital sinus, located behind the murine eye, and is used to detect specific antibody activity by means of serum immunoglobulins.

5.2.3.2. Bronchoalveolar lavage

To obtain inflammatory cells from the bronchial and alveolar zones of the lung, we performed bronchoalveolar lavage. Practically, a tracheal cannula is inserted and a salt solution is instilled and withdrawn gently, resulting in 2 fractions. After centrifugation, the cell-free supernatant of the first fraction is stored to assess cytokine levels by means of ELISA (enzyme-linked immunosorbant assay) or multiplex assays (Merck Millipore, Brussels, Belgium) (CBA, Becton Dickinson, Erembodegem, Belgium), whereas the cell pellets of the first and second fraction together are used to enumerate and differentiate cells by means of cytospin (***Figure 10***) and flow cytometry (***Chapter 5.2.2.5***).

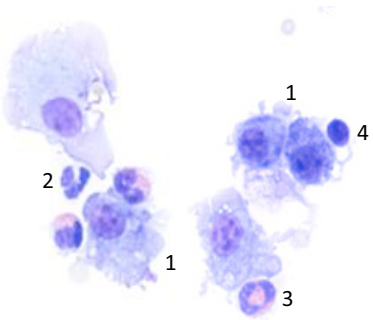


Figure 10: *Cytospins of BAL fluid stained with May-Grünwald-Giemsa.*
1. macrophage; 2. neutrophil; 3. eosinophil; 4. lymphocyte.

Cytospins stained with May-Grünwald-Giemsa (Sigma-Aldrich, Bornem) (VWR, Leuven) are used to perform differential cell counts, based on standard morphological criteria of the cells. ***Macrophages*** are large mononuclear cells with abundant cytoplasm, enriched with numerous cytoplasmic granules. ***Neutrophils***, also called polymorphonuclear neutrophilic leukocytes, have a fragmented multi-lobed nucleus (usually three fragments), with barely stained cytoplasm after May-Grünwald Giemsa. ***Eosinophils*** are characterized by their doughnut-shaped nucleus and their pink cytoplasm, indicative of eosinophilic granules. ***Lymphocytes*** are generally smaller than the previous leukocytes and have a rather big round-shaped nucleus with a high nuclear to cytoplasmic ratio (***Figure 10***).

5.2.3.3. Single cell suspensions

To perform lymph node cultures (***Chapter 5.2.2.4***) or flow cytometry (***Chapter 5.2.2.5***) of the lungs, single cell suspensions are required. Practically, removed tissues are incubated with 1 mg/ml collagenase type 2 (Worthington Biochemical, Lakewood, NY) and 0.02 mg/ml DNase I (grade II from bovine pancreas, Boehringer Mannheim, Brussels, Belgium) for 45 min at 37°C and 5% CO_2. Red blood cells are lysed using ammonium chloride buffer () and finally, cell suspensions are filtered through a 50 µm nylon mesh to remove undigested organ fragments.

5.2.3.4. Lymph node culture

To determine the cytokine expression profile within lung draining lymph nodes, mediastinal lymph nodes (***Figure 9***) are removed and digested as described before (***Chapter 5.2.2.3***). 2 x 10^5 cells are cultured in 96-well plates, with or without 15 µg HDM extract/ml culture medium. After 5 days in a humidified 5% CO_2 incubator at 37°C, culture supernatant is harvested to measure typical Th1, Th2 or Th17 cytokines.

5.2.3.5. Flow cytometry

Flow cytometry is a technique used to characterize different cell populations based on cell-specific phenotypic markers, on or inside the cell. Briefly, antibodies conjugated to fluorescent dyes are used to label specific markers on each single cell. When laser light of the flow cytometer strikes the fluorescent dye, a fluorescent signal with a certain wavelength (read: 'color') is emitted and measured by the flow cytometer.

To perform flow cytometry, single cell suspensions from BAL or the third lobe of the right lung (see ***Chapter 5.2.2.3***) are pre-incubated with FcR-blocking antibody (anti-CD16/CD32, clone 2.4G2) to minimize non-specific binding of the antibodies. The combination of various cell-specific monoclonal antibodies with different fluorescent dyes, allows researchers to fully characterize differential cell populations within BAL and lung.

The cells described in the thesis are characterized as illustrated in ***Figure 11***. Macrophages are defined as high autofluorescent, CD11c^{+}cells (***Figure 11A***). DCs are low autofluorescent, CD11c^{+} MHCII^{+} cells and CD11b^{+} or CD11b^{-} DCs can be distinguished (***Figure 11B***). T-lymphocytes are characterized as small, CD3^{+} cells and various subpopulations exist. In this thesis, we identify CD4^{+} and CD8^{+} T-lymphocytes in BAL and lung and we further characterize the activation status of lung T-cells using CD69 as activation marker (***Figure 11C***).

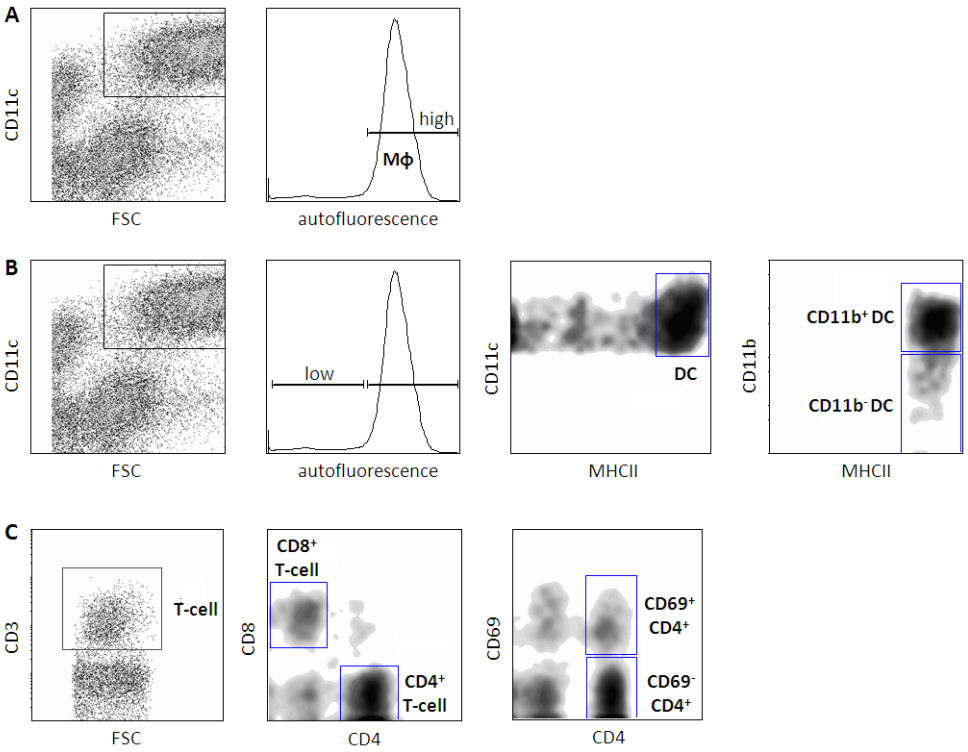


Figure 11*: Gating strategy for the characterization of inflammatory cells in BAL and lung.*
A: *macrophages;* ***B:*** *CD11b^{+} DCs;* ***C:*** *CD4^{+} and CD8^{+} T-lymphocytes, activated (CD69^{+}) or not (CD69^{-}).*

5.5.3.6. Lung homogenate

Lung tissue homogenates are prepared to determine the cytokine profile of the lung by means of ELISA or multiplex. Practically, the middle lobe of the right lung is minced mechanically (TissueRuptor, Qiagen, Hilden, Germany) into 1 ml T-Per tissue protein

extraction reagent completed with 10 µl Protease Inhibitor Cocktail Kit (Thermo Fisher Scientific, Waltham, MA, USA). The total protein concentration is defined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and lung homogenates are diluted until a final protein concentration of 500 µg/ml is attained.

5.5.3.7. Real time RT-PCR

Real-time reverse transcriptase (RT)-PCR has proven to be a powerful tool to quantify gene expression profiles. Briefly, total lung mRNA is extracted from the first lobe of the right lung, using the RNeasy Mini kit (Qiagen, Hilden, Germany). The expression of IL-13, MIP-3α, CXCL13, CCL19 or CCL20 mRNA relative to the housekeeping gene Hprt (hypoxanthine guanine phosphoribosyl transferase) is analysed, using Assay-on-Demand gene expression products (Applied Biosystems, Halle, Belgium) or custom designed probes synthesized on demand [13]. RT-PCR is performed on a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) with murine leukemia virus reverse transcriptase (Applied Biosystems, Halle) under previously described conditions [13,14].

5.5.3.8. Histological evaluation

After the left lung is fixed in 4% paraformaldehyde (Klinipath, Olen, Belgium) and embedded in paraffin, transversal sections of 3 µm are subjected to chemical (periodic acid-Schiff (PAS), Congo Red, toluidin blue staining) or immunohistochemical stainings (α-smooth muscle actin, E-cadherin, hyaluronic acid, CD3/B220 double staining).
Quantitative measurements are performed in a blinded fashion, using a Zeiss KS400 Image Analyzer (Oberkochen, Germany). The following morphometric parameters are marked manually on the digital representation of the airway (***Figure 12***): the length of the basement membrane (Pbm), the area defined by the basement membrane (Abm), and the area defined by the total adventitial perimeter (Ao). Furthermore, the total bronchial wall area (WAt) is calculated as WAt = Ao – Abm and normalized to the square length of the Pbm. All airways with a 800 µm < Pbm < 2000 µm and cut in reasonable cross-sections (defined by a ratio of minimal to maximal internal diameter > 0.5) are included. Measurements are performed on at least 5 airways per mouse.

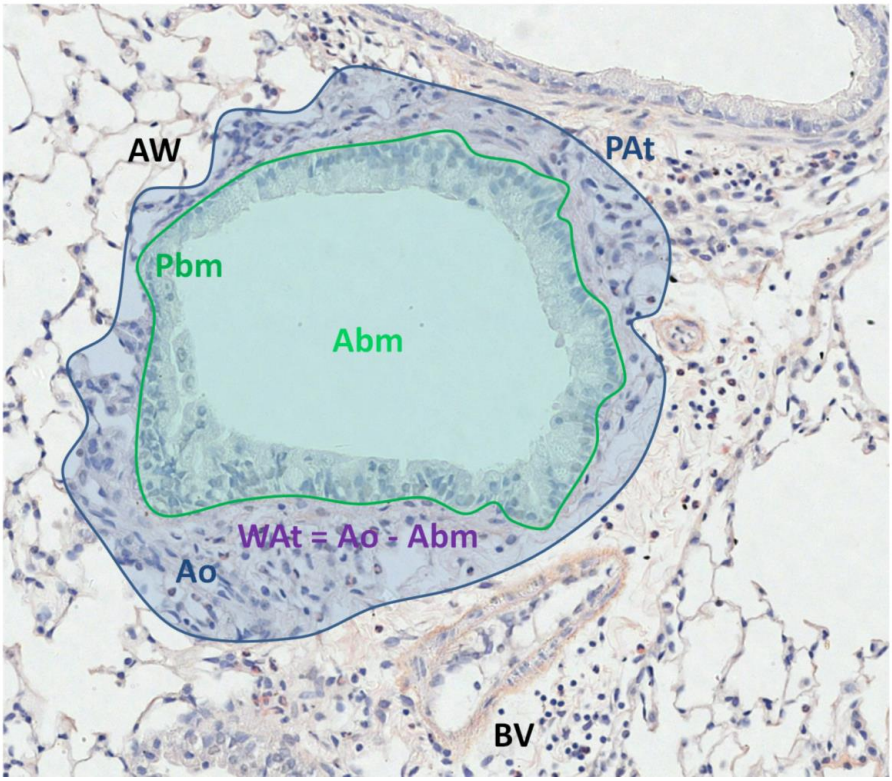


***Figure 12**: Morphometrical parameters defined on a digital representation of the airway. **AW**: airway; **BV**: blood vessel; **Pbm**: perimeter basement membrane; **PAt**: total adventitial perimeter; **Abm**: area defined by the basement membrane; **Ao**: area defined by the total adventitial perimeter; **WAt**: total bronchial wall area.*

Goblet cells are quantified after ***PAS*** staining (Klinipath, Olen) and the number of PAS-positive cells are expressed per millimeter Pbm. Peribronchial infiltration of eosinophils is evaluated after ***Congo Red*** (Klinipath, Olen) staining and determined as the total number per mm^2 bronchial wall area (WAt). Mast cells are highlighted after ***toluidin blue*** staining (Fluka, Bornem) and counted per field (whole left lung lobe). Quantification of ***α-smooth muscle actin*** (Abcam, Cambridge, UK), ***E-cadherin*** (BD Biosciences, Erembodegem, Belgium) or ***hyaluronic acid*** deposition (Seikagaku, Tokyo, Japan) within the airway wall is performed using color recognition, determined by the KS400 software and normalized to Pbm. To quantify lymphoid follicles, lung sections are immunostained with a ***CD3/B220 double-staining*** (DAKO, Heverlee, Belgium) (BD, Erembodegem, Belgium). Dense accumulations of at least 50 cells are defined as lymphoid follicles and their number is normalized for the amount of bronchovascular bundles per lung section.

5.5.3.9. Pulmonary resistance

Airway hyperresponsiveness is a characteristic feature of asthma and implies an increased responsiveness of the airways to a provocative agent. In mice, airway resistance can be measured using direct triggering agents, e.g. carbachol [15]. Carbachol is a muscarinic receptor agonist, which directly binds and activates the acetylcholine receptor on airway smooth muscle cells, hence inducing bronchoconstriction.

Murine airway resistance is measured invasively in tracheostomized anaesthetized mice using the FlexiVent System (SCIREQ, Montreal, QC, Canada) (***Figure 13***). Therefore, the femoral artery and the jugular vein are catheterized to monitor blood pressure and drug administration respectively. The animals are placed on a 37°C heated blanket and are ventilated with an average breathing frequency of 150 breaths/min. Neuromuscular blockade is induced by injecting pancuronium bromide (1 mg/kg) (Inresa, Freiburg, Germany) intravenously. To measure airway hyperresponsiveness, mice are challenged with increasing doses of carbachol (0, 5, 10, 20, 40, 80, 160 and 320 µg/kg) (Sigma-Aldrich, Bornem). A "snapshot perturbation" maneuver is imposed to measure the (dynamic) resistance of the whole respiratory system (airways, lung and chest wall). For each concentration, 12 "snapshot perturbations" are performed and the % increase in lung resistance is calculated relative to the baseline resistance. For each mouse, a dose-response curve is generated and the area under the curve is calculated.

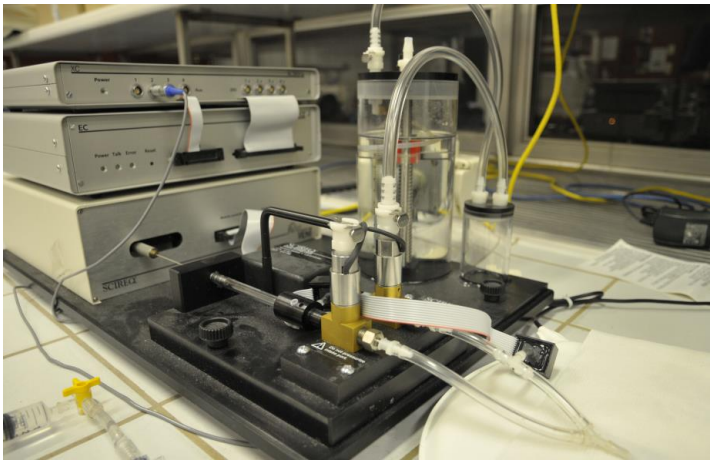


Figure 13*: Flexivent for invasive lungfunction measurement in small laboratory animals.*

Reference List

1. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005; 26: 204-13.
2. D'hulst AI, Maes T, Bracke KR, Demedts IK, Tournoy KG, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respir. Res.* 2005; 6: 147.
3. D'hulst AI, Bracke KR, Maes T, De Bleecker JL, Pauwels RA, Joos GF, Brusselle GG. Role of tumour necrosis factor-alpha receptor p75 in cigarette smoke-induced pulmonary inflammation and emphysema. *Eur. Respir. J.* 2006; 28: 102-12.
4. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res.* 2006; 7: 49.
5. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J. Immunol.* 2004; 173: 6384-92.
6. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am. J. Respir. Crit Care Med.* 2004; 169: 378-85.
7. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.
8. Hellings PW, Hens G, Meyts I, Bullens D, Vanoirbeek J, Gevaert P, Jorissen M, Ceuppens JL, Bachert C. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to Staphylococcus aureus enterotoxin B. *Clin. Exp. Allergy* 2006; 36: 1063-71.
9. Bracke KR, D'hulst AI, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice. *Clin. Exp. Allergy* 2007; 37: 1467-79.
10. Macdonald G, Kondor N, Yousefi V, Green A, Wong F, Aquino-Parsons C. Reduction of carboxyhaemoglobin levels in the venous blood of cigarette smokers following the administration of carbogen. *Radiother. Oncol.* 2004; 73: 367-71.
11. Yee BE, Ahmed MI, Brugge D, Farrell M, Lozada G, Idupaganthi R, Schumann R. Second-hand smoking and carboxyhemoglobin levels in children: a prospective observational study. *Paediatr. Anaesth.* 2010; 20: 82-9.
12. Southam DS, Dolovich M, O'Byrne PM, Inman MD. Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. *Am. J. Physiol Lung Cell Mol. Physiol* 2002; 282: L833-L839.
13. Chen SC, Vassileva G, Kinsley D, Holzmann S, Manfra D, Wiekowski MT, Romani N, Lira SA. Ectopic expression of the murine chemokines CCL21a and CCL21b induces the formation of lymph node-like structures in pancreas, but not skin, of transgenic mice. *J. Immunol.* 2002; 168: 1001-8.
14. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, Brusselle GG. Cigarette Smoke-Induced Pulmonary Inflammation and Emphysema Are Attenuated in CCR6-Deficient Mice. *J Immunol.* 2006; 177: 4350-9.

15. Kips JC, Anderson GP, Fredberg JJ, Herz U, Inman MD, Jordana M, Kemeny DM, Lotvall J, Pauwels RA, Plopper CG, Schmidt D, Sterk PJ, van Oosterhout AJ, Vargaftig BB, Chung KF. Murine models of asthma. *Eur. Respir. J.* 2003; 22: 374-82.

CHAPTER 6: PUBLICATIONS

6

6.1. CONCOMITANT INHALATION OF CIGARETTE SMOKE AND AEROSOLIZED PROTEIN ACTIVATES AIRWAY DENDRITIC CELLS AND INDUCES ALLERGIC AIRWAY INFLAMMATION IN A TLR-INDEPENDENT WAY

Lander L. Robays, Ellen A. Lanckacker, Katrien B. Moerloose, Tania Maes, PhD, Ken R. Bracke, PhD, Guy G. Brusselle, MD, PhD, Guy F. Joos, MD, PhD, Karim Y. Vermaelen, MD, PhD

Journal of Immunology. 2009 Aug 15;183(4):2758-66.

1. Abstract

Cigarette smoking is associated with the development of allergic asthma. In mice, exposure to cigarette smoke sensitizes the airways towards co-inhaled OVA, leading to OVA-specific allergic inflammation. Pulmonary dendritic cells (DCs) are professional antigen-presenting cells involved in immunosurveillance and implicated in the induction of allergic responses in lung. We investigated the effects of smoking on some of the key features of pulmonary DC biology, including population dynamics and cellular activation status in different lung compartments. We found that cigarette smoke inhalation greatly amplified DC-mediated transport of inhaled antigens to mediastinal lymph nodes, a finding supported by the upregulation of CCR7 on airway DCs. Pulmonary plasmacytoid DCs, which have been involved in inhalational tolerance, were reduced in number after smoke exposure. In addition, combined exposure to cigarette smoke and OVA-aerosol increased surface expression of MHC Class II, CD86 and PD-L2 on airway DCs, while ICOS-L was strongly downregulated. Although endotoxins present in cigarette smoke have been shown to act as DC activators and Th2-skewing sensitizers, TLR4-deficient and MyD88-knockout mice did not show impaired eosinophilic airway inflammation after concomitant exposure to smoke and OVA. From these data, we conclude that cigarette smoke activates the pulmonary DC network in a pattern that favors allergic airway sensitization towards co-inhaled inert protein. The TLR-independency of this phenomenon suggests that alternative immunological adjuvants are present in cigarette smoke.

2. Introduction

Increasing epidemiological and experimental data now support an active role of cigarette smoking in the development and severity of asthma [1,2]. Active smoking interacts with the asthmatic phenotype causing more severe allergic symptoms, a greater decline in lung function, and impaired therapeutic responses to corticosteroids [3,4]. In addition, smoke exposure is considered as a risk factor for allergen sensitization and is associated with the onset of asthma [5-7].

In order to better understand these effects, animal models studying the interaction between cigarette smoke and allergic responses have been developed [8-16]. We and others recently established a mouse model in which concomitant inhalation of aerosolized OVA together with mainstream cigarette smoke -without prior immunization- induces features of allergic airway inflammation [16,17]. This allergic response was absent after exposure to OVA alone, suggesting a prominent role for cigarette smoking in the establishment of a OVA-specific Th2 immune response. Indeed, synthesis of IL-5, a Th2-derived cytokine critical for the recruitment and survival of eosinophils, was detected in mediastinal lymph node (MLN) cell suspensions of OVA/smoke-exposed animals only. However, not all studies supported a positive contribution of cigarette smoke exposure to the allergic phenotype and sensitization phenomenon, possibly as a result from the variable experimental conditions used among different laboratory (e.g. the use of environmental versus mainstream smoke, whole-body versus nose-only exposure, etc.) [11,12,14,15]. One outstanding observation was that the effects of smoke exposure on allergic responses appear to be dose-dependent [15].

Pulmonary dendritic cells (DCs) are specialized antigen-presenting cells and have emerged as central players in the immunological balance of the airways [18,19]. A continuous flow of migrating DCs interacting with resident airway cells constantly assesses whether inhaled material should be confronted with an active immune response, or whether homeostatic tolerance should be maintained [20]. The outcome of adaptive immune responses depends on the context in which the antigen is encountered by airway DCs and is predominantly defined by presence or absence of danger signals at the exposed site, such as pathogen-associated molecular patterns (PAMPs) betraying the presence of bacterial, fungal or viral pathogens and damage-associated molecular patterns (DAMPs) released by stressed or damaged host tissue cells [21-23]. TLRs expressed on DCs are essential for the integration of these alarm

signals, and different pulmonary DC subsets have been shown to express different TLRs, potentially leading to different immunological outcomes [24]. Triggering of TLRs leads to DC activation, manifested by the upregulation of a specific set of costimulatory molecules, enhanced migration towards draining LNs and efficient priming of T cells, which will recirculate to the periphery in an attempt to neutralize the threat. In the setting of allergic airway inflammation, DCs mount an inappropriate Th2-type inflammatory immune response against otherwise non-pathogenic inhaled material [25,26]. The immunological mechanisms by which DCs induce this aberrant response are being unraveled. A working hypothesis is that environmental factors acting as DAMPs affect airway DCs in a way that breaks tolerance towards co-inhaled inert antigen [18,23]. In this study, we asked ourselves whether cigarette smoke inhalation affects pulmonary DCs in a way that would facilitate allergic sensitization to co-inhaled ovalbumin, a model inert protein antigen. As TLR triggering by trace amounts of endotoxin has been shown to spark allergic sensitization to OVA [27-29], and as cigarette smoke has been shown to contain bioactive endotoxins [30], we verified whether TLR4 and MyD88-dependent TLR signaling signaling contributes to the phenomenon of smoke-induced allergic sensitization.

3. Methods

Mice

Balb/c mice (7-9 weeks old) were obtained from Harlan (The Netherlands). TLR4-deficient mice (C.C3-*Tlr4Lps-d*/J, 8-12 weeks old) and control Balb/c mice (7-9 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). MyD88-knockout mice on a Balb/c background were obtained from the laboratory of G. Lauvau (INSERM, University of Sofia Antipolis, Valbonne, France), bred in our facility, and 8 week old mice were kindly provided by Dr Kryszko (Ear-Nose-Throat Research Laboratory, Ghent University Hospital). Control Balb/c mice were purchased from Harlan. The local Ethical Committee (ECD Faculty of Medicine and Health Sciences, Ghent, Belgium) approved all in vivo manipulations.

Cigarette smoke exposure and aerosol challenge

Groups of 8 mice were subjected to whole body mainstream cigarette smoke exposure of 5 Kentucky Reference cigarettes (2R4F without filters, University of Kentucky, Lexington, KY, USA) for 7 min, 4 times/day, 5 days/week in a plexiglas chamber (17x28x14 cm) with an inlet for pressurized air (1.25 l/min). For the MyD88 experiment, 3R4F Kentuky cigarettes were used. In the experiments where cigarette smoke and aerosol exposure were performed simultaneously, the smoking chamber was additionally connected to an ultrasonic aerosol generator. Concurrent aerosol challenge with PBS or 1% OVA (Grade III, Sigma, Belgium) in PBS was performed for 7 min, 4 times/day, 5 days/week. The amount of contaminating LPS found in the grade III OVA-preparation was measured by the *Limulus* amebocyte lysate (LAL) assay and determined at 5,82 ng bioactive LPS/mg protein.

Bronchoalveolar lavage and cytospins

Twenty-four hours after the last exposure, mice were sacrificed by a lethal dose of pentobarbital. A tracheal cannula was inserted and bronchoalveolar lavage (BAL) was performed by instillation of 3 x 300 µl HBSS supplemented with BSA for cytokine analysis. Three additional instillations with 1 ml HBSS + EDTA were performed to achieve maximal recovery of BAL cells. 50000 BAL cells were processed for cytospins and were stained with May-Grunwald-Giemsa for differential cell counting. Remaining cells were used for FACS-analysis.

Preparation of single-cell suspensions

Lungs were perfused with saline plus EDTA through the pulmonary artery to remove contaminating blood cells. Lungs and mediastinal LNs (MLN) were removed and digested as detailed previously [31]. Briefly, minced lung pieces and LNs were incubated with 1 mg/ml collagenase and 20 µg/ml DNAse I for 45 min at 37°. Red blood cells were lysed using ammonium chloride buffer. Finally, cell suspensions were filtered through a 50 µm nylon mesh to remove undigested organ fragments.

Flow cytometry

All staining procedures were carried out in calcium and magnesium-free PBS containing 10 mM EDTA, 1% BSA (Dade Behring, Germany) and 0.1% sodium azide. One million cells were preincubated with anti-CD16/CD32 (2.4G2) to block Fc-receptors. Monoclonal antibodies used to identify cell surface molecules were anti-CD11c (clone HL3), anti-CD11b (clone M1/70), anti-MHCII (I-A/I-E, clone M5/114) and anti-CD86 (clone GL1), all from BDBiosciences, Erembodegem, Belgium); anti-ICOS-L (anti-CD275, clone HK5.3), anti-PDL1 (anti-CD274, clone MIH5), anti-PDL2 (anti-CD273, clone 122), anti-OX40L (anti-CD134L, clone RM134L), anti-GITRL (clone YGL386) and anti-CCR7 (CD194, clone 4B12) were from eBioscience, San Diego, CA, USA; the PDCA-1 monoclonal antibody (clone JF05-1C2.4.1) was from Miltenyi Biotec (Germany).

Evaluation of dendritic cell migration by intratracheal instillation of fluorescent OVA

After 3 weeks of smoke exposure, mice were anesthetized with avertin (1 mg tribromethanol per ml t-amylalcohol in 2.5% in PBS). Dose of the avertin solution was carefully determined according to the body weight of each mouse separately so that no respiratory depression was observed. 70 µl of fluorescein-conjugated OVA (OVA-FITC, Invitrogen, Belgium) diluted in sterile PBS (5 mg/ml) was injected intratracheally using pyrogen-free catheders in order not to cause any local pulmonary inflammation, as described previously [31]. Mice were further exposed to smoke until they were sacrificed at indicated time points after instillation. M LNs were removed and processed as described above to obtain single cell suspensions. The number of OVA-bearing airway-derived DCs (AW-DC) was calculated by flow-cytometry as the fraction of FITC-positive CD11c+ $MHCII^{high}$ LN cells multiplied by total number of LN cells. PBS-instilled mice were used as a control to eliminate background fluorescence.

Histology

Left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Transversal sections of 3 µm were stained with Congo-Red (Klinipath, Belgium) for the visualization of eosinophils or with periodic acid-Schiff (PAS) to identify mucopolysaccharides in tissue and counterstained with hematoxyline (Sigma, Belgium). PAS-positive cells were identified as goblet cells and quantified in airway walls with a perimeter basement membrane between 800 and 2000 µm.

Measurement of OVA-specific IgE

Blood was collected by cardiac puncture for measurement of total and OVA-specific IgE with a home-made ELISA as described before [17].

RNA preparation and RT-PCR

RNA from lung tissue was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Real-time RT-PCR was performed on a Lightcycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) with Assays-on-Demand™ Gene Expression Products (Applied Biosystems, USA) starting from 20 ng of cDNA. Monitoring of the RT-PCR occurred in real-time using a FAM/TAMRA probe. For IL-13, a 2-step RT-PCR protocol was used. Reverse transcription was performed by 10 min at 25°C, 60 min at 42°C and 10 min at 70°C, using random hexameres and RevertAid M-MuLV Reverse transcriptase (Fermentas, Canada). After 10 min incubation at 95°C for denaturation of RNA-DNA heteroduplexes, a DNA-amplification was performed with 50 cycles of 95°C for 15 sec and 60°C for 60 sec, using the Taqman Universal Primer Mix No AmpErase UNG (Applied Biosystems). Expression of the mRNA of gene of interest relative to hypoxanthine guanine phosphoribosyl transferase (*hprt*) mRNA was calculated.

Statistical analysis

Reported values are expressed as mean ± SEM. Statistical analysis was performed with SPSS software (version 15.0) using non-parametric tests (Kruskal-Wallis and Mann-Whitney U-test) without Bonferroni corrections. Values of $p < 0.05$ were considered significant (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$).

4. Results

4.1. Combined exposure to cigarette smoke and OVA induces allergic airway inflammation

The effect of cigarette smoking on the development of allergic responses was illustrated by exposing mice to a combination of OVA-aerosol and cigarette smoke without prior immunization to OVA [17]. As we observed earlier, 3 weeks of simultaneous exposure induced features of allergic airway inflammation, demonstrated by accumulation of eosinophils, neutrophils and lymphocytes and in the BAL-fluid (*Fig. 1A*), production of OVA-specific IgE in serum (*Fig. 1A*), eosinophilic infiltrates in the peribronchial tissue (*Fig. 1B*) and marked airway goblet cell hyperplasia on histological airway sections (*Fig 1C*), fully consistent with earlier observations in this model [17]. In line with the goblet cell hyperplasia, we observed a strong induction of pulmonary IL-13 expression in mice concomitantly exposed to OVA and cigarette smoke compared to OVA alone (*Fig 1C*). IL-13 protein levels in the BAL fluid were, however, below the detection limit (data not shown). We also showed earlier that this allergic response was associated with OVA-specific Th2-cytokine production in the draining LNs [17]. Taken together, this suggests that cigarette smoke possessed adjuvant properties toward concomitantly inhaled OVA that favored the development of mucosal Th2 responses.

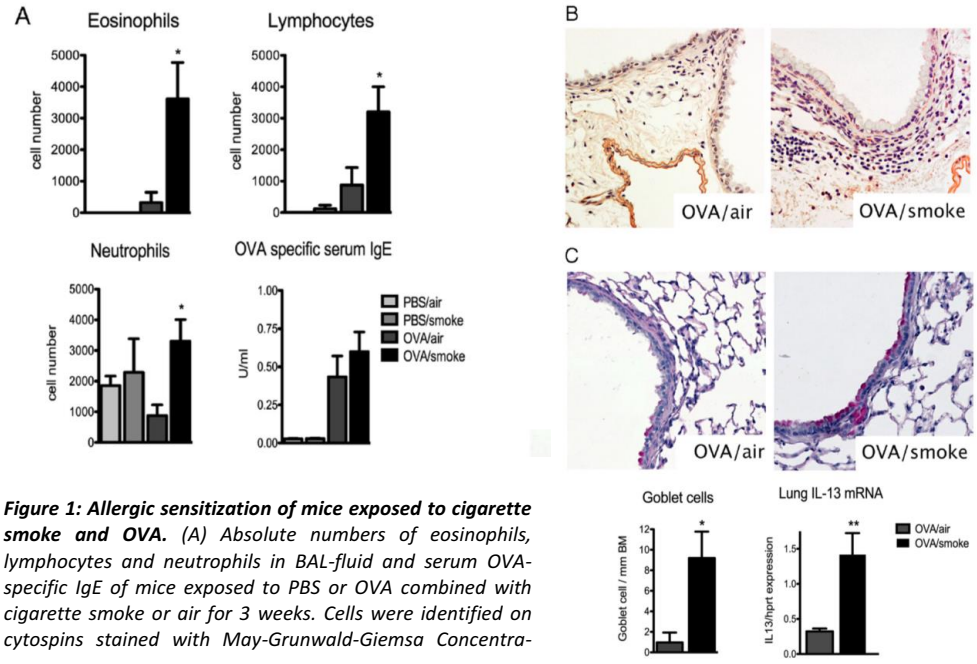


Figure 1: Allergic sensitization of mice exposed to cigarette smoke and OVA. *(A) Absolute numbers of eosinophils, lymphocytes and neutrophils in BAL-fluid and serum OVA-specific IgE of mice exposed to PBS or OVA combined with cigarette smoke or air for 3 weeks. Cells were identified on cytospins stained with May-Grunwald-Giemsa Concentra-*

*tion of OVA-specific IgE in serum was determined by ELISA. (B) Histological evaluation of eosinophilic peribronchial infiltrates on Congo Red stained lung tissue sections counterstained with hematoxylin. (C) Histological evaluation of goblet cell hyperplasia on periodic acid-Schiff (PAS) stained lung tissue sections. Quantification of the number of goblet cells per mm perimeter basement membrane (BM) of airway walls with a perimeter between 800 and 2000 µm of mice exposed to OVA with or without cigarette smoke. mRNA transcripts for IL-13 relative to a housekeeping gene (hprt) were measured in whole lung homogenates by real time RT-PCR. A representative experiment (n=8 mice per group) of 3 independent experiments is shown. *represents a significant difference (p<0.05) between the OVA/air and OVA/smoke group.*

4.2. Effects of cigarette smoke exposure on pulmonary DC dynamics and antigen transport

Pulmonary DC trafficking can be divided into a recruitment phase towards the lung, progressing to an emigration phase towards MLNs. Combined exposure to cigarette smoke and OVA induced a strong elevation in DC numbers in the BAL compartment, indicating that the developing allergic inflammation in these mice stimulated DC recruitment to the airways (*Fig. 2A),* which is consistent with earlier observations using i.p. alum as an adjuvant [32]. Airway-antigen specific T cell priming relies on the capture and transport of inhaled antigen by DCs migrating to the MLN. Hence, we investigated whether smoke inhalation as such could affect airway DC trafficking and antigen transport. We delivered fluorescently labeled OVA into the airways once after 3 weeks of smoke exposure alone. At various time points after intratracheal challenge, FITC+ cells were detected within CD11c+ $MHCII^{high}$ airway-derived DCs in the MLNs (AW-DCs), according to previous studies [31] (*Fig. 2B, upper graph*). Time course of these FITC+ AW-DCs in LNs of sham-exposed animals illustrated steady-state kinetics characterized by a peak of DCs infiltrating the LNs at 24h after antigen challenge, slowly declining thereafter (*Fig. 2B, lower graph*). By contrast, smoke-exposed animals showed a marked amplification of DC-mediated antigen transport to LNs, i.e. more antigen-loaded DCs reaching the draining LNs (*Fig. 2B, lower graph*). This large wave of DCs carrying antigens to the LNs during smoke-exposure peaked at 24-48h after antigen challenge and rapidly declined thereafter. Accordingly, we found that cigarette smoke exposure selectively upregulated surface expression of CCR7 on airway DCs, i.e. the population primarily involved in airway antigen capture (*Fig. 2C*). No CCR7 could, however, be detected on lung parenchymal DCs.

Last, different subsets of DCs have been found in the lung with conventional and plasmacytoid DCs (pDCs) representing the main subsets. Pulmonary pDCs were shown to actively contribute to tolerance towards inhaled antigen [33]. Hence we evaluated how the numbers of pDCs were affected by cigarette smoke exposure. Remarkebly, numbers of

CD11c^{int} MHCII$^{low/int}$ PDCA-1+ cells (generally regarded as pDCs) were reduced in lung and MLNs of smoke-exposed mice (*Fig 2D*).

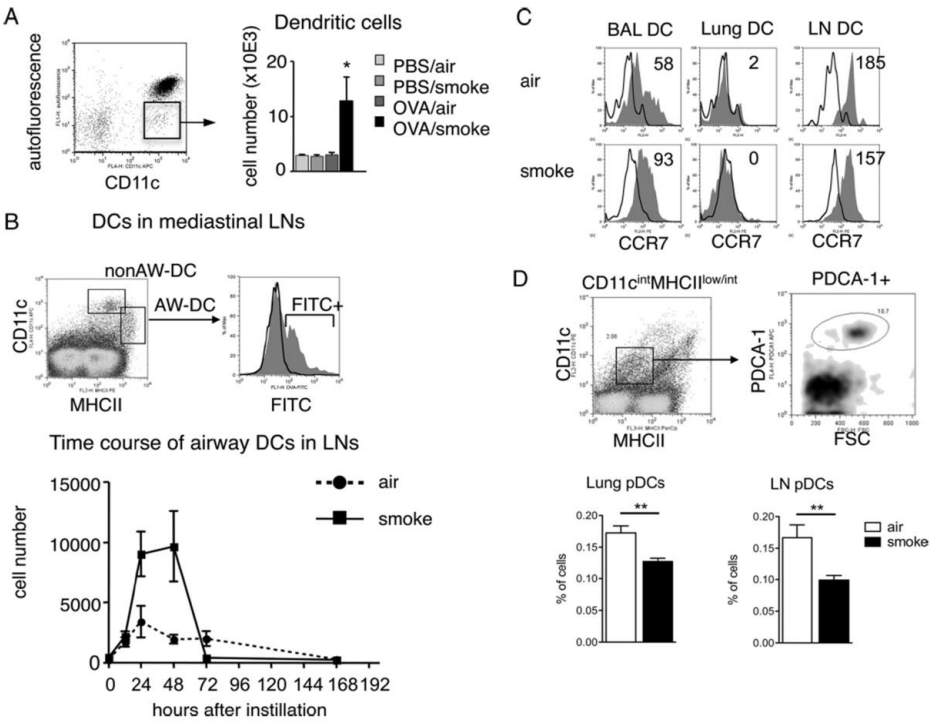


Figure 2: Migration of pulmonary dendritic cells during cigarette smoke exposure. *(A) Numbers of DCs in the BAL of mice exposed to a combination of cigarette smoke and aerosolized OVA for 3 weeks. DCs were identified as low-autofluorescent CD11c^{high} cells in the cellular fraction of BAL fluid. * represents a significant difference (p<0.05) between the OVA/air and OVA/smoke group. (B) Kinetics of DC emigration to the draining LNs. Mice were exposed to mainstream cigarette smoke (smoke) or sham-exposed (air). After 3 weeks exposure, 70 µl of an OVA-FITC solution in sterile PBS was instilled into the airways and MLNs were isolated at various time points after instillation. Single cell suspensions were prepared from LNs by enzymatic digestion and labeled with CD11c and MHCII. FITC+ cells were exclusively found within the fraction of CD11c+ MHCII^{high} airway-derived dendritic cells (AW-DC). Graph depicts time course of absolute numbers of FITC+ AW-DC at various time points after instillation between cigarette smoke-exposed mice (smoke) and sham-exposed mice (air). (C) CCR7 expression on pulmonary DCs from 3 weeks cigarette smoke-exposed mice (smoke) and sham-exposed mice (air). Single cell suspensions derived from BAL fluid and enzymatically digested lung tissue and MLNs were stained with monoclonal antibodies for CD11c, MHCII and CCR7. DCs were identified as low-autofluorescent CD11c^{high} cells in BAL-fluid (BAL DC) and lung tissue (lung DC) and as CD11c+ MHCII^{high} cells in LNs (LN DC). Representative histogram of CCR7 expression on DCs is shown (grey shaded) relative to isotype control staining (black line). Cells of 3 mice (n=9 mice per group) were pooled before staining. (D) Numbers of plasmacytoid DCs (pDCs) characterized as CD11c^{int} MHCII$^{low/int}$ PDCA-1+ cells in lung digests and MLNs of cigarette smoke versus air exposed mice (for 4 weeks).*

4.3. Effects of inhaled cigarette smoke and/or aerosolized OVA on pulmonary dendritic cell activation and costimulatory molecule expression

Costimulatory molecules expressed on DCs during antigen presentation contribute to the outcome of T cell stimulation and polarization. We therefore examined expression levels of various costimulatory molecules on the cell surface of pulmonary DCs during smoke and/or OVA antigen exposure. We focused on accessory molecules with documented involvement in the establishment of tolerance versus Th2-development. Examination of the expression levels of MHCII and CD86 showed that the majority of pulmonary DCs from airway compartment and lung tissue of OVA/smoke-exposed mice exhibited a mature or activated phenotype (*Fig. 3*). This was also observed on DCs from PBS/smoke-exposed mice in the airway compartment and peripheral lung tissue, consistent with our previous results [34,35].

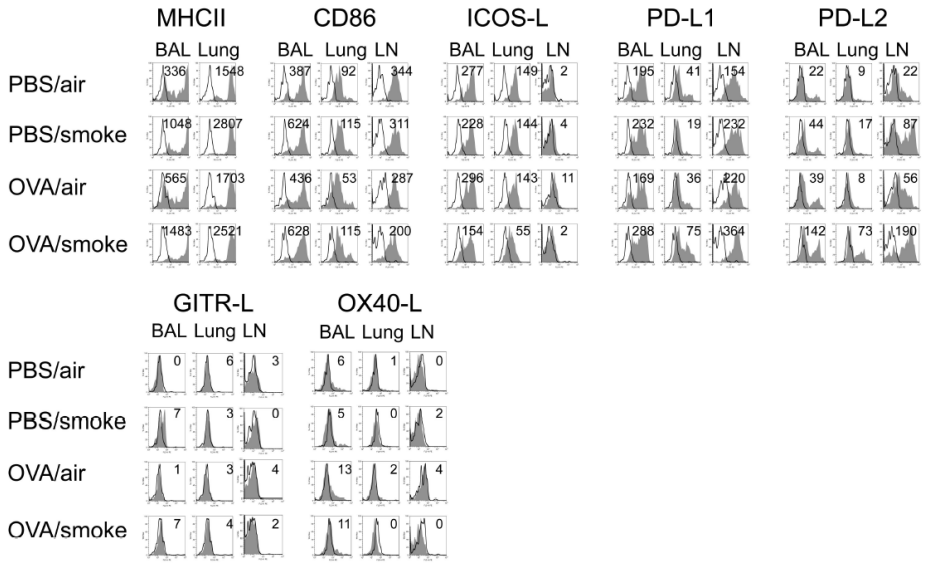


Figure 3: Costimulatory molecule profile of pulmonary dendritic cells during cigarette smoke and/or aerosolized OVA exposure. *Mice were exposed to cigarette smoke (smoke) or sham-exposed (air) together with a PBS- or OVA-aerosol for 3 weeks. Single cell suspensions were prepared from BAL-fluid and enzymatically digested lung tissue and MLNs and stained with monoclonal antibodies. DCs were identified as low-autofluorescent CD11c^{high} cells in BAL-fluid (BAL DC) and lung tissue (lung DC) and as CD11c+ MHCII^{high} cells in LNs (LN DC). Expression levels of various costimulatory markers (grey histograms) are shown relative to isotype control staining (black line). Relative mean fluorescence intensity (MFI antibody corrected for MFI isotype control) is indicated. Cells of 3 mice (n=9 mice per group) were pooled to obtain sufficient cells for staining. Representative histograms of 2 independent experiments are shown.*

Expression of inducible costimulator ligand (ICOS-L, CD275), a member of the B7 family and a negative regulator of immune responses [36], was downregulated from the cell surface of airway and lung DCs of OVA/smoke DCs compared to other groups. Alternatively, expression of two other B7 family members, programmed death ligand-1 (PD-L1, CD274) and PD-L2 (CD273), both ligands for the inhibitory T cell receptor programmed death receptor-1 (PD-1, CD279), was increased on DCs from OVA/smoke-exposed mice in the airway compartment and lung tissue. Remarkably, PD-L2 was clearly upregulated on airway-derived DCs in MLNs of combined OVA/smoke and PBS/smoke-exposed mice. We were not able to detect significant amounts of GITR-L or OX40-L (CD134L) on pulmonary DCs by flow-cytometry (compared to OX40-L positive control staining on splenocytes stimulated with LPS; data not shown).

4.4. Toll-like receptor independency of cigarette smoke-induced allergic sensitization

TLR4 has previously been reported to play a pivotal role in the allergic sensitization of airways towards inhaled OVA [27,28]. Accordingly, we examined whether the observed allergic sensitization could be due to endotoxin-like effects of cigarette smoke. Therefore, we challenged TLR4-deficient mice with cigarette smoke and OVA simultaneously for 3 weeks and measured the degree of eosinophilic inflammation. Remarkably, TLR4-deficient mice were clearly able to develop a Th2-response comparable to the levels observed in Balb/c control mice, as measured by BAL eosinophilia, BAL lymphocytes, BAL DCs and serum OVA-specific IgE (*Fig. 4A*). In addition, histological evaluation of lung tissue showed peribronchial eosinophilic infiltrates in both TLR4-deficient and control mice exposed to cigarette smoke and OVA simultaneously (*Fig. 4B*).

To exclude the contribution of alternative TLRs in cigarette-smoke induced allergic airway sensitization, we repeated the experiment in Myd88-gene deficient animals. MyD88 is an essential adaptor molecule broadly shared among members of the TLR/IL-1R family. OVA/smoke-exposed Myd88-knockout mice did not show any significant reduction in eosinophilic airway inflammation, lymphocytic BAL infiltration and airway DC recruitment (*Fig. 4C*). In contrast, and consistent with earlier observations in murine models of cigarette smoke-induced lung inflammation [37], neutrophil and macrophage recruitment into the airways in response to OVA/smoke inhalation was clearly impaired in the absence of MyD88.

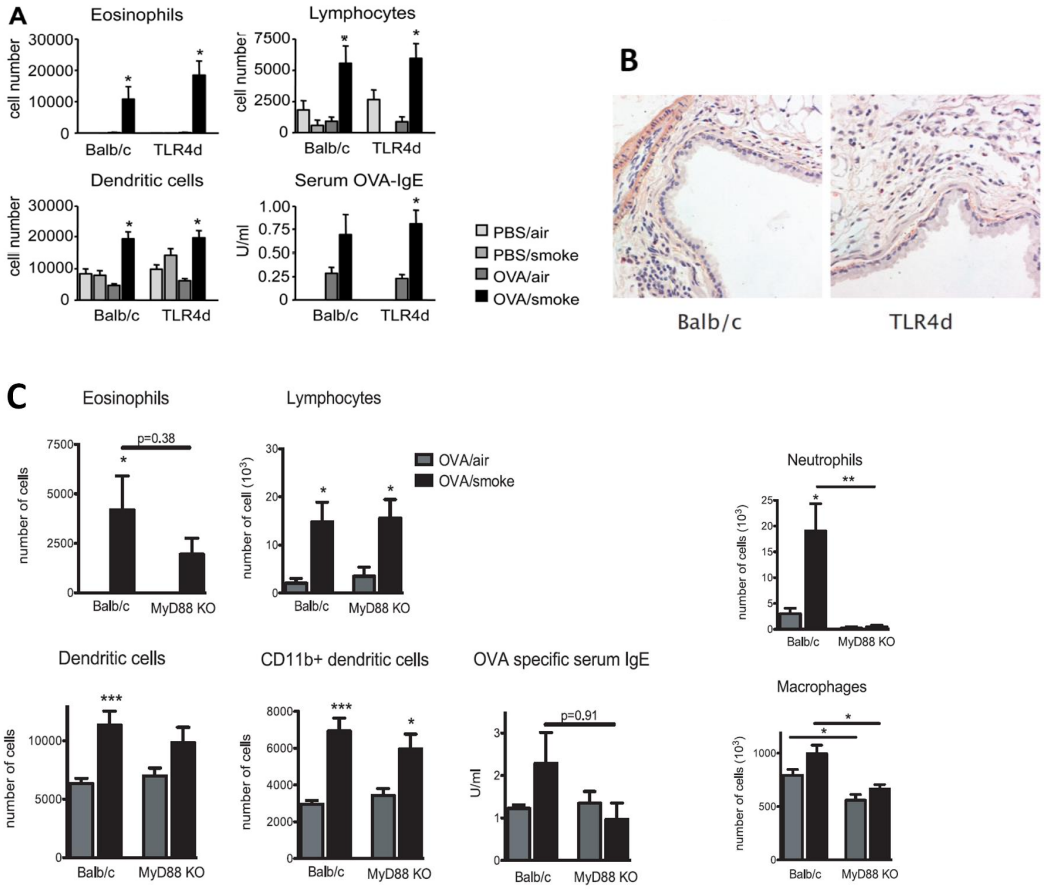


Figure 4: Allergic sensitization of TLR4-deficient mice exposed to cigarette smoke and OVA. *(A) Absolute numbers of eosinophils, lymphocytes and DCs in the bronchoalveolar lavage (BAL) fluid and concentration of OVA-specific IgE in serum of control mice (Balb/c) or TLR4-deficient mice (TLR4d) exposed to PBS or OVA combined with cigarette smoke or air for 3 weeks. Eosinophils and lymphocytes are identified on cytospins stained with May-Grunwald-Giemsa, while DCs are identified by flow-cytometry as CD11c^{high} low-autofluorescent cells. n = 9 mice per group. (B) Histological evaluation of eosinophilic peribronchial infiltrates on Congo Red stained lung tissue sections counterstained with hematoxylin of Balb/c mice and TLR4-deficient mice. (C) Numbers of eosinophils, lymphocytes, neutrophils, DCs and macrophages in the BAL-fluid of MyD88-knockout or Balb/c control mice after 3 weeks of OVA-aerosol with (black bars) or without (grey bars) cigarette smoke simultaneously. Eosinophils, lymphocytes and neutrophils were identified on cytospins, whereas DCs (CD11c^{high} low-autofluorescent cells), CD11b+ DCs (CD11b+ CD11c^{high} low-autofluorescent cells) and macrophages (CD11c^{high} high-autofluorescent cells) were differentiated using FACS analysis. OVA-specific IgE is determined using ELISA. n = 7 mice per group. * represents a significant difference (p<0.05) between the OVA/air and OVA/smoke group, unless otherwise indicated.*

5. Discussion

Concomitant inhalation of aerosolized OVA and cigarette smoke in mice induces a Th2-type airway inflammation that is absent after exposure to either agent alone. This aberrant immune response has high clinical relevance, as illustrated by the interaction between smoking habits and the development of asthma in predisposed subjects. The present study was designed to investigate the triggering of allergic airway inflammation by cigarette smoke from a pulmonary DC point of view. Key features of pulmonary DC biology are (i) recruitment to the antigen exposed lung, (ii) sampling of inhaled antigen and transport towards draining MLNs, (iii) maturation (i.e. increase in costimulatory molecule expression), which is necessary to generate antigen-specific effector T cells. Subsequently, we sought to gain mechanistic insight in the observed phenomenon: based on previous knowledge, we focused on potential endotoxin-like activity in cigarette smoke.

We first confirmed that combined smoke and OVA protein aerosol exposure induces a strong increase in DC numbers in the airway compartment, which is consistent with observations made previously in OVA/alum-sensitized, OVA-aerosol challenged mice [32,38]. Transport of antigens from the airways to the T cell areas of draining LNs represents an additional key step in the antigen-presenting function of airway DCs. Here, we reveal that there is strongly amplified DC-mediated transport of inhaled protein antigen towards MLN in the presence of co-inhaled cigarette smoke. In accordance with this observation, we detected an upregulation of the LN-homing chemokine receptor CCR7 on airway DCs. Migration of DCs to the LNs is enabled by CCR7 expression on the cell surface of DCs interacting with the chemokines CCL21 and CCL19 displayed on lung lymphatics and in T cell zones of LNs. An alternative explanation for the enhanced capture and transport of airway antigen by DCs could be the recently described alteration of airway epithelial tight junctions by mainstream cigarette smoke [39]. The resulting increase in epithelial permeability could lead to enhanced penetration of airborne antigen and promote the activation of the subepithelial DC network [40]. In addition to changes in DC trafficking to the LNs, perturbation of the epithelial homeostasis could also affect DCs in a way that can trigger Th2-oriented sensitization. GM-CSF for instance, a product of the airway epithelium which is increased in smoke-exposed airways [13,17,41], has been shown to induce allergic sensitization to inhaled OVA by means of airway DC activation, in the absence of any other adjuvant [42]. Furthermore, allergic

sensitization by cigarette smoking was recently found to be, at least partially, GM-CSF dependent [16]. Thymic stromal lymphopoietin (TSLP) is another candidate cytokine which can be secreted by the respiratory epithelium and is both a powerful activator of DCs leading to a Th2-polarized immune response in an OX40-OX40L-dependent manner [43]. Interestingly, intanasal administration of cigarette smoke-extract induced TSLP expression in the mouse lung and blocking TSLP activity inhibited OVA-specific Th2 responses and airway inflammation after concomittant cigarette smoke-extract and OVA exposure [44]. However, in our experiments, we could not detect significant modulation of GM-CSF or TSLP expression after either smoke or concomitant OVA/smoke inhalation, nor did we observe upregulation of OX40L on pulmonary DC populations (data not shown).

The pattern of accessory or costimulatory molecule expression on the antigen-transporting DCs is a known determinant in the outcome of immune responses. Our data show that DCs in the airway compartment of OVA/smoke-exposed lungs display an increased expression of accessory molecules previously known for their role in modulating allergic immune responses. In line with our earlier observations [34,35], cigarette smoke inhalation induces upregulation of CD86 (B7-2) on airway DCs. This costimulatory molecule has been clearly shown to be involved in the priming of Th2 responses and the subsequent development of allergic airway inflammation towards inhaled protein [32,45], and could thus be a means through which cigarette smoke exerts it's Th2-skewing properties in this model. The striking downregulation of ICOS-L on airway DCs of mice concomitantly exposed to cigarette smoke and OVA is also consistent with our previous observations in the OVA-alum-i.p./OVA-aerosol asthma model [32]. Expression of ICOS-L on DCs during initial priming of T cells drives the formation of regulatory T cells in lung draining LNs and has been involved in the establishment of respiratory tolerance [36]. Withdrawal of this inhibitory signal might contribute to the preferential Th2 priming towards inhaled antigens. An intriguing observation is the upregulation of PD-L2, another member of the B7 family, on airway-derived DCs in the draining MLNs of mice with OVA/smoke-induced eosinophilic airway inflammation. This is reminiscent of the previously described increase in PD-L2 on airway DCs of OVA-alum-sensitized/OVA-aerosol-challenged allergic mice [46], and might thus be a characteristic feature of allergic immune responses. PD-L2 costimulation during allergen priming and challenge was shown to exacerbate immune and inflammatory allergic responses in mice, supporting a Th2-potentiating function [47]. It has also been proposed that

Th2 cells specifically regulate PD-L2 expression on DCs via reverse signaling [48,49]. The novel finding in our study, however, is a striking upregulation of PD-L2 on airway-derived LN DCs after cigarette smoke inhalation alone. Whether this creates a context prone to Th2-polarized sensitization towards co-inhaled protein is a tempting hypothesis. Further investigations using e.g. blocking antibodies or local delivery of siRNA may help to elucidate the role of DC-expressed PD-L2 in cigarette smoke-induced allergic airway inflammation.

Interestingly, cigarette smoke exposure resulted in a reduction of pDC numbers in the lungs and thoracic lymph nodes. pDCs have been recently described for their capacity to maintain inhalational tolerance: selective depletion of pulmonary pDCs was able to spark Th2-polarized airway inflammation towards inhaled inert antigen, in the absence of any adjuvant [33]. Together with the activation of conventional airway DCs and the increase in DC-mediated transport of inhaled antigen towards lymph nodes, the reduced number of pDCs in smoke-exposed lungs might thus establish a general climate of reduced inhalational tolerance.

In contrast to our current findings, a recent study by Robbins et al. reported that cigarette smoke leads to a decrease in lung DC numbers, a decrease in DC activation markers and no change in migration towards MLNs [50]. These findings are likely attributable to fundamental methodological differences. The most important element (not obvious in the abovementioned study) is the exposure system used, i.e. nose-only vs. whole-body, as this leads to large differences in dose intensity as reflected by carboxyhemoglobin (HbCO) levels. The protocol we used involves more frequent exposures and results in HbCO levels comparable to those measured in human smokers [51,52]. In addition, we report surface expression of costimulatory molecules on DCs as shifts in mean fluorescence intensity (MFI), which is more accurate than % positive cells for these types of markers. Finally, in our study we aim to discriminate effects on lung parenchymal DCs obtained by enzymatic digestion versus airway DCs harvested by BAL. This allows us to detect the confinement of smoke effects (e.g. the upregulation of accessory molecules and CCR7) to the DCs in the airway compartment.

We sought to gain mechanistic insight in the Th2-skewing adjuvant properties of cigarette smoke co-inhaled with inert protein. We hypothesized that this effect would rely on the presence of endotoxin-like activity within the smoke itself. Indeed, whereas endotoxin-free OVA induces inhalational tolerance, co-inhalation of OVA and low-dose endotoxin has been shown to trigger OVA-specific allergic airway inflammation [27]. This phenomenon, along with

the activation and enhanced migration of airway DCs, appeared TLR4-dependent [28]. TLR4 also appears to be a determining factor in the initiation of allergic responses against inhaled house dust mite allergen [53,54]. Furthermore, our group and others previously showed that acute cigarette smoke exposure induces airway DC maturation in a TLR4-dependent fashion [34,37]. Finally, both mainstream and sidestream cigarette smoke are known to contain bioactive endotoxin [30]. Therefore, we were surprised to observe unhampered development of allergic airway inflammation in TLR4-deficient mice exposed to cigarette smoke and OVA. Any endotoxin potentially contaminating the cigarette smoke and/or the OVA used in these studies would have its pro-allergic effects greatly diminished in the absence of functional TLR4. cigarette smoke exposure could also activate alternative TLRs, such as TLR2 which can bind heat shock protein-70, an endogenous danger signal known to be released in smoke-exposed airways [37]. However, smoke-induced allergic sensitization was unhampered in Myd88-gene deficient animals, thereby ruling out the contribution of TLR2 and several other members of the TLR/IL-1R family of innate immune sensors. This is in contrast to airway neutrophilia that disappeared completely in our model (consistent with [37]) and the Myd88-dependency of allergic responses observed after house dust mite challenge, intranasal challenge of OVA with low-dose LPS or in the presence of bacterial infection [29,55,56].

The fact that in our experiments the development of allergic inflammation is unaffected by TLR4/MyD88-deficiency suggests immunological adjuvant mechanisms of a different kind. The cellular stress inflicted by cigarette smoke in the airways, for instance, could lead to the release of compounds that trigger TLR/MyD88-independent immunogenic pathways. One of these endogenous compounds could be ATP, which was recently reported to trigger purinergic receptors on the airway DC network, leading to a DC-dependent allergic sensitization towards inhaled inert protein [57]. Furthermore, alum, a prototypical Th2-skewing adjuvant, was shown to exert its adjuvant effect in a TLR4-independent way and activate the NLRP3 inflammasome pathway either directly, or through the release of uric acid from the local tissue micro-environment [58,59]. It is worth noting that aluminum has been detected at high concentrations in cigarette smoke, potentially precipitating as alum-like hydroxy-salts in the epithelial lining fluid overlying the airway DC network [60]. In support of this, MyD88-signaling is not always necessary for alum to act as an adjuvant for humoral adaptive responses [61]. Hence, cytotoxic compounds in cigarette smoke could act in a TLR/MyD88-independent manner by activating airway DCs either directly or by means of

endogenous danger signals released by stressed airway cells (reviewed in [62]). Whether this could result in a deviation of immune homeostasis towards an aberrant Th2-driven inflammation, is a compelling working hypothesis for further investigation.

6. Acknowledgements

The authors thank E. Castrique, C. Snauwaert, G. Barbier, K. De Saedeleer, I. De Borle, A. Neesen, M. Mouton, E. Spruyt and P. Degryze for their excellent technical support. We also thank Dr Krysko for providing the MyD88-knockout mice.

Reference List

1. Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *Eur. Respir. J.* 2004; 24: 822-33.
2. Gilmour MI, Jaakkola MS, London SJ, Nel AE, Rogers CA. How exposure to environmental tobacco smoke, outdoor air pollutants, and increased pollen burdens influences the incidence of asthma. *Environ. Health Perspect.* 2006; 114: 627-33.
3. Thomson NC, Spears M. The influence of smoking on the treatment response in patients with asthma. *Curr. Opin. Allergy Clin. Immunol.* 2005; 5: 57-63.
4. Lazarus SC, Chinchilli VM, Rollings NJ, Boushey HA, Cherniack R, Craig TJ, Deykin A, DiMango E, Fish JE, Ford JG, Israel E, Kiley J, Kraft M, Lemanske RF, Jr., Leone FT, Martin RJ, Pesola GR, Peters SP, Sorkness CA, Szefler SJ, Wechsler ME, Fahy JV. Smoking affects response to inhaled corticosteroids or leukotriene receptor antagonists in asthma. *Am. J. Respir. Crit Care Med.* 2007; 175: 783-90.
5. Kumar R. Prenatal factors and the development of asthma. *Curr. Opin. Pediatr.* 2008; 20: 682-7.
6. Gilliland FD, Islam T, Berhane K, Gauderman WJ, McConnell R, Avol E, Peters JM. Regular Smoking and Asthma Incidence in Adolescents. *Am. J Respir Crit Care Med.* 2006; 174: 1094-100.
7. Piipari R, Jaakkola JJ, Jaakkola N, Jaakkola MS. Smoking and asthma in adults. *Eur. Respir. J.* 2004; 24: 734-9.
8. Seymour BW, Pinkerton KE, Friebertshauser KE, Coffman RL, Gershwin LJ. Second-hand smoke is an adjuvant for T helper-2 responses in a murine model of allergy. *J. Immunol.* 1997; 159: 6169-75.
9. Moerloose KB, Pauwels RA, Joos GF. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. *Am. J. Respir. Crit Care Med.* 2005; 172: 168-72.
10. Robbins CS, Pouladi MA, Fattouh R, Dawe DE, Vujicic N, Richards CD, Jordana M, Inman MD, Stampfli MR. Mainstream cigarette smoke exposure attenuates airway immune inflammatory responses to surrogate and common environmental allergens in mice, despite evidence of increased systemic sensitization. *J Immunol.* 2005; 175: 2834-42.
11. Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, Van Der Strate BW, Kerstjens HA, Timens W, Hylkema MN. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am. J. Respir. Cell Mol. Biol.* 2004; 30: 880-5.
12. Melgert BN, Timens W, Kerstjens HA, Geerlings M, Luinge MA, Schouten JP, Postma DS, Hylkema MN. Effects of 4 months of smoking in mice with ovalbumin-induced airway inflammation. *Clin. Exp. Allergy* 2007; 37: 1798-808.
13. Rumold R, Jyrala M, Diaz-Sanchez D. Secondhand smoke induces allergic sensitization in mice. *J. Immunol.* 2001; 167: 4765-70.
14. Bowles K, Horohov D, Paulsen D, Leblanc C, Littlefield-Chabaud M, Ahlert T, Ahlert K, Pourciau S, Penn A. Exposure of adult mice to environmental tobacco smoke fails to enhance the immune response to inhaled antigen. *Inhal. Toxicol.* 2005; 17: 43-51.
15. Thatcher TH, Benson RP, Phipps RP, Sime PJ. High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function. *Am. J. Physiol Lung Cell Mol. Physiol* 2008; 295: L412-L421.

16. Trimble NJ, Botelho FM, Bauer CM, Fattouh R, Stampfli MR. Adjuvant and anti-inflammatory properties of cigarette smoke in murine allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 2009; 40: 38-46.
17. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res.* 2006; 7: 49.
18. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 2008; 8: 193-204.
19. Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* 2008; 8: 142-52.
20. Vermaelen K, Pauwels R. Pulmonary dendritic cells. *Am. J Respir Crit Care Med.* 2005; 172: 530-51.
21. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 2004; 5: 987-95.
22. Shimizu K, Fujii S. An adjuvant role of in situ dendritic cells (DCs) in linking innate and adaptive immunity. *Front Biosci.* 2008; 13: 6193-201.
23. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* 2001; 13: 114-9.
24. Demedts IK, Bracke KR, Maes T, Joos GF, Brusselle GG. Different roles for human lung dendritic cell subsets in pulmonary immune defense mechanisms. *Am. J. Respir. Cell Mol. Biol.* 2006; 35: 387-93.
25. Holt PG, Upham JW. The role of dendritic cells in asthma. *Curr. Opin. Allergy Clin. Immunol.* 2004; 4: 39-44.
26. Lambrecht BN, Hammad H. Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat. Rev. Immunol.* 2003; 3: 994-1003.
27. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 2002; 196: 1645-51.
28. Dabbagh K, Dahl ME, Stepick-Biek P, Lewis DB. Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. *J. Immunol.* 2002; 168: 4524-30.
29. Piggott DA, Eisenbarth SC, Xu L, Constant SL, Huleatt JW, Herrick CA, Bottomly K. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *J. Clin. Invest* 2005; 115: 459-67.
30. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999; 115: 829-35.
31. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 2001; 193: 51-60.
32. Vermaelen K, Pauwels R. Accelerated airway dendritic cell maturation, trafficking, and elimination in a mouse model of asthma. *Am. J. Respir. Cell Mol. Biol.* 2003; 29: 405-9.
33. de Heer HJ, Hammad H, Soullie T, Hijdra D, Vos N, Willart MA, Hoogsteden HC, Lambrecht BN. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J. Exp. Med.* 2004; 200: 89-98.
34. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA, Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int. Arch. Allergy Immunol.* 2006; 141: 354-68.

35. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005; 26: 204-13.
36. Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH, Berry G, DeKruyff RH, Umetsu DT. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat. Med.* 2002; 8: 1024-32.
37. Doz E, Noulin N, Boichot E, Guenon I, Fick L, Le Bert M, Lagente V, Ryffel B, Schnyder B, Quesniaux VF, Couillin I. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J. Immunol.* 2008; 180: 1169-78.
38. van Rijt LS, Prins JB, Leenen PJ, Thielemans K, de Vries VC, Hoogsteden HC, Lambrecht BN. Allergen-induced accumulation of airway dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma. *Blood* 2002; 100: 3663-71.
39. Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C. Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. *Inhal. Toxicol.* 2007; 19: 13-22.
40. Gangl K, Reininger R, Bernhard D, Campana R, Pree I, Reisinger J, Kneidinger M, Kundi M, Dolznig H, Thurnher D, Valent P, Chen KW, Vrtala S, Spitzauer S, Valenta R, Niederberger V. Cigarette smoke facilitates allergen penetration across respiratory epithelium. *Allergy* 2009; 64: 398-405.
41. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, Lilja A, Hansen MJ, Gualano RC, Irving L, Anderson GP. Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *Am. J. Physiol Lung Cell Mol. Physiol* 2006; 290: L931-L945.
42. Stampfli MR, Wiley RE, Neigh GS, Gajewska BU, Lei XF, Snider DP, Xing Z, Jordana M. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest* 1998; 102: 1704-14.
43. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt RW, Omori M, Zhou B, Ziegler SF. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu. Rev. Immunol.* 2007; 25: 193-219.
44. Nakamura Y, Miyata M, Ohba T, Ando T, Hatsushika K, Suenaga F, Shimokawa N, Ohnuma Y, Katoh R, Ogawa H, Nakao A. Cigarette smoke extract induces thymic stromal lymphopoietin expression, leading to T(H)2-type immune responses and airway inflammation. *J. Allergy Clin. Immunol.* 2008; 122: 1208-14.
45. van Rijt LS, Vos N, Willart M, Kleinjan A, Coyle AJ, Hoogsteden HC, Lambrecht BN. Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of TH2 effector responses in a mouse model of asthma. *J. Allergy Clin. Immunol.* 2004; 114: 166-73.
46. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* 2005; 201: 981-91.
47. Oflazoglu E, Swart DA, Anders-Bartholo P, Jessup HK, Norment AM, Lawrence WA, Brasel K, Tocker JE, Horan T, Welcher AA, Fitzpatrick DR. Paradoxical role of programmed death-1 ligand 2 in Th2 immune responses in vitro and in a mouse asthma model in vivo. *Eur. J. Immunol.* 2004; 34: 3326-36.

48. Loke P, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc. Natl. Acad. Sci. U. S. A* 2003; 100: 5336-41.
49. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, Azuma M, Yagita H. Expression of programmed death 1 ligands by murine T cells and APC. *J. Immunol.* 2002; 169: 5538-45.
50. Robbins CS, Franco F, Mouded M, Cernadas M, Shapiro SD. Cigarette smoke exposure impairs dendritic cell maturation and T cell proliferation in thoracic lymph nodes of mice. *J. Immunol.* 2008; 180: 6623-8.
51. Bracke KR, D'hulst AI, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice. *Clin. Exp. Allergy* 2007; 37: 1467-79.
52. Macdonald G, Kondor N, Yousefi V, Green A, Wong F, Aquino-Parsons C. Reduction of carboxyhaemoglobin levels in the venous blood of cigarette smokers following the administration of carbogen. *Radiother. Oncol.* 2004; 73: 367-71.
53. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.
54. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, Karp CL. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009; 457: 585-8.
55. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, Mattes J, Barry J, Davidson S, Oreo K, Smith L, Mansell A, Matthaei KI, Foster PS. Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 responses. *Am. J. Respir. Crit Care Med.* 2009; 179: 883-93.
56. Schroder NW, Crother TR, Naiki Y, Chen S, Wong MH, Yilmaz A, Slepenkin A, Schulte D, Alsabeh R, Doherty TM, Peterson E, Nel AE, Arditi M. Innate immune responses during respiratory tract infection with a bacterial pathogen induce allergic airway sensitization. *J. Allergy Clin. Immunol.* 2008; 122: 595-602.
57. Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC, Jr., Lambrecht BN. Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat. Med.* 2007; 13: 913-9.
58. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 2008; 453: 1122-6.
59. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, Hoogsteden HC, Hammad H, Lambrecht BN. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* 2008; 205: 869-82.
60. Exley C, Begum A, Woolley MP, Bloor RN. Aluminum in tobacco and cannabis and smoking-related disease. *Am. J. Med.* 2006; 119: 276-11.
61. Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, Nemazee D. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science* 2006; 314: 1936-8.
62. Robays LJ, Maes T, Joos GF, Vermaelen KY. Between a cough and a wheeze: dendritic cells at the nexus of tobacco smoke-induced allergic airway sensitization. *Mucosal. Immunol.* 2009; 2: 206-19.

6.2

6.2. SHORT CIGARETTE SMOKE EXPOSURE FACILITATES SENSITIZATION AND ASTHMA DEVELOPMENT IN MICE

Ellen A. Lanckacker, Kurt G. Tournoy, MD, PhD, Hamida Hammad, PhD, Gabriele Holtappels, Bart N. Lambrecht, MD, PhD, Guy F. Joos, MD, PhD, Tania Maes, PhD

European Respiratory Journal. 2012 Aug 16. [Epub ahead of print]

1. Abstract

Objective: Epidemiological studies indicate that cigarette smoke (CS) exposure is a risk factor for increased sensitization and asthma development. The aim of the study was to examine the impact of CS on sensitization and allergic airway inflammation, in response to a low dose of house dust mite (HDM), and to obtain potential mechanistic insights.

Methods: Mice were exposed to low doses of HDM extract combined with air or CS exposure, either during allergen sensitization or during development of allergic airway disease.

Results: Mice concomitantly exposed to low dose HDM, combined with CS for 3 weeks, demonstrated an asthmatic phenotype with significantly increased airway eosinophilia, goblet cell metaplasia, airway hyperresponsiveness and a rise in HDM-specific serum IgG1, compared to sole HDM or CS exposure. In addition, short CS inhalation, during the initial contact with HDM allergens, was sufficient to facilitate sensitization and development of a complete asthmatic phenotype after rechallenge with HDM. Mechanistically, short CS exposure amplified DC-mediated transport of FITC-labelled HDM allergens to the intrathoracic lymph nodes and generated a local Th2 response.

Conclusion: Short CS exposure is sufficient to facilitate allergic sensitization and the development of low dose HDM-induced allergic asthma, possibly through affecting dendritic cell function.

2. Introduction

Most asthma begins in early childhood after sensitization and re-exposure to ubiquitous environmental allergens, like house dust mites (HDM), moulds, plant pollen or animal dander. The risk for sensitization is strongly dependent on the level of allergen exposure [1]. Over the last decades, the incidence of asthma has increased worldwide, especially in industrialized countries. This strong rise in asthma prevalence emphasizes an important role for environmental and socio-economic conditions [2].

One of the main environmental risk factors, associated with asthma is the exposure to cigarette smoke (CS) [3]. Epidemiological studies have shown that smoking is responsible for higher asthma severity scores [4,5], diminished lung function [5,6] and poorer asthma control [7,8]. Smoking is even associated with increased sensitization to HDM allergens [9], and appears to be a risk factor for new onset asthma among children and adults [10-13]. The mechanisms mediating the adverse effects of smoking on asthma pathogenesis remain to be elucidated. Most murine models, designed to characterize the complex interaction between smoking and allergic airway inflammation, used to rely on the sensitization to the "surrogate" allergen ovalbumin (OVA) [14-19]. As OVA is an intrinsically inert protein, the role of CS might have been overestimated in the past. Moreover, the differences in biochemical character between OVA and real-life allergens remain an undeniable limitation of the previously used OVA models.

To explain the observed increase in asthma prevalence due to CS exposure, we hypothesized that CS may lower the threshold for sensitization to HDM allergens. We developed a clinically relevant murine model of allergic asthma, using low doses of HDM as real-life aeroallergen, combined with CS. We examined the effect of CS on both sensitization and ensuing asthma development and checked whether a few days of smoke exposure are sufficient to prime local sensitization in the lymph nodes. Finally, we looked for potential mechanistic insights.

3. Methods

Mice

Male Balb/c mice (6-8 weeks old) were purchased from Harlan (Zeist, the Netherlands). All *in vivo* manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Cigarette smoke (CS) exposure and house dust mite (HDM) administration

Mice (n = 10 per group) were subjected to whole body CS (3R4F Kentucky Reference cigarettes) as described before [20]. Control mice were exposed to air. 30 minutes after the last smoke exposure, 25 µg HDM extract (*Dermatophagoides pteronyssinus*) (Greer Laboratories, Lenoir, NC, USA) (25,27 µg Der p1/mg protein; 8.43 endotoxin U/mg) or PBS was administered intranasally in isoflurane anesthetized mice on days 0, 7 and 14 and mice were analysed on day 17. To evaluate the impact of CS on the development of HDM-induced allergic asthma, we performed *Protocol 1* (*Figure 1*). To unravel the impact of CS during the sensitization phase, mice were subjected to HDM and CS according to *Protocol 2 or 4* (*Figure 1*). The impact of CS during the challenge phase was evaluated using *Protocol 3* (*Figure 1*).

Bronchoalveolar lavage (BAL) and cytospins

24 hours after the last smoke exposure and 72 hours after the last HDM application, mice were euthanized with an overdose of pentobarbital (Sanofi-Ceva, Paris, France). BAL, cytospins and cell differentiation were performed as described previously [20]. Remaining cells were used for FACS-analysis.

Lung and mediastinal lymph node (mLN) single-cell suspensions

Lungs were perfused with saline plus EDTA through the pulmonary artery to remove contaminating blood cells. Lungs and mLN were removed and digested as described before [21].

Flow cytometry

Staining procedures, data acquisition and analysis were performed as described previously [20]. Monoclonal antibodies (mAbs) used to identify mouse DC populations were anti-CD11c (clone HL3), anti-MHC class II (I-A/I-E, clone M5/114.15.2) and anti-CD11b (clone M1/70). $CD11b^+$ DCs were defined as CD11c-bright, low autofluorescent cells which strongly express

MHC class II and CD11b on their surface. The following mAbs were used to stain mouse T-cell subpopulations: anti-CD3 (clone 145-2C11), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7) and anti-CD69 (clone H1.2F3) (all mAbs from BD Pharmingen, San Diego, CA, USA).

MLN cell culture

Paratracheal and parathymic intrathoracic LNs were collected into sterile tubes containing cold (4°C) tissue culture medium (TCM) and digested (see above) to obtain a single cell suspension. TCM was prepared using RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin/streptomycin and β-mercaptoethanol (all from Gibco BRL; Invitrogen Corp). Cells were then transferred in triplicate to round-bottom, 96-well plates (Becton Dickinson (BD), BD, CA, USA) with or without 15 µg HDM extract/ml culture medium, at a density of 2 x 10^5 cells per well and incubated in a humidified 37°C incubator with 5% CO_2. After 5 days, supernatants were harvested and frozen for cytokine measurements.

Histology

The left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Transversal sections of 3 µm were stained with Congo Red (Klinipath, Olen, Belgium) to highlight eosinophils, with periodic acid-Schiff (PAS) to identify goblet cells or immunostained for α-smooth muscle actin to evaluate airway smooth muscle content. Quantitative measurements were performed using a Zeiss KS400 Image Analyzer platform (Oberkochen, Germany) as described earlier [22]. To identify mast cells, lungs were stained with toluidin blue. The number of mast cells was counted per field (whole left lung lobe). Only those located in the airway wall were included for analysis.

Protein measurements

Total IgE was measured using coated microtiter plates and biotinylated polyclonal rabbit anti-mouse IgE (S. Florquin, ULB, Brussels, Belgium). For the detection of HDM-specific IgG1, microtiter plates were coated with HDM extract. Serum was added, followed by a horseradish peroxidase (HRP) conjugated polyclonal goat anti-mouse IgG1 antibody (Bethyl Laboratories, Montgomery, USA). Levels of HDM-specific IgG1 were reported in optical densities (OD). MLN supernatants were assayed for IL-4, IL-5, IL-13, IL-10 and IFN-gamma by means of multiplex (Merck Millipore, Brussels, Belgium). In the supernatants of crushed

lungs, IL-25, IL-33, TSLP, GM-CSF and IL-1β were measured with ELISA (R&D Systems, Abingdon, UK) following the manufacturer's instructions.

Assessment of airway responsiveness

Airway responsiveness was measured in tracheostomized anaesthetized mice using the FlexiVent System (SCIREQ, Montreal, QC, Canada). Neuromuscular blockade was induced by injecting pancuronium bromide (1 mg/kg) intravenously. To check for airway hyperresponsiveness, mice were challenged with increasing doses of carbachol (0, 5, 10, 20, 40, 80, 160 and 320 µg/kg). A "snapshot perturbation" manoeuvre was imposed to measure the (dynamic) resistance (R) of the whole respiratory system (airways, lung and chest wall). For each mouse, a dose-response curve was generated and the area under the curve (AUC) was calculated.

Evaluation of DC migration by intratracheal instillation of fluorescent HDM

To obtain fluorescently labelled HDM, HDM extract (Greer Laboratories, Lenoir, NC, USA) was dialysed against a carbonate-bicarbonate buffer (pH 9.5), overnight at 4°C. 10mg/ml FITC in DMSO (Sigma Aldrich, Bornem, Belgium) was added to the HDM solution and rotated for 1 hour at room temperature. The whole procedure was performed under dark, sterile conditions. After 4 days of CS exposure, anesthetized mice (i.p. ketamine (80 mg/kg; Ketamine 1000 CEVA; Ceva Sante Animale, Brussels, Belgium) – xylazine (8 mg/kg; Rompun 2%; Bayer AG, Leverkusen, Germany)) were held vertically and 50 µl of FITC conjugated HDM (100 µg) or PBS was pipetted just above their vocal cords. Mice were sacrificed 0, 24 and 48 hours after instillation. MLN were removed and processed as described above. Discrimination in the mLN between the airway derived (AW-DCs) ($CD11c^{int\text{-}high}/MHCII^{high}$) and non-airway derived DCs (NAW-DCs) ($CD11c^{+}/MHCII^{int}$) was performed using the method published by Vermaelen *et al* [21]. The % of HDM-bearing airway-derived DCs (AW-DCs) was determined by flow cytometry as the fraction of $FITC^{+}$ $MHCII^{high}$ $CD11c^{int\text{-}high}$ cells. The results were expressed as the % of $FITC^{+}$ DCs regarding to the total DC population.

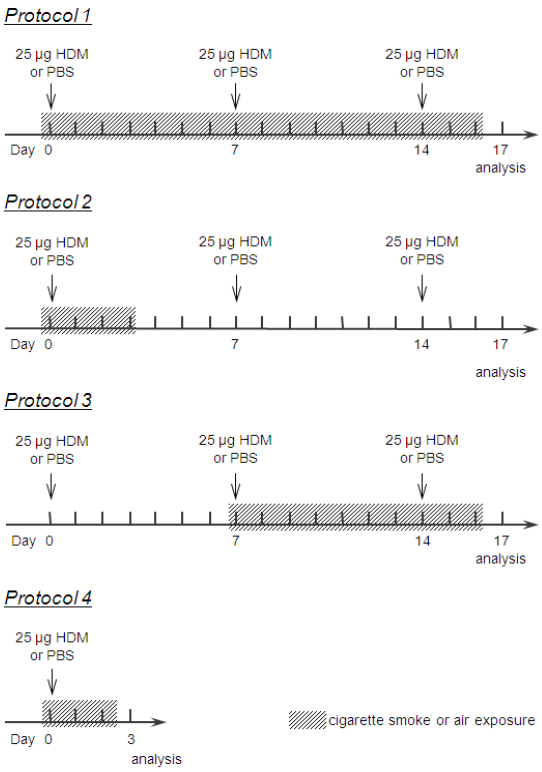
Statistical analysis

Statistical analysis was performed with PASW Statistics 18 using nonparametric tests. The different experimental groups were compared by a Kruskall-Wallis test for multiple

comparisons. Pairwise comparisons were made by means of a Mann-Whitney U-test. A p-value $p \leq 0.05$ was considered significant. Reported values are expressed as mean ± SEM.

4. Results

4.1. CS facilitates the development of new onset allergic asthma



To evaluate the role of CS in the pathogenesis of HDM-induced allergic asthma, Balb/c mice were exposed to CS or air for 3 weeks and instilled with HDM (25 µg) or PBS once a week (*Figure 1 – Protocol 1*). We instilled 4 times less HDM compared with our previously described model [23], in order to limit the biological effects of HDM on its own.

Figure 1: Exposure protocols. *4 groups were included in our experimental set-up: PBS/air, PBS/CS (sole CS), HDM/air (sole HDM) and HDM/CS. Abbreviations: PBS = phosphate buffered saline, HDM = house dust mite, CS = cigarette smoke*

4.1.1. Exposure to both HDM and CS aggravates the allergic response in BAL fluid and lung

The intranasal delivery of sole HDM elicited a faint asthmatic phenotype with increased eosinophils, $CD11b^+$ DCs and $CD4^+$ and $CD8^+$ T lymphocytes as compared to PBS exposed control mice (*Figure 2*). CS exposure as such enhanced the amount of total cells and macrophages (data not shown), neutrophils, $CD11b^+$ DCs and $CD4^+$ and $CD8^+$ T lymphocytes in the BAL fluid compared to air exposed mice (*Figure 2*). The concomitant exposure to HDM and CS amplified the allergic phenotype considerably. This is reflected by a 10-fold increase in the number of eosinophils and a rise in $CD11b^+$ DCs and $CD4^+$ and $CD8^+$ T lymphocytes in BAL fluid as compared to the other groups. No further increase in macrophages (data not shown) or neutrophils was observed (*Figure 2*).

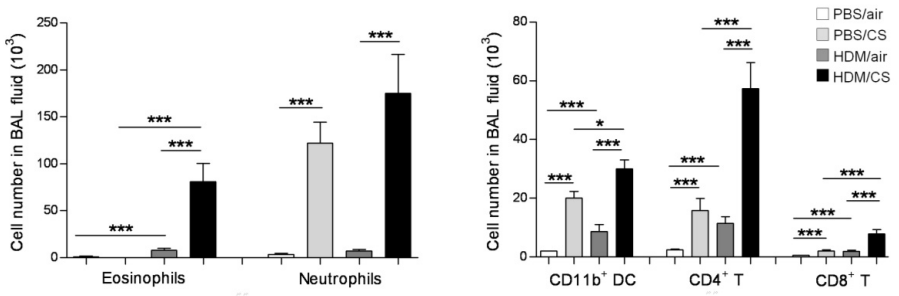


Figure 2: Inflammatory response from mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM), combined with air or cigarette smoke (CS) for 3 weeks. *Cell differentiation of bronchoalveolar lavage (BAL) fluid. Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

In line, lung single cell suspensions had more CD11b$^+$ DCs and activated CD4$^+$CD69$^+$ and CD8$^+$CD69$^+$ T cells as compared to sole HDM or CS (*Figure 3a*). Histological analysis revealed peribronchovascular eosinophilic inflammation and mucin-producing goblet cells in HDM exposed mice, compared with naive control mice (*Figure 3b,c*). However, exposure to both CS and HDM, resulted in a further increase of eosinophils, goblet cells and mast cells (*Figure 3b,c,d*). Other features of airway wall remodelling, such as quantification of airway smooth muscle content, revealed no differences between the 4 groups (data not shown).

4.1.2. Combined exposure to HDM and CS increases HDM-specific IgG1 production in serum

Total serum IgE did not differ between the 4 groups (data not shown), but HDM exposed mice showed significantly elevated allergen-specific IgG1 titers in the serum, compared with naive control mice. This level was further increased after combined exposure to HDM and CS (*Figure 4a*). HDM-specific IgE measurements were below detection limit.

4.1.3. Concomitant exposure to HDM and CS amplifies the production of Th2 cytokines

HDM restimulated mLN cells from the sole HDM group showed increased production of the inflammatory cytokines IL-4, IL-5, IL-13 and IL-10, together with decreased levels of the typical Th1 cytokine IFN-γ (*Figure 4b*). The HDM/CS combination yielded in a further increase in IL-4, IL-5, IL-13 and IL-10 (*Figure 4b*). IFN-γ had a further tendency to decrease (*Figure 4b*). The role of the epithelium was investigated by measuring innate pro-Th2 cytokines in lung homogenate. Exposure to both HDM and CS, resulted in more IL-25, IL-33 and IL-1β (F*igure 4c*). TSLP and GM-CSF were below detection limit.

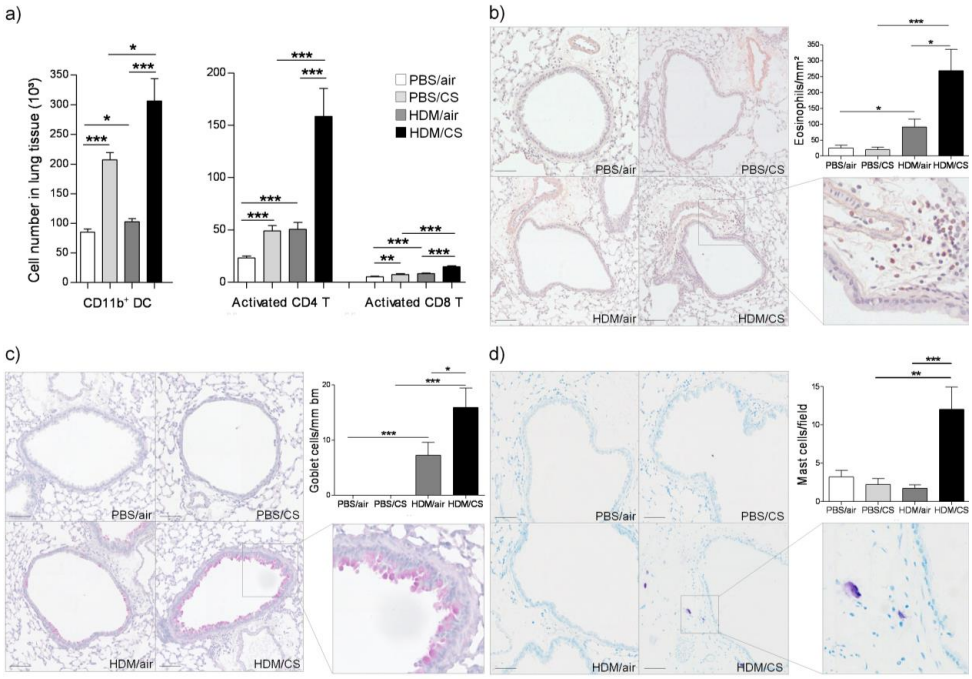


Figure 3: Histopathological evaluation from mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM), combined with air or cigarette smoke (CS) for 3 weeks. *a) Cell differentiation of lung digest. b) Photomicrographs of eosinophilic peribronchial infiltrates. c) Photographs of goblet cells. d) Total number of mast cells. Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

4.1.3. Concomitant exposure to HDM and CS amplifies the production of Th2 cytokines

HDM restimulated mLN cells from the sole HDM group showed increased production of the inflammatory cytokines IL-4, IL-5, IL-13 and IL-10, together with decreased levels of the typical Th1 cytokine IFN-γ (Figure 4b). The HDM/CS combination yielded in a further increase in IL-4, IL-5, IL-13 and IL-10 (Figure 4b). IFN-γ had a further tendency to decrease (Figure 4b). The role of the epithelium was investigated by measuring innate pro-Th2 cytokines in lung homogenate. Exposure to both HDM and CS, resulted in more IL-25, IL-33 and IL-1β (Figure 4c). TSLP and GM-CSF were below detection limit.

4.1.4. CS exposure exacerbates HDM-induced airway hyperresponsiveness

Figure 4d shows the dose-response curve of the *in vivo* reactivity of all 4 groups. HDM or CS exposed mice were slightly responsive towards the highest carbachol dose. Mice concomitantly exposed to both stimuli were more responsive (Figure 4d).

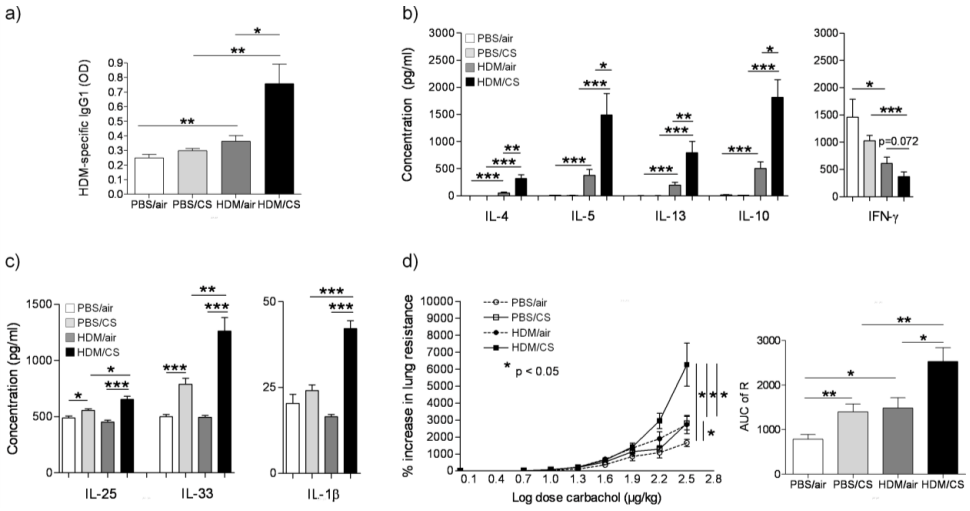


Figure 4: Immunoglobulins, cytokines and airway hyperresponsiveness of mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM), combined with air or cigarette smoke (CS) for 3 weeks. *a) HDM-specific IgG1. b) Protein levels of IL-4, IL-5, IL-13, IL-10 and IFN-γ in the supernatant of HDM restimulated lymph node cells. c) Measurements of IL-25, IL-33 and IL-1β in supernatant of crushed lungs. d) Airway hyperresponsiveness to carbachol. Dose-response curve and area under the curve (AUC). Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

4.2. CS exposure during sensitization and not during allergen challenge is important for subsequent asthma development

To investigate the putative role of CS during sensitization, Balb/c mice were briefly exposed to CS (for 4 consecutive days) and instilled with HDM (25 µg) once a week during 3 consecutive weeks (Figure 1 – *Protocol 2*). In the sera of HDM/CS exposed mice, enhanced allergen-specific IgG1 titers were detected (Figure 5a). In contrast to *Protocol 1*, where mice received CS for 3 weeks, we observed no neutrophils after limited CS exposure in *Protocol 2*. (Figure 5b). Mice concomitantly exposed to HDM and only 4 days of CS showed more BAL $CD11b^+$ DCs, $CD4^+$ and $CD8^+$ T-lymphocytes in comparison to all other groups and had a 20-fold increase in the number of BAL eosinophils (Figure 5b). Compared to *Protocol 1*, the

inflammation in BAL fluid was less pronounced and even in lung single cell suspensions, the number of DCs and T-lymphocytes no longer increased upon combined HDM/CS exposure (Figure 5c). Histological examination revealed however significantly more eosinophils (Figure 5d) and goblet cells in the airway wall (Figure 5e) together with increased airway hyperresponsiveness in concomitantly exposed mice (Figure 6a).

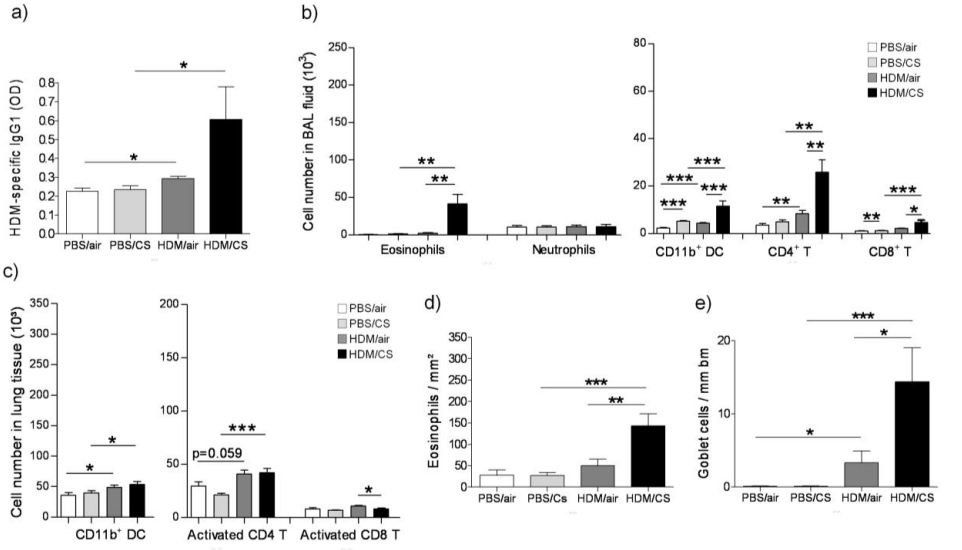


Figure 5: Immunoglobulins, inflammatory response and histopathological evaluation of mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM) and 4 days of air or cigarette smoke (CS). *a) HDM-specific IgG1. b) Cell differentiation of bronchoalveolar lavage (BAL) fluid and c) lung digest. d) Quantification of eosinophils. e) Measurement of goblet cells. Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

Altogether, these Th2 associated airway responses were accompanied by increases in IL-4, IL-5, IL-13 and IL-10 in the supernatant of restimulated mLN cultures of HDM/CS exposed mice (Figure 6b), but no differences in IL-25, IL-33 and IL-1β production could be observed between all 4 groups (Figure 6c).

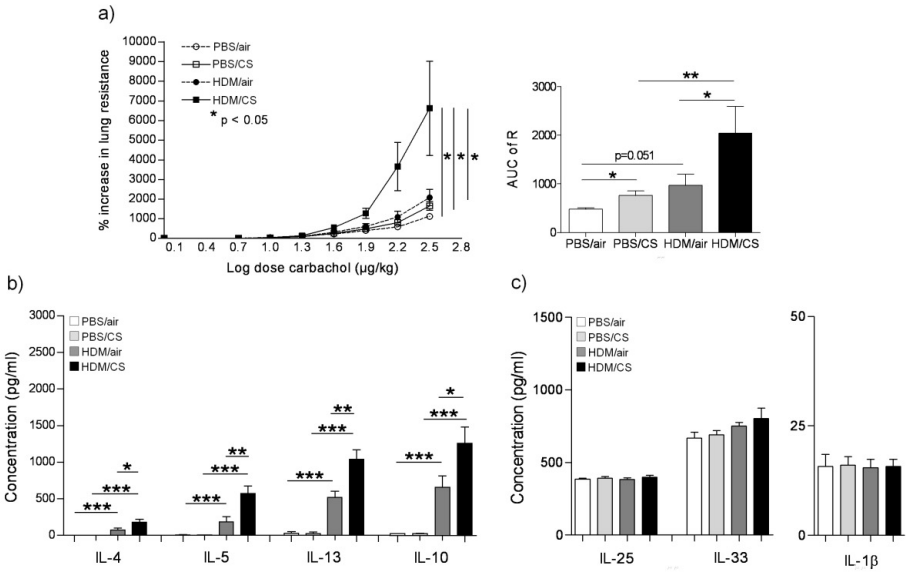


Figure 6: Airway hyperresponsiveness to carbachol and cytokine responses of mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM) and 4 days of air or cigarette smoke (CS). *a) Dose-response curve to carbachol and area under the curve (AUC). b) Protein levels of IL-4, IL-5, IL-13 and IL-10 in supernatant of HDM restimulated lymph node cells. c) Measurements of IL-25, IL-33 and IL-1β in supernatant of crushed lungs. Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

On the contrary, exposure to CS exclusively during the HDM challenge phase (Figure 1 – *Protocol 3*) was unable to induce an allergic phenotype and did not show increased HDM specific IgG1 (Figure 7a), nor elevated numbers of eosinophils, $CD11b^+$ DCs or T lymphocytes in BAL fluid (Figure 7b) and lung tissue, except for a rise of $CD4^+CD69^+$ T cells in the lung (Figure 7c). Additionally, we found no increase in the number of goblet cells (Figure 7d) or airway hyperresponsiveness in these mice (Figure 7e).

4.3. Short exposure to CS enhances HDM uptake and DC migration to the mLN and facilitates sensitization to common aeroallergens

To investigate if 4 days of smoke inhalation already affects airway DC trafficking and antigen transport to the draining LNs, we delivered fluorescently labelled HDM intratracheally to CS or air exposed mice. FITC+ DCs were exclusively found within the population of $CD11c^{int-high}/MHCII^{high}$ AW-DCs. At various time points after HDM instillation (24h, 48h), CS exposed mice showed a marked increase in DC-mediated HDM transport to the draining LNs, compared to air exposed mice (Figure 8a).

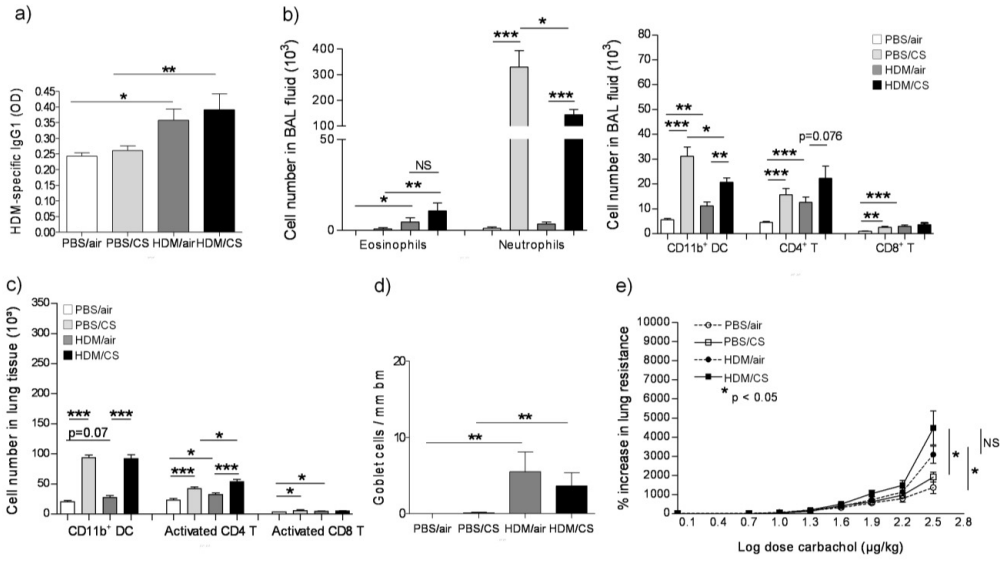


Figure 7: Immunoglobulins, inflammatory response, histopathological evaluation and airway hyperresponsiveness of mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM) and air or cigarette smoke (CS) during the allergen challenge phase. *a) HDM-specific IgG1. b) Cell differentiation of bronchoalveolar lavage (BAL) fluid and c) lung digest. d) Quantification of goblet cells. e) Airway hyperresponsiveness to carbachol. Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

To characterize the role of CS during initial sensitization towards common aeroallergens, we exposed Balb/c mice to CS for 3 consecutive days and instilled HDM allergens on the first day (25µg) (Figure 1 – *Protocol 4*). This short interaction between CS and HDM allergens significantly increased the number of $CD11b^+$ DCs in BAL fluid and lung tissue (Figure 8b,c), with enhanced activation of BAL DCs, as read out by the expression of CD86 (Figure 8b). At the functional level, we found that brief exposure to CS during the first contact with HDM allergens was sufficient to induce sensitization in the mLN, characterized by a pronounced Th2 cytokine profile in HDM/CS exposed mice (Figure 8d). To explain this heightened state of allergen-specific sensitization, we measured typical DC-activating cytokines, released by bronchial epithelial cells early in the sensitization process. TSLP and GM-CSF were below detection limit, but IL-25, IL-33 and IL-1β were elevated in CS exposed mice (Figure 8e). IL-1β increased further after concomitant exposure to HDM and CS.

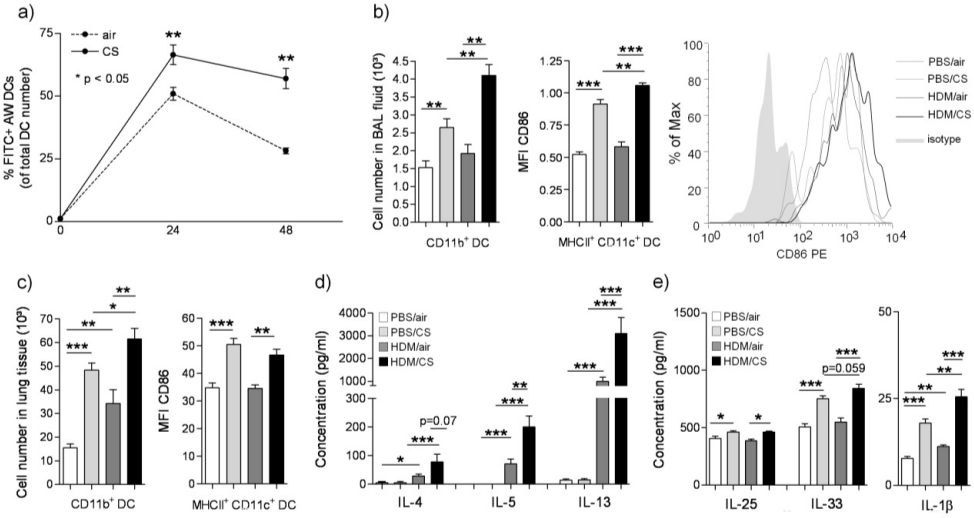


Figure 8: Migration, recruitment and maturation of pulmonary DCs and corresponding cytokine profiles upon phosphate buffered saline (PBS) or house dust mite (HDM) and acute air or cigarette smoke (CS) exposure. *a) Dendritic cell (DC) migration to the mediastinal lymph nodes. b) CD11b$^+$ DCs and expression of CD86 on BAL DCs c) and lung DCs (calculated within the population of low autofluorescent, CD11c$^+$, MHCII$^+$ DCs). d) Protein levels of IL-4, IL-5 and IL-13 in HDM restimulated lymph node cells. e) Measurements of IL-25, IL-33 and IL-1ß in supernatant of crushed lungs. Results are expressed as means ± SEM; n=10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

5. Discussion

In this paper, we demonstrate that cigarette smoke (CS) exposure can play a determining role during allergic sensitization and asthma development. (i) We show that concomitant exposure to CS and a low dose of house dust mite (HDM) results in a pronounced Th2-related asthmatic phenotype, which is hardly present when sole HDM is used. In addition, we demonstrate that, (ii) in the presence of allergens, acute CS exposure is sufficient to cause sensitization and subsequent asthma development, (iii) possibly by the amplified allergen transport of airway DCs towards the mLNs.

The strongest predictive factor for asthma development is the sensitization to common environmental allergens, like house dust mites (HDMs), grass pollen or animal dander. Epidemiological studies provide indirect clinical evidence that smoking is associated with increased sensitization to HDM allergens [24]. Smoke exposure is even correlated with higher asthma incidence and severity of the disease. Especially children become more susceptible due to smoke exposure, as illustrated by the increased wheeze and asthma prevalence among children and young adolescents upon passive smoke inhalation [25].

Within our lab, we created a murine model, supporting the findings from epidemiological studies and using HDM as clinically relevant allergen. HDM is the most significant source of indoor allergens, responsible for atopic symptoms in 10% of individuals. It is a complex mixture of various protein allergens and non-protein compounds, with some allergens having the natural capacity to induce mucosal sensitization through the respiratory tract [24]. Although the content of commercially available preparation of HDM extract can vary extensively [26], these extracts might be a good representation of the indoor HDM allergens, present in our homes. To examine whether CS can lower the threshold for asthma development, we first created a mild murine asthma model by down-titration of the HDM protein content until almost no asthmatic phenotype could be elicited (data not shown). Such models are relevant to evaluate potential synergistic effects upon CS inhalation.

In this paper, we demonstrate that CS exposure facilitates and aggravates the asthmatic disease, as illustrated by the increased eosinophils and neutrophils in BAL. These findings are in agreement with our previous work, investigating the role of CS in asthma development with ovalbumin (OVA) as “surrogate” allergen [15,27]. However, OVA is no

naturally occurring allergen and prolonged exposure elicits inhalation tolerance, rather than allergic inflammation in mice [28].

An important novelty of our study, using adolescent mice of 6-8 weeks old, is the striking result that only 3 days of CS exposure during the initial allergen contact are sufficient to prime HDM-specific Th2 cells in the LNs. This process might be driven by the enhanced HDM transport of the airway DCs. Our findings suggest that adolescent smokers and young children, may become more susceptible to allergic sensitization and ensuing asthma development, due to (short) CS inhalation. To our opinion, these results can be extrapolated to humans, since our murine CS exposure protocol reaches carboxyhemoglobin (COHb) levels comparable to those in human smokers [29,30] and since COHb levels of young children (aged 1-2) exposed to parental smoke are similar to those measured in adult smokers [31].

Furthermore, we focussed on the release of innate pro-allergic cytokines, known to instruct DCs to mount Th2-mediated cell responses in the lung [32]. Three weeks of HDM and CS exposure resulted in significantly more IL-25, IL-33 and IL-1β. In contrast, when stopping CS exposure after the initial sensitization, no differences in these cytokines were found 2 weeks after smoke cessation. This suggests a synergistic role for CS, particularly during the ongoing allergic response and illustrates the direct impact of CS on airway epithelial cells and on the release of innate pro-Th2 cytokines, the driving force in activating DCs and ensuing asthma development. IL-1β and IL-33 were even increased after 1 HDM administration concomitant with 3 days of smoke exposure, suggesting a role for these cytokines during the sensitization phase and in facilitating the sensitization process due to CS. This idea is supported by Willart *et al.* confirming the role of these cytokines early in asthma development. Neutralizing IL-1β during HDM sensitization reduced the production of Th2 cytokines, whereas blocking IL-33 signalling at the time of sensitization decreased the number of eosinophils and lymphocytes in the BAL [33].

To our knowledge, this is the first *in vivo* model showing unambiguous synergy between CS and HDM allergens. In line with our observation, Rusznak *et al.* found increased inflammatory mediator release from primary *in vitro* cultures of human bronchial epithelial cells, after exposure to CS and *Der p* allergens [34]. Blacquière *et al.* examined the effect of maternal smoking during pregnancy [35]. Upon HDM exposure, mice offspring of smoking mothers showed increased airway wall remodelling and AHR, but no increase in

inflammatory response or elevated Th2 cytokines could be demonstrated in HDM/CS exposed mice. Mitchell *et al.* investigated the role of progesterone and, or CS in exacerbating allergic airway disease. Although the difference in experimental setup, they found some indications for increased allergic inflammation due to CS, however less pronounced than in our model [36,37]. In contrast to our current findings, a recent study by Botelho *et al.* [38] using a murine model of established allergic asthma, reported significantly attenuated eosinophilia in BAL fluid of mice exposed to both HDM and CS, together with decreased mucus production and no difference in $CD4^+$ T cell activation nor AHR between HDM and, or CS exposed mice.

Disparities between our data and the studies mentioned above, might be related to fundamental differences in allergen and CS exposure protocol, such as the use of alum as Th2 skewing adjuvant [36,37] or the timing, duration and intensity of smoke exposure. In addition, the variability between commercially available preparations of HDM extract (e.g. endotoxin content) may affect the development of potential Th2 responses in the lung [38].

Compared to the previous studies, our model is innovative since we show synergy using a mild asthma model and a short CS exposure protocol. The clear distinction between the sensitization and allergen challenge phase, made it possible to examine the impact of CS on different phases of asthma pathogenesis. Because of the diversity of asthma phenotypes, preclinical mouse models of combined exposures to allergens and environmental pollutants will become more important in the future. Combination models mimic more closely the real-life situation in humans and are therefore more reliable to provide mechanistic insights and to test potential therapeutic strategies.

In conclusion and in agreement with epidemiological studies, we provide biological mechanistic data, supporting the hypothesis that environmental factors such as cigarette smoke, are risk factors for sensitization and ensuing asthma development. We found that even short-term cigarette smoke exposure can lower the threshold for allergen sensitization, making individuals more vulnerable to future asthma development.

6. Acknowledgement

We would like to thank Greet Barbier, Eliane Castrique, Indra De Borle, Philippe De Gryze, Katleen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, Ann Neessen, Christelle Snauwaert and Evelyn Spruyt for their excellent technical assistance.

The present study was supported by the Concerted Research Action of the University of Ghent (BOF/GOA 01251504) and the Interuniversity Attraction Poles (IUAP) Program, Belgian State, Belgian Science Policy, Project P6/35. K.G.T. is a senior clinical investigator with a grant from FWO-Vlaanderen.

Reference List

1. Tovey ER, Marks GB. It's time to rethink mite allergen avoidance. *J Allergy Clin. Immunol.* 2011; 128: 723-7.
2. D'Amato G, Liccardi G, D'Amato M, Holgate S. Environmental risk factors and allergic bronchial asthma. *Clin. Exp. Allergy* 2005; 35: 1113-24.
3. Pietinalho A, Pelkonen A, Rytila P. Linkage between smoking and asthma. *Allergy* 2009; 64: 1722-7.
4. Polosa R, Russo C, Caponnetto P, Bertino G, Sarva M, Antic T, Mancuso S, Al-Delaimy WK. Greater severity of new onset asthma in allergic subjects who smoke: a 10-year longitudinal study. *Respir. Res.* 2011; 12: 16.
5. Comhair SA, Gaston BM, Ricci KS, Hammel J, Dweik RA, Teague WG, Meyers D, Ampleford EJ, Bleecker ER, Busse WW, Calhoun WJ, Castro M, Chung KF, Curran-Everett D, Israel E, Jarjour WN, Moore W, Peters SP, Wenzel S, Hazen SL, Erzurum SC. Detrimental Effects of Environmental Tobacco Smoke in Relation to Asthma Severity. *PLoS. One.* 2011; 6: e18574.
6. Boulet LP, Lemiere C, Archambault F, Carrier G, Descary MC, Deschesnes F. Smoking and asthma: clinical and radiologic features, lung function, and airway inflammation. *Chest* 2006; 129: 661-8.
7. Thomson NC, Spears M. The influence of smoking on the treatment response in patients with asthma. *Curr. Opin. Allergy Clin. Immunol.* 2005; 5: 57-63.
8. Chaudhuri R, McSharry C, McCoard A, Livingston E, Hothersall E, Spears M, Lafferty J, Thomson NC. Role of symptoms and lung function in determining asthma control in smokers with asthma. *Allergy* 2008; 63: 132-5.
9. Jarvis D, Chinn S, Luczynska C, Burney P. The association of smoking with sensitization to common environmental allergens: results from the European Community Respiratory Health Survey. *J. Allergy Clin. Immunol.* 1999; 104: 934-40.
10. Oberg M, Jaakkola MS, Woodward A, Peruga A, Pruss-Ustun A. Worldwide burden of disease from exposure to second-hand smoke: a retrospective analysis of data from 192 countries. *Lancet* 2011; 377: 139-46.
11. Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C. Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. *Inhal. Toxicol.* 2007; 19: 13-22.
12. Piipari R, Jaakkola JJ, Jaakkola N, Jaakkola MS. Smoking and asthma in adults. *Eur. Respir. J.* 2004; 24: 734-9.
13. Polosa R, Knoke JD, Russo C, Piccillo G, Caponnetto P, Sarva M, Proietti L, Al-Delaimy WK. Cigarette smoking is associated with a greater risk of incident asthma in allergic rhinitis. *J. Allergy Clin. Immunol.* 2008; 121: 1428-34.
14. Rumold R, Jyrala M, Diaz-Sanchez D. Secondhand smoke induces allergic sensitization in mice. *J. Immunol.* 2001; 167: 4765-70.
15. Robays LJ, Lanckacker EA, Moerloose KB, Maes T, Bracke KR, Brusselle GG, Joos GF, Vermaelen KY. Concomitant inhalation of cigarette smoke and aerosolized protein activates airway dendritic cells and induces allergic airway inflammation in a TLR-independent way. *J. Immunol.* 2009; 183: 2758-66.
16. Moerloose KB, Pauwels RA, Joos GF. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. *Am. J. Respir. Crit Care Med.* 2005; 172: 168-72.

17. Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, Van Der Strate BW, Kerstjens HA, Timens W, Hylkema MN. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am. J. Respir. Cell Mol. Biol.* 2004; 30: 880-5.
18. Van Hove CL, Moerloose K, Maes T, Joos GF, Tournoy KG. Cigarette smoke enhances Th-2 driven airway inflammation and delays inhalational tolerance. *Respir. Res.* 2008; 9: 42.
19. Thatcher TH, Benson RP, Phipps RP, Sime PJ. High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function. *Am. J. Physiol Lung Cell Mol. Physiol* 2008; 295: L412-L421.
20. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, Brusselle GG. Cigarette Smoke-Induced Pulmonary Inflammation and Emphysema Are Attenuated in CCR6-Deficient Mice. *J Immunol.* 2006; 177: 4350-9.
21. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 2001; 193: 51-60.
22. Van Hove CL, Maes T, Cataldo DD, Gueders MM, Palmans E, Joos GF, Tournoy KG. Comparison of acute inflammatory and chronic structural asthma-like responses between C57BL/6 and BALB/c mice. *Int. Arch. Allergy Immunol.* 2009; 149: 195-207.
23. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.
24. Ishii A, Takaoka M, Matsui Y. Production of guinea pig reaginic antibody against the house dust mite extract, Dermatophagoides pteronyssinus, without adjuvant. *Jpn. J Exp. Med.* 1977; 47: 377-83.
25. Burke H, Leonardi-Bee J, Hashim A, Pine-Abata H, Chen Y, Cook DG, Britton JR, McKeever TM. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics* 2012; 129: 735-44.
26. Geissler W, Maasch HJ, Winter G, Wahl R. Kinetics of allergen release from house dust mite Dermatophagoides pteronyssinus. *J Allergy Clin. Immunol.* 1986; 77: 24-31.
27. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res.* 2006; 7: 49.
28. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged Inhaled Allergen Exposure Can Induce Persistent Tolerance. *Am. J Respir Cell Mol. Biol.* 2007; 36: 573-84.
29. Bracke KR, D'hulst AI, Maes T, Demedts IK, Moerloose KB, Kuziel WA, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice. *Clin. Exp. Allergy* 2007; 37: 1467-79.
30. Macdonald G, Kondor N, Yousefi V, Green A, Wong F, Aquino-Parsons C. Reduction of carboxyhaemoglobin levels in the venous blood of cigarette smokers following the administration of carbogen. *Radiother. Oncol.* 2004; 73: 367-71.
31. Yee BE, Ahmed MI, Brugge D, Farrell M, Lozada G, Idupaganthi R, Schumann R. Second-hand smoking and carboxyhemoglobin levels in children: a prospective observational study. *Paediatr. Anaesth.* 2010; 20: 82-9.
32. Hammad H, Lambrecht BN. Dendritic cells and airway epithelial cells at the interface between innate and adaptive immune responses. *Allergy* 2011; 66: 579-87.

33. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* 2012; 209: 1505-17.
34. Rusznak C, Sapsford RJ, Devalia JL, Shah SS, Hewitt EL, Lamont AG, Davies RJ, Lozewicz S. Interaction of cigarette smoke and house dust mite allergens on inflammatory mediator release from primary cultures of human bronchial epithelial cells. *Clin. Exp. Allergy* 2001; 31: 226-38.
35. Blacquiere MJ, Timens W, Melgert BN, Geerlings M, Postma DS, Hylkema MN. Maternal smoking during pregnancy induces airway remodelling in mice offspring. *Eur. Respir. J.* 2009; 33: 1133-40.
36. Mitchell VL, Van Winkle LS, Gershwin LJ. Environmental Tobacco Smoke and Progesterone Alter Lung Inflammation and Mucous Metaplasia in a Mouse Model of Allergic Airway Disease. *Clin. Rev. Allergy Immunol.* 2011.
37. Mitchell VL, Gershwin LJ. Progesterone and environmental tobacco smoke act synergistically to exacerbate the development of allergic asthma in a mouse model. *Clin. Exp. Allergy* 2007; 37: 276-86.
38. Botelho FM, Llop-Guevara A, Trimble NJ, Nikota JK, Bauer CM, Lambert KN, Kianpour S, Jordana M, Stampfli MR. Cigarette smoke differentially affects eosinophilia and remodeling in a model of house dust mite asthma. *Am. J Respir. Cell Mol. Biol.* 2011; 45: 753-60.

6.3

6.3. MECHANISTIC INSIGHTS INTO THE ROLE OF CIGARETTE SMOKE AS RISK FACTOR FOR ALLERGIC SENSITIZATION AND ASTHMA DEVELOPMENT IN MICE – A PRELIMINARY REPORT

Ellen A. Lanckacker, Ken Bracke, PhD, Sijranke Post, Mieke A. Dentener, PhD, Irene H. Heijink, PhD, Guy G. Brusselle, MD, PhD, Kurt G. Tournoy, MD, PhD, Guy F. Joos, MD, PhD, Tania Maes, PhD

In preparation

1. Abstract

Objective: Cigarette smoke (CS) exposure is associated with increased asthma development in children and adults. The aim of the study was to obtain mechanistic insights into the role of CS as a risk factor for allergic sensitization and subsequent asthma development, with the main focus on the airway epithelium.

Methods: To unravel the impact of CS during mucosal sensitization or asthma development, mice were subjected to whole body CS (3 times/day, 5 days/week) or air, combined with house dust mite extract (HDM) or PBS (intranasal, 1/week) for 3 days or 3 weeks.

Results: Expression analysis of E-cadherin in mice exposed to HDM or CS for 3 days showed no significant changes in epithelial integrity. However, the induction of early Th2 immunity as well as the inflammatory response upon acute HDM/CS exposure were IL1RI dependent. In addition, investigation of danger-associated molecular pattern molecules, revealed uric acid as well as hyaluronan to be additionally increased upon acute and, or prolonged concomitant HDM/CS exposure.

Conclusion: Preliminary results point to a role for IL1RI signaling, uric acid and hyaluronan during increased allergic sensitization or asthma development upon CS inhalation. However, additional experiments are needed to further elucidate the airway epithelial function.

2. General introduction

Although individuals with asthma are often genetically predisposed, the interaction with environmental factors is critical for the expression of the disease [1]. Epidemiological studies have demonstrated that cigarette smoke (CS) exposure is a major risk factor in the development or aggravation of asthma [2,3]. To obtain mechanistic insights into the role of CS as adjuvant during allergic sensitization and ensuing asthma development, we previously designed a murine model of facilitated allergic inflammation after acute (3 days) and prolonged (3 weeks) concomitant house dust mite (HDM) and CS inhalation [4]. We demonstrated that acute CS exposure during primary allergen sensitization may induce local Th2 immunity in the lymph nodes. Because respiratory epithelial cells play a pivotal role in asthma pathogenesis [5], we focused on the airway epithelium as a key factor in promoting early mucosal sensitization in the lung. More precisely, we investigated potential contributions of the airway epithelial barrier function, IL1RI signaling and damage associated molecular pattern (DAMP) molecules.

3. The role of the airway epithelial barrier function during allergic sensitization

3.1. Introduction

The **airway epithelium** acts as a physical barrier between the internal and external milieu of the lungs. To protect against inhalants, the epithelial integrity depends on tight junction (e.g. occludin, claudin, ZO-1) and adherens junction molecules (e.g. **E-cadherin**), which form adhesive forces between adjacent epithelial cells [6]. In asthma, the airway epithelium is often compromised, possibly the result of proteolytic activity from inhaled aeroallergens or as a result of irritant compounds. Indeed, HDM allergens, as well as CS, have been shown to damage airway epithelial cells, by decreasing the E-cadherin expression [7,8] or disrupting tight junction proteins [8-11]. To explain the observed increase in allergic sensitization due to CS inhalation, we hypothesize that CS may synergize with HDM allergens to further decrease the epithelial barrier function. Decreased epithelial integrity may facilitate transepithelial delivery of HDM allergens to antigen-presenting cells, hence increasing the sensitization risk.

3.2. Preliminary results

3.2.1. Analysis of epithelial integrity after acute HDM/CS exposure

To investigate the role of the epithelial barrier function during CS-induced facilitated sensitization, we exposed mice to CS for 3 days, instilled HDM on the first day (*Figure 1*) and measured the E-cadherin expression on lung tissue sections. We observed no significant difference in E-cadherin expression between all 4 groups, although the E-cadherin level of some individual subjects within the PBS/air groups appeared to be higher. (*Figure 2a,b*).

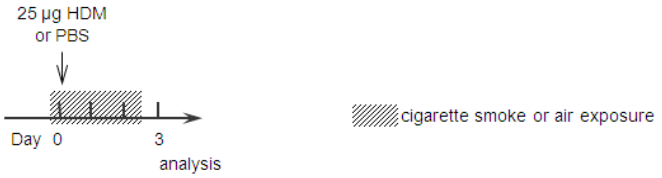


Figure 1: Exposure protocol 1. *To analyse whether short cigarette smoke inhalation during primary allergen contact, facilitates allergic sensitization, we exposed male mice (n= 8 à 10) for 3 days to cigarette smoke and instilled HDM (Greer) at the first day. 4 groups were included in our experimental set-up: PBS/air, PBS/CS (sole CS), HDM/air (sole HDM) and HDM/CS.*

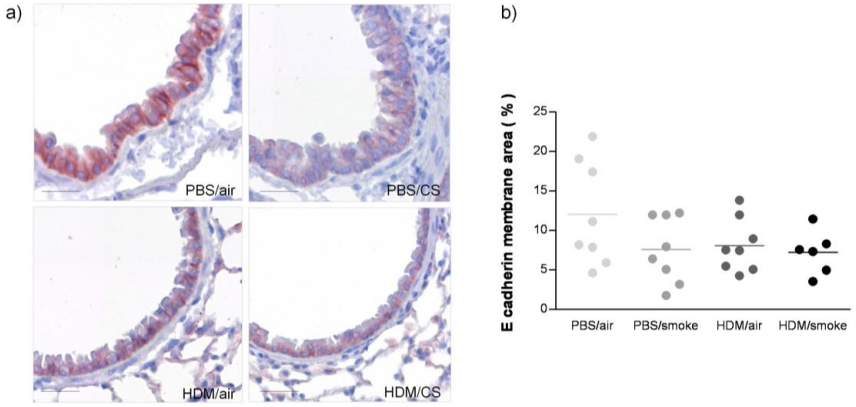


Figure 2: E-cadherin membrane expression in Balb/c mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM) and 3 days of air or cigarette smoke (CS). *a) Photomicrographs of E-cadherin staining between airway epithelial cells. b) Quantification of E-cadherin positive staining, with levels expressed as the percentage of E-cadherin positivity relative to the total surface area of the airway epithelium. Each individual point refers to the mean E-cadherin staining per mouse, measured on at least 7 lung sections. Means are shown ; n=8 à 10 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

3.3. Discussion

We investigated the role of the airway epithelial barrier function during CS-induced allergic sensitization in mice. Although we did not find significant differences between all 4 groups, some individual E-cadherin values within the PBS/air control group seemed to be higher, which is in line with previous findings from the literature. De Boer *et al*. demonstrated decreased E-cadherin expression in bronchial biopsies from atopic asthmatic patients compared with non-atopic controls [8]. In addition, *in vitro* studies on human bronchial epithelial cells revealed a transient fall in E-cadherin after HDM stimulation [7,12] and reduced the expression of epithelial tight junction proteins upon exposure to CS extract [13].

Future experiments will be performed on larger experimental groups to increase the power of the E-cadherin analysis. Moreover, because possible differences in E-cadherin expression may become more explicit after a more prolonged exposure period, the expression analysis will also be performed in a more extended 3 week exposure protocol [4].

4. The role of IL1RI signaling during CS-induced allergic sensitization

4.1. Introduction

Activation of airway epithelial cells has been shown to release innate pro-allergic cytokines, such as thymic stromal lymphopoietin (TSLP), granulocyte macrophage colony-stimulating factor (GM-CSF) or interleukin (IL)-1 [14-16]. We previously showed that IL-1β is significantly increased during facilitated sensitization due to CS inhalation [4]. IL-1α and IL-1β are both pro-inflammatory isoforms, acting on the same IL1RI receptor. Although IL-1α is generally released as an 'alarmin' [17], both cytokines are enhanced in response to inflammatory stimuli. IL-1α and IL-1β are both synthesized as precursor proteins and unlike IL-1α, the IL-1β precursor form requires proteolytic cleavage to become fully activated (generally performed by caspase-1) [18]. Upon IL1RI activation, transcription factors NF-κB or AP-1 are stimulated to further express pro-inflammatory genes. Because the IL-1 signaling pathway is involved in many inflammatory disorders, such as rheumatoid arthritis or asthmatic disease, we are interested whether the IL1RI signaling is important to facilitate allergic sensitization upon CS inhalation.

4.2. Preliminary results

4.2.1. IL-1β but not IL-1α is increased after acute HDM/CS exposure

Because IL-1α and IL-1β are both cytokines with shared biological activity, we were interested whether IL-1α, like IL-1β (see ***Chapter 6.2.***), is significantly increased after acute HDM/smoke exposure (*Figure 1*). 3 days of CS exposure increased the level of IL-1α in lung homogenates, however in contrast to IL-1β, no additional increase could be found after exposure to HDM and CS together (*Figure 3a,b*).

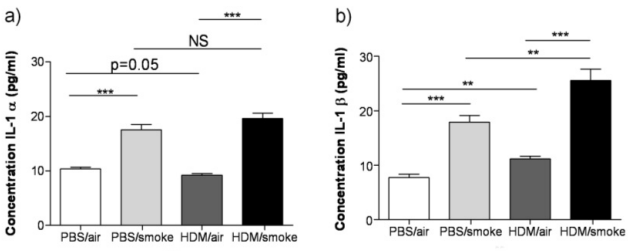


Figure 3: Pulmonary levels of IL-1α and IL-1β in mice concomitantly exposed to phosphate buffered saline (PBS) or house dust mite (HDM) and 3 days of air or cigarette smoke (CS). *a) IL-1a and b) IL-1b in lung homogenate of Balb/c mice, measured by ELISA. Results are expressed as means ± SEM; n=8 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

4.2.2. IL1RI is implicated in acute HDM/CS-induced pulmonary inflammation

To further unravel the role of IL-1 during CS-induced facilitated allergic sensitization, we concomitantly exposed WT and IL1RI KO mice to CS for 3 days and instilled HDM on the first day (*Figure 1*). In accordance with our previous results [4], C57Bl/6 WT mice exposed to a combination of HDM and CS had significantly more BAL and lung $CD11b^+$ DCs (*Figure 4a,b*), and additionally more BAL neutrophils (*Figure 4c*). In contrast, HDM/CS treated IL1RI KO mice were protected against this acute increase of inflammatory cells (*Figure 4a,b,c*). At this short time point, no eosinophils could be enumerated.

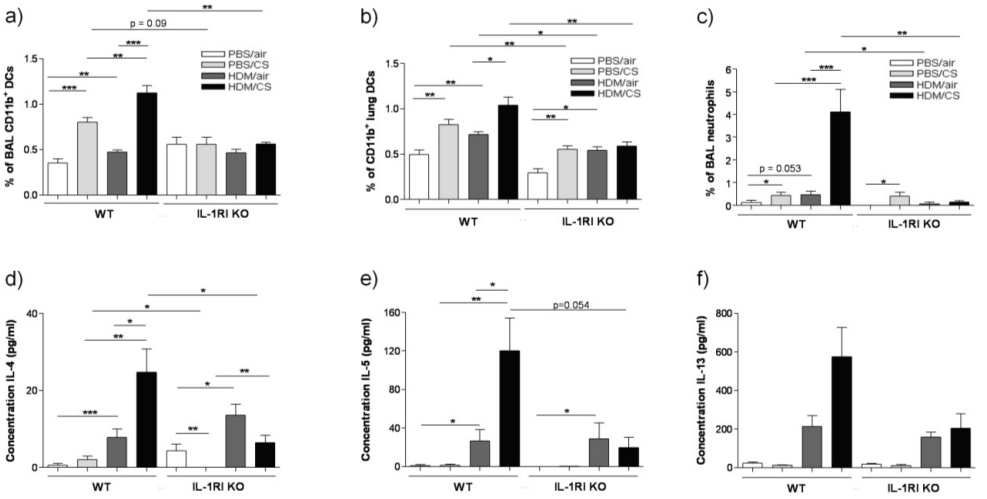


Figure 4: The effect of IL1RI deficiency on pulmonary inflammation and the development of local Th2 immunity in C57/Bl6 mice concomitantly exposed to phosphate buffered saline (PBS) or house dust mite (HDM) and 3 days of air or cigarette smoke (CS). *a) % of CD11b+ dendritic cells (DC) in bronchoalveolar lavage (BAL) and b) lung digest using flow cytometry. c) % of neutrophils in BAL fluid quantified on cytospins. d) Protein levels of IL-4, e) IL-5 and f) IL-13 in the supernatant of HDM restimulated lymph node cells, measured by ELISA. Results are expressed as means ± SEM; n=8 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

4.2.3. IL1RI is involved in the development of local Th2 sensitization in the lymph nodes

To check whether IL1RI is necessary to prime local Th2 responses in the draining lymph nodes, mediastinal lymph node cells from WT and IL1RI KO mice were cultured *in vitro* and restimulated with HDM. Culture supernatant of HDM/CS treated WT mice revealed more IL-4, IL-5 and IL-13 compared to all control groups. However, in contrast, IL1RI KO mice were protected against CS-induced allergic sensitization, as illustrated by lower levels of Th2 cytokines (*Figure 4d,e,f*).

4.3. Discussion

We evaluated the role of IL1RI during CS-induced facilitated sensitization. Using knockout mice, we showed that both the cellular influx as well as the induction of early Th2 immunity were IL1RI dependent. Previous work already demonstrated the IL1RI dependence during CS- [19-21] or HDM-induced inflammation [15,22], however we are the first to display the importance after combined HDM/CS inhalation during the early sensitization phase. In conflict with Willart *et al*, HDM-treated IL1RI-/- mice were not fully protected against Th2 sensitization, a discrepancy that might be explained because of differences in exposure protocol and timing of analysis. Furthermore, in line with Pauwels *et al*, 3 days of CS inhalation already enhanced the level of IL-1α and IL-1β in lung homogenate, though concomitant HDM/CS inhalation only additionally increased IL-1β. This finding suggests a potential role for the IL-1β – IL-1RI pathway during CS-induced facilitated sensitization, however, neutralization with blocking antibodies is required to confirm this assumption.

5. The role of DAMP molecules during sensitization and subsequent asthma development

5.1. Introduction

Upon epithelial injury or cellular stress, **damage associated molecular pattern** (DAMP) molecules or 'alarmins' are released to alert the host. DAMP molecules are sensed by the innate immune system through pathogen recognition receptors (PRRs) and are known to orchestrate airway inflammation by the recruitment of innate inflammatory cells. Uric acid, as well as, high-mobility group box 1 or hyaluronic acid are a few examples of DAMP molecules, which are markedly increased in pulmonary secretions of patients with asthma [23-26]. Endogenous **uric acid** (UA) crystals are the metabolic breakdown product of purine nucleotides. UA is released from dying or stressed cells and is known to promote Th2 immunity through the activation of inflammatory DCs [23]. **High-mobility group box 1** (HMGB-1) is a highly conserved chromatin binding protein [27], which enhances the production of pro-inflammatory cytokines by activating neutrophils and DCs [28,29]. **Hyaluronic acid** (HA) is a high molecular weight (HMW >1 x 10^6) polysaccharide, ubiquitously present in the extracellular matrix of all vertebrates. Upon tissue damage or inflammation, HMW HA breaks down into small low molecular weight (LMW) fragments (average molecular mass of 25 x 10^4 Da) with various pro-inflammatory properties, such as enhanced maturation of DCs [30] or activation of alveolar macrophages [31]. HA synthase genes *Has1* and *Has2* are responsible for the production of HMW HA, while *Has3* creates LMW fragments. In addition, hyaluronidase *Hyal2* degrades HMW HA into LMW fragments, which are further degraded into oligosaccharides by *Hyal1* encoded enzymes [32]. Since DAMP molecules are released upon tissue damage or stress, we hypothesize that CS may synergize with HDM allergens to further increase epithelial injury and the release of DAMP molecules.

5.2. Preliminary results

5.3.1. Uric acid is increased after acute and prolonged HDM/CS exposure

3 days of CS inhalation and one HDM application (*Figure 1*) significantly increased the level of uric acid (UA) in lung homogenate, compared to sole HDM or CS exposed mice (*Figure 5a*). The difference in UA concentration between the experimental groups, became even more pronounced after 3 weeks of concomitant HDM/CS exposure (*Figure 5b*) (*Figure 6*), meaning that UA might be an important trigger during CS-induced facilitated sensitization and

subsequent asthma development. No difference in BAL uric acid concentrations were measured between the groups (data not shown).

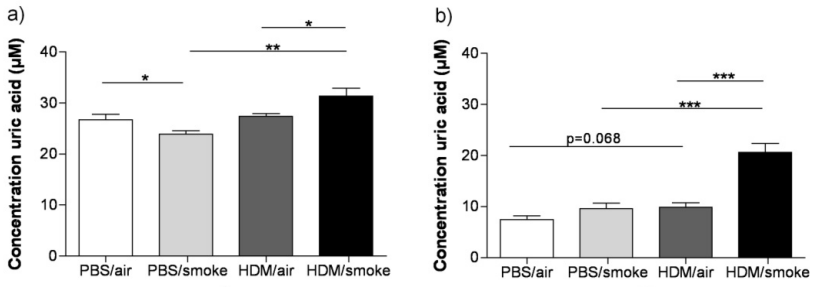


Figure 5: Pulmonary levels of uric acid *a) in Balb/c mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM) combined with air or cigarette smoke (CS) for 3 days or b) 3 weeks. Results are expressed as means ± SEM; n=8 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

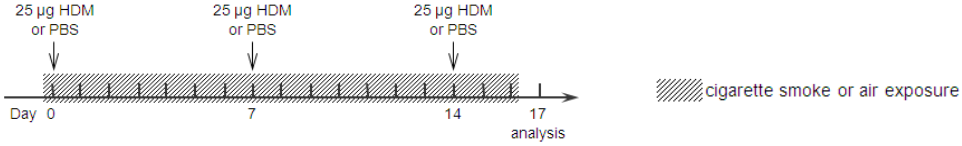


Figure 6: Exposure protocol 2. *To evaluate the effect of cigarette smoke on the development of HDM-induced allergic asthma, we exposed mice to cigarette smoke for 3 consecutive weeks and instilled HDM extract once a week. 4 groups were included in our experimental set-up: PBS/air, PBS/CS (sole CS), HDM/air (sole HDM) and HDM/CS.*

5.3.2. Hyaluronan is increased after prolonged, concomitant HDM and CS exposure

The level of total hyaluronan in BAL fluid was enhanced after 3 days of CS exposure (*Figure 1*) (*Figure 7a*), but even further increased after a more prolonged and concomitant exposure to HDM and CS for 3 weeks (*Figure 6*) (*Figure 7b*). Within the 3 week exposure model, we quantified the deposition of HA in the airway wall after immunohistochemical staining of lung tissue sections. Using color recognition, quantification revealed no difference between all 4 groups (*Figure 7c*). To find an explanation for the enhanced concentration of HA in BAL fluid after 3 week of HDM and CS, we studied the expression of the HA synthase and hyaluronidase genes on total lung. RT-PCR revealed a significant decrease of the mRNA expression of *Has1* after sole HDM or CS exposure and an increased expression of *Has3* after CS inhalation (*Figure 7d,e*). In addition, no difference in the expression profile of *Has2*, *Hyal1* and *Hyal2* was found between the groups (data not shown).

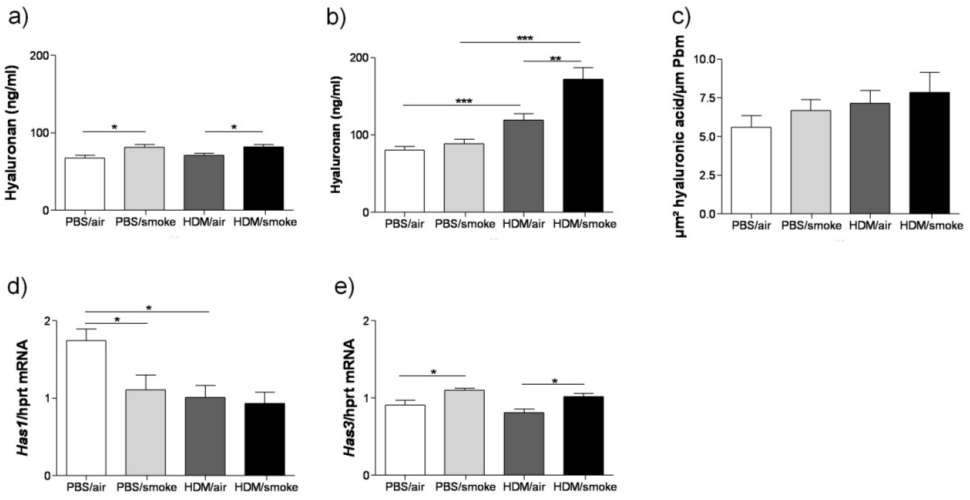


Figure 7: The role of hyaluronic acid (HA) during concomitant exposure to phosphate buffered saline (PBS) or house dust mite (HDM) combined with air or cigarette smoke (CS). *a) Protein levels of HA in Balb/c mice exposed to PBS or HDM, combined with air or CS for 3 days. b) Protein levels of HA in mice exposed to PBS or HDM, combined with air or CS for 3 weeks. c) Quantitative measurement of the HA deposition in lung tissue upon 3 weeks of PBS or HDM and air or CS exposure. d) mRNA expression of HA modulating enzymes Has1 and e) Has3 in mice concomitantly exposed to PBS or HDM and air or CS exposure of 3 weeks. Results are expressed as means ± SEM; n=8 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

5.3.3. High-mobility group box 1 is not enhanced after simultaneous HDM/CS exposure

The evaluation of the HMGB-1 level in BAL fluid after acute HDM/CS exposure revealed no difference between the 4 groups (*Figure 8a*). HMGB-1 was significantly enhanced after 3 weeks of sole HDM or CS exposure, but not additionally after concomitant exposure to both stimuli (*Figure 8b*).

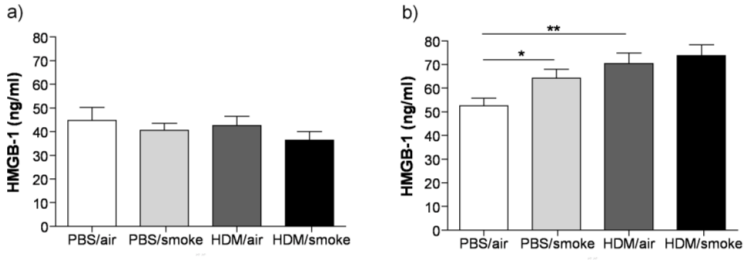


Figure 8: Bronchoalveolar lavage (BAL) levels of high-mobility group box 1 (HMGB-1) *a) in Balb/c mice simultaneously exposed to phosphate buffered saline (PBS) or house dust mite (HDM) combined with air or cigarette smoke (CS) for 3 days or b) 3 weeks and measured by ELISA. Results are expressed as means ± SEM; n=8 animals/group. *p<0.05; **p<0.01; ***p<0.001.*

5.3. Discussion

We examined the production of DAMP molecules during allergic sensitization and asthma development. We observed a significantly higher release of uric acid (UA) upon acute and prolonged HDM/CS exposure, suggesting a potential role for this molecule during CS-induced facilitated sensitization and asthma progression. UA as been identified as a promoter of mucosal Th2 sensitization by amplifying the epithelial production of innate pro-Th2 cytokines and the activation of DCs [23]. Kool *et al.* elegantly demonstrated the release of endogenous UA upon HDM administration [23] or alum injection [33]. Because alum has been detected at high concentrations in CS [34], the presence of this adjuvant may explain the additional release of UA upon concomitant HDM/CS exposure and may clarify the observed increase in allergic sensitization upon CS inhalation. Furthermore, UA is a potent antioxidant which is produced in response to oxidative stress [35], originating from environmental factors such as HDM allergens or CS [36,37].

In line with previous reports [38,39], we demonstrated increased pulmonary hyaluronic acid (HA) after acute CS inhalation. Moreover, BAL levels of HA further enhanced upon prolonged and concomitant HDM/CS exposure, illustrating the synergistic effect of CS during ongoing allergic inflammation. The observed increase in HA after 3 weeks of concomitant HDM/CS exposure, could not be explained from the gene expression profile of HA modulating enzymes on total lung. However, mRNA expression of *Has1* and *Has3* rather suggested a net production of pro-inflammatory LMW fragments. Importantly, the possible difference in gene expression between various groups, can become lost in total lung. Therefore, it might be useful to examine the expression of HA modulating enzymes in more specific cells types, such as fibroblasts or airway epithelial cells.

These preliminary results point to a role for DAMP molecules during enhanced CS-induced allergic sensitization and asthma development in mice, however additional experiments are needed to unravel their impact into more detail.

6. Methods

Mice

Balb/c mice (6-8 weeks old) were purchased from Harlan (Zeist, the Netherlands). Homozygous breeding pairs of IL1RI knockout (KO) mice and C57Bl/6 control mice (6-10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were bred in the animal facility at the Faculty of Medicine and Health Sciences, Ghent University (Ghent, Belgium). Animals were maintained in standard conditions under a 12 hour light/dark cycle, provided a standard diet and chlorinated tap water *ad libitum*. All *in vivo* manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Exposure protocol

To unravel the impact of CS on the early sensitization phase or on asthma development, mice (n = 8 mice per group) were subjected to whole body CS (3R4F Kentucky Reference cigarettes), for 3 days (*Figure 1*) or 3 consecutive weeks (*Figure 6*) respectively. Control mice were exposed to air. 30 minutes after the last smoke exposure, 25 µg HDM extract (*Dermatophagoides pteronyssinus*) (Greer Laboratories, Lenoir, NC, USA) or PBS was administered intranasally, once a week. 24 hours after the last smoke exposure and 72 hours after the last HDM application, mice were euthanized with an overdose of pentobarbital (Sanofi-Ceva, Paris, France).

Bronchoalveolar lavage and cytospins

A tracheal cannula was inserted and BAL was performed by instillation of 3 x 300 µl of HBSS supplemented with 1% BSA (Dade Behring, Eschborn, Germany). The supernatant of the recovered BAL fluid was used for cytokine analysis. Three additional instillations with 1 ml of HBSS plus EDTA were performed to achieve maximal recovery of BAL cells. A total cell count was performed in a Bürker chamber and differential cell counts (on at least 400 cells) were performed on cytocentrifuge preparations using standard morphologic criteria after May-Grünwald-Giemsa staining. Remaining cells were used for FACS-analysis.

Lung and mediastinal lymph nodes single-cell suspensions

Lungs were perfused with saline plus EDTA through the pulmonary artery to remove contaminating blood cells. Lungs and mediastinal lymph nodes (mLN) were removed and digested as described previously [40]. Briefly, minced lung pieces and LNs were incubated with 1 mg/ml collagenase type 2 (Worthington Biochemical, Lakewood, NY) and 0.02 mg/ml DNase I (grade II from bovine pancreas, Boehringer Mannheim, Brussels, Belgium) for 45 min at 37°C and 5% CO_2. Red blood cells were lysed using ammonium chloride buffer. Finally, cell suspensions were filtered through a 50 µm nylon mesh to remove undigested organ fragments.

Flow cytometry

All staining procedures were performed in PBS without Ca^{2+} or Mg^{2+} containing 5mM EDTA and 1% BSA. To minimize non-specific binding, single-cell suspensions were pre-incubated with Fc-blocking antibody (anti-CD16/CD32, clone 2.4G2). Monoclonal antibodies (mAbs) used to identify mouse DC populations were anti-CD11c (clone HL3), anti-I-Ab (clone AF6-120.1) and anti-CD11b (clone M1/70). In a last step before analysis, cells were incubated with 7-aminoactinomycin D (or viaprobe; BD Pharmingen) to check cell viability. Flow cytometry data acquisition was performed on a FACSCalibur™ running CellQuest™ software (BD Biosciences, San Jose, CA, USA). FlowJo software was used for data analysis (TreeStar Inc., Ashland, OR, USA).

Mediastinal lymph node cell culture

MLN were collected into sterile tubes containing cold (4°C) tissue culture medium (TCM) and digested to obtain a single cell suspension. TCM was prepared using RPMI 1640 supplemented with 5% fetal bovine serum, L-glutamine, penicillin/streptomycin and β-mercaptoethanol (all from Gibco BRL; Invitrogen Corp). Cells were then transferred in triplicate to round-bottom, 96-well plates (Becton Dickinson (BD), BD, CA, USA) with or without 15 µg HDM extract/ml culture medium, at a density of 2×10^5 cells per well and incubated in a humidified 37°C incubator with 5% CO_2. After 5 days, supernatants were harvested and frozen for cytokine measurements.

Cytokine and DAMP measurements

Uric acid was evaluated in BAL supernatant, using the Amplex Red Uric Acid/Uricase Assay Kit (Invitrogen, Merelbeke, Belgium). BAL hyaluronan (HA) and HMGB-1 were measured using commercially available ELISA kits (Tebu-bio, Boechout, Belgium) (Gentaur, Kampenhout, Belgium). IL-α and IL-1β were determined in the supernatant of crushed lungs with ELISA (R&D Systems, Abingdon, UK). Within the supernatant of MLN cultures, IL-4, IL-5 and IL-13 were assayed by means of ELISA (R&D Systems, Abingdon, UK) following the manufacturer's instructions.

Histology

The left lung was fixated by intratracheal infusion of 4% paraformaldehyde. Histolocalization of HA was determined on paraffin sections using biotin-labeled HA-binding protein (HABP-b) (Seikagaku, Tokyo, Japan). Sections were subjected to deparaffinization followed by rehydration and were stained with HABP-b (2 µg/ml) at room temperature for 1 hour. After washing, the DAKO Cytomation Streptavidine ABComplex/HRP system was used according to the manufacturer's instructions (DAKO, Glostrup, Denmark). Enzymatic reactivity was visualized with the Vector NovaRED peroxidase substrate kit (Vector, Burlingame, CA). Sections were lightly counterstained with hematoxylin and mounted in Faramount (DAKO). The HABP-b staining in the airway walls was quantified, using a Zeiss KS400 image analyser platform (KS400, Zeiss, Oberkochen, Germany). For the visualization of E-cadherin, lung sections were stained with mouse-anti-E-cadherin (1/800, BD Biosciences, Erembodegem, Belgium) as described before [12]. Immunostains were developed using 3-amino-9-ethylcarbazole (AEC) substrate and quantifications were performed using color recognition by the KS400 software.

RT-PCR

Total lung RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time PCR reactions were performed in duplicate using diluted cDNA (dilution 1:25), 0.6 pmol/primer (custom designed) and IQ SYBR Green Supermix I dye (Bio-Rad, Hercules, CA) in a total volume of 20 µl as described in reference [38]. RT-PCR was performed on a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland). The expression of the hyaluronan synthase (*Has1, Has2, Has3*) and hyaluronidase genes (*Hyal1, Hyal2*) was corrected by a

normalization factor, calculated based on the expression of three reference genes (*Hprt1, Ppia, Rpl13a*).

Statistical analysis

Reported values are expressed as mean ± SEM. Statistical analysis was performed with PASW Statistics 18 using non-parametric tests. The different experimental groups were compared by a Kruskal-Wallis test for multiple comparisons. Only when a p-value ≤ 0.05 was obtained with the Kruskal-Wallis test, we performed *post hoc* pairwise comparisons by means of a Mann-Whitney U test. A p-value ≤ 0.05 was considered significant.

7. Acknowledgement

We would like to thank Greet Barbier, Eliane Castrique, Indra De Borle, Philippe De Gryze, Katleen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, Ann Neessen, Christelle Snauwaert and Evelyn Spruyt for their excellent technical assistance.

The present study was supported by the Concerted Research Action of the University of Ghent (BOF/GOA 01251504) and the Interuniversity Attraction Poles (IUAP) Program, Belgian State, Belgian Science Policy, Project P6/35. K.G.T. is a senior clinical investigator with a grant from FWO-Vlaanderen.

Reference List

1. Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE. The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. *Proc. Am. Thorac. Soc.* 2009; 6: 655-9.
2. Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *Eur. Respir. J.* 2004; 24: 822-33.
3. Gilmour MI, Jaakkola MS, London SJ, Nel AE, Rogers CA. How exposure to environmental tobacco smoke, outdoor air pollutants, and increased pollen burdens influences the incidence of asthma. *Environ. Health Perspect.* 2006; 114: 627-33.
4. Lanckacker EA, Tournoy KG, Hammad H, Holtappels G, Lambrecht BN, Joos GF, Maes T. Short cigarette smoke exposure facilitates sensitization and asthma development in mice. *Eur. Respir. J.* 2012.
5. Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat. Med.* 2012; 18: 684-92.
6. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: gatekeeper of airway mucosa and allergic sensitization. *Trends Immunol.* 2011; 32: 248-55.
7. Heijink IH, van OA, Kapus A. Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. *Eur. Respir. J.* 2010; 36: 1016-26.
8. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Can. J. Physiol Pharmacol.* 2008; 86: 105-12.
9. Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C. Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. *Inhal. Toxicol.* 2007; 19: 13-22.
10. Rusznak C, Sapsford RJ, Devalia JL, Justin JR, Hewitt EL, Lamont AG, Wood AJ, Shah SS, Davies RJ, Lozewicz S. Cigarette smoke potentiates house dust mite allergen-induced increase in the permeability of human bronchial epithelial cells in vitro. *Am. J. Respir. Cell Mol. Biol.* 1999; 20: 1238-50.
11. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi HM, Vernon-Wilson E, Sammut D, Bedke N, Cremin C, Sones J, Djukanovic R, Howarth PH, Collins JE, Holgate ST, Monk P, Davies DE. Defective epithelial barrier function in asthma. *J. Allergy Clin. Immunol.* 2011; 128: 549-56.
12. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, Heijink IH. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* 2011.
13. Heijink IH, Brandenburg SM, Postma DS, van Oosterhout AJ. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. *Eur. Respir. J.* 2012; 39: 419-28.
14. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.

15. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* 2012; 209: 1505-17.
16. Hastie AT, Everts KB, Cho SK, Zangrilli J, Shaver JR, Pollice MB, Fish JE, Peters SP. IL-1 beta release from cultured bronchial epithelial cells and bronchoalveolar lavage cells from allergic and normal humans following segmental challenge with ragweed. *Cytokine* 1996; 8: 730-8.
17. Eigenbrod T, Park JH, Harder J, Iwakura Y, Nunez G. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J. Immunol.* 2008; 181: 8194-8.
18. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 2009; 27: 519-50.
19. Pauwels NS, Bracke KR, Dupont LL, Van Pottelberge GR, Provoost S, Vanden Berghe T, Vandenabeele P, Lambrecht BN, Joos GF, Brusselle GG. Role of IL-1alpha and the Nlrp3/caspase-1/IL-1beta axis in cigarette smoke-induced pulmonary inflammation and COPD. *Eur. Respir. J.* 2011; 38: 1019-28.
20. Botelho FM, Bauer CM, Finch D, Nikota JK, Zavitz CC, Kelly A, Lambert KN, Piper S, Foster ML, Goldring JJ, Wedzicha JA, Bassett J, Bramson J, Iwakura Y, Sleeman M, Kolbeck R, Coyle AJ, Humbles AA, Stampfli MR. IL-1alpha/IL-1R1 expression in chronic obstructive pulmonary disease and mechanistic relevance to smoke-induced neutrophilia in mice. *PLoS. One.* 2011; 6: e28457.
21. Botelho FM, Nikota JK, Bauer CM, Morissette MC, Iwakura Y, Kolbeck R, Finch D, Humbles AA, Stampfli MR. Cigarette smoke-induced accumulation of lung dendritic cells is interleukin-1alpha-dependent in mice. *Respir. Res.* 2012; 13: 81.
22. Nakae S, Komiyama Y, Yokoyama H, Nambu A, Umeda M, Iwase M, Homma I, Sudo K, Horai R, Asano M, Iwakura Y. IL-1 is required for allergen-specific Th2 cell activation and the development of airway hypersensitivity response. *Int Immunol.* 2003; 15: 483-90.
23. Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa, Hammad H, Lambrecht BN. An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity.* 2011; 34: 527-40.
24. Shim EJ, Chun E, Lee HS, Bang BR, Kim TW, Cho SH, Min KU, Park HW. The role of high-mobility group box-1 (HMGB1) in the pathogenesis of asthma. *Clin. Exp. Allergy* 2012; 42: 958-65.
25. Sahu S, Lynn WS. Hyaluronic acid in the pulmonary secretions of patients with asthma. *Biochem. J.* 1978; 173: 565-8.
26. Liang J, Jiang D, Jung Y, Xie T, Ingram J, Church T, Degan S, Leonard M, Kraft M, Noble PW. Role of hyaluronan and hyaluronan-binding proteins in human asthma. *J. Allergy Clin. Immunol.* 2011; 128: 403-11.
27. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* 2005; 5: 331-42.
28. Park JS, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY, Choe KH, Strassheim D, Pitts TM, Tracey KJ, Abraham E. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am. J. Physiol Cell Physiol* 2003; 284: C870-C879.

29. Yang D, Chen Q, Yang H, Tracey KJ, Bustin M, Oppenheim JJ. High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin. *J. Leukoc. Biol.* 2007; 81: 59-66.
30. Termeer CC, Hennies J, Voith U, Ahrens T, Weiss JM, Prehm P, Simon JC. Oligosaccharides of hyaluronan are potent activators of dendritic cells. *J. Immunol.* 2000; 165: 1863-70.
31. McKee CM, Penno MB, Cowman M, Burdick MD, Strieter RM, Bao C, Noble PW. Hyaluronan (HA) fragments induce chemokine gene expression in alveolar macrophages. The role of HA size and CD44. *J. Clin. Invest* 1996; 98: 2403-13.
32. Stern R, Kogan G, Jedrzejas MJ, Soltes L. The many ways to cleave hyaluronan. *Biotechnol. Adv.* 2007; 25: 537-57.
33. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, Hoogsteden HC, Hammad H, Lambrecht BN. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* 2008; 205: 869-82.
34. Exley C, Begum A, Woolley MP, Bloor RN. Aluminum in tobacco and cannabis and smoking-related disease. *Am. J. Med.* 2006; 119: 276-11.
35. Vorbach C, Harrison R, Capecchi MR. Xanthine oxidoreductase is central to the evolution and function of the innate immune system. *Trends Immunol.* 2003; 24: 512-7.
36. Rahman I, Adcock IM. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur. Respir. J.* 2006; 28: 219-42.
37. Fukunaga M, Gon Y, Nunomura S, Inoue T, Yoshioka M, Hashimoto S, Ra C. Protease-mediated house dust mite allergen-induced reactive oxygen species production by neutrophils. *Int Arch. Allergy Immunol.* 2011; 155 Suppl 1: 104-9.
38. Bracke KR, Dentener MA, Papakonstantinou E, Vernooy JH, Demoor T, Pauwels NS, Cleutjens J, van Suylen RJ, Joos GF, Brusselle GG, Wouters EF. Enhanced deposition of low-molecular-weight hyaluronan in lungs of cigarette smoke-exposed mice. *Am. J. Respir. Cell Mol. Biol.* 2010; 42: 753-61.
39. Churg A, Zhou S, Wang X, Wang R, Wright JL. The role of interleukin-1beta in murine cigarette smoke-induced emphysema and small airway remodeling. *Am. J. Respir. Cell Mol. Biol.* 2009; 40: 482-90.
40. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 2001; 193: 51-60.

6.4

6.4. EXACERBATION OF CIGARETTE SMOKE-INDUCED PULMONARY INFLAMMATION BY *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN B IN MICE

Wouter Huvenne, MD, Ellen A. Lanckacker, Olga Krysko, PhD, Ken R Bracke, PhD, Tine Demoor, PhD, Peter W Hellings, MD, PhD, Guy G Brusselle, MD, PhD, Guy F Joos, MD, PhD, Claus Bachert, MD, PhD, Tania Maes, PhD

Respiratory Research. 2011;27;12:69.

1. Abstract

Background: Cigarette smoke (CS) is a major risk factor for the development of COPD. CS exposure is associated with an increased risk of bacterial colonization and respiratory tract infection, because of suppressed antibacterial activities of the immune system and delayed clearance of microbial agents from the lungs. Colonization with Staphylococcus aureus results in release of virulent enterotoxins, with superantigen activity which causes T cell activation.

Objective: To study the effect of *Staphylococcus aureus* enterotoxin B (SEB) on CS-induced inflammation, in a mouse model of COPD.

Methods: C57/Bl6 mice were exposed to CS or air for 4 weeks (5 cigarettes/exposure, 4x/day, 5 days/week). Endonasal SEB (10 µg/ml) or saline was concomitantly applied starting from week 3, on alternate days. 24 h after the last CS and SEB exposure, mice were sacrificed and bronchoalveolar lavage (BAL) fluid and lung tissue were collected.

Results: Combined exposure to CS and SEB resulted in a raised number of lymphocytes and neutrophils in BAL, as well as increased numbers of CD8+ T lymphocytes and granulocytes in lung tissue, compared to sole CS or SEB exposure. Moreover, concomitant CS/SEB exposure induced both IL-13 mRNA expression in lungs and goblet cell hyperplasia in the airway wall. In addition, combined CS/SEB exposure stimulated the formation of dense, organized aggregates of B- and T- lymphocytes in lungs, as well as significant higher CXCL-13 (protein, mRNA) and CCL19 (mRNA) levels in lungs.

Conclusions: Combined CS and SEB exposure aggravates CS-induced inflammation in mice, suggesting that *Staphylococcus aureus* could influence the pathogenesis of COPD.

2. Introduction

Cigarette smoking is associated with an increased risk of bacterial colonization and respiratory tract infection, because of suppressed antibacterial activities of the immune system and delayed clearance of microbial agents from the lungs [1]. This is particularly relevant in COPD patients, where bacterial colonization in the lower respiratory tract has been shown [2]. These bacteria are implicated both in stable COPD and during exacerbations, where most commonly pneumococci, *Haemophilus influenza, Moraxella catarrhalis* and *Staphylococcus aureus (S. aureus)* are found [3]. Interestingly, colonization with *S. aureus* may embody a major source of superantigens as a set of toxins are being produced including *S. aureus* enterotoxins (SAEs) [4]. These toxins activate up to 20% of all T cells in the body by binding the human leukocyte antigen (HLA) class II molecules on antigen-presenting cells (APCs) and specific V beta regions of the T cell receptor [5]. Between 50 and 80% of *S. aureus* isolates are positive for at least one superantigen gene, and close to 50% of these isolates show superantigen production and toxin activity [6].

During the last few years, it became increasingly clear that SAEs are known to modify airway disease [7], like allergic rhinitis [8], nasal polyposis [9] and asthma [10]. Furthermore, studies have shown a putative role for SAEs in patients suffering from the atopic eczema/dermatitis syndrome (AEDS), where colonization with *S. aureus* is found more frequently (80-100%) compared to healthy controls (5-30%) [11], and *S. aureus* isolates secrete identifiable enterotoxins like *Staphylococcus aureus* enterotoxin A and B (SEA, SEB) and toxic shock syndrome toxin (TSST)-1. Until now, evidence for SAE involvement in the pathogenesis of upper airway disease like chronic rhinosinusitis with nasal polyposis (CRSwNP), arises from the finding that IgE against SEA and SEB has been demonstrated in nasal polyps [12] and levels of SAE-specific IgE in nasal polyposis correlated with markers of eosinophil activation and recruitment [13]. Similarly, in COPD patients, a significantly elevated IgE to SAE was found, pointing to a possible disease modifying role in COPD, similar to that in severe asthma [14]. Moreover, we have recently demonstrated the pro-inflammatory effect of SEB on human nasal epithelial cells *in vitro*, resulting in augmented granulocyte migration and survival [15].

In murine research, the role of SAEs as inducer and modifier of disease has been demonstrated in models of airway disease [16,17], allergic asthma [18], atopic dermatitis [19] and food allergy [20]. These findings highlight the important pathological consequences of SAE

exposure, as these superantigens not only cause massive T-cell stimulation, but also lead to activation of B-cells and other pro-inflammatory cells like neutrophils, eosinophils, macrophages and mast cells [21].

To date, the exact pathomechanisms of COPD are not yet elucidated. Cigarette smoking is a primary risk factor for the development of COPD, but only 20% of smokers actually develop the disease, suggesting that genetic predisposition plays a role [22]. However, understanding the impact of toxin-producing bacteria on cigarette-smoke induced inflammation might provide novel insights into the pathogenesis of smoking-related disease such as COPD. Therefore, we investigated the effects of concomitant *Staphylococcus aureus* Enterotoxin B (SEB) application on a well established mouse model of cigarette-smoke (CS) induced inflammation [23]. We evaluated inflammatory cells and their mediators in bronchoalveolar lavage (BAL) fluid and lung tissue, looked at systemic effects by measuring serum immunoglobulins, and evaluated goblet cell hyperplasia and lymphoid neogenesis.

3. Methods

Experimental protocol

Male C57BL/6 mice (n=8), 6–8 weeks old were purchased from Charles River Laboratories (Brussels, Belgium). Mice were exposed to the tobacco smoke of five cigarettes (Reference Cigarette 2R4F without filter, University of Kentucky, Lexington, KY, USA) four times per day with 30 min smoke-free intervals [24]. The animals were exposed to mainstream cigarette smoke (CS) by whole body exposure, 5 days per week for 4 weeks. Control groups (8 age-matched male C57BL/6 mice) were exposed to air. Starting from day 14 of the CS exposure, mice received concomitant endonasal application of SEB (50 µL – 10 µg/mL - Sigma-Aldrich, LPS content below detection limit) or Saline, on alternate days. This dose was chosen based on Hellings *et al*. [18]. For the application, mice were slightly anaesthetized with isoflurane, and six applications were performed as depicted in *Figure 1*. All experimental procedures were approved by the local ethical committee for animal experiments (Faculty of Medicine and Health Sciences, Ghent University). The results section contains data from one representative experiment out of three independent experiments.

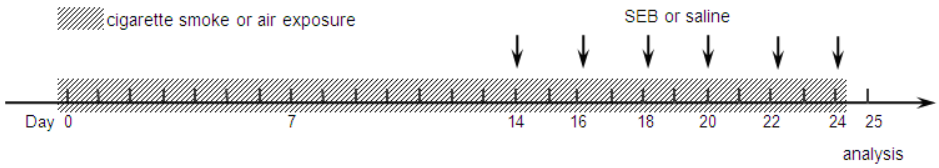


Figure 1: Experimental protocol. *Male C57BL/6 mice (n = 8) were exposed to cigarette smoke(CS) of five cigarettes, four times per day with 30 min smoke-free intervals. Controls were exposed to air. Starting from day 14 of the CS exposure, mice received concomitant endonasal application of SEB (50 µL - 10 µg/mL) or saline, on alternate days.*

Bronchoalveolar lavage and cytospins

Twenty-four hours after the last cigarette smoke (CS) exposure and endonasal application, mice were sacrificed by a lethal dose of pentobarbital (Sanofi-Synthelabo). A cannula was inserted in the trachea, and BAL was performed by instillation of 3 x 300 µl of HBSS supplemented with BSA for cytokine measurements. Three additional instillations with 1 ml of HBSS plus EDTA were performed to achieve maximal recovery of BAL cells. A total cell count was performed in a Bürker chamber. Approximately fifty thousand BAL cells were

processed for cytospins and were stained with May-Grünwald-Giemsa for differential cell counting. The remaining cells were used for FACS analysis.

Preparation of lung single-cell suspensions

Blood was collected via retro-orbital bleeding. Then, the pulmonary and systemic circulation was rinsed to remove contaminating blood cells. Lungs were taken and digested as described previously [24]. Briefly, minced lung pieces were incubated with 1 mg/ml collagenase and 20 µg/ml DNase I for 45 min at 37°C. Red blood cells were lysed using ammonium chloride buffer. Finally, cell suspensions were filtered through a 50-µm nylon mesh to remove undigested organ fragments.

Flow cytometry

All staining procedures were conducted in calcium- and magnesium-free PBS containing 10 mM EDTA, 1% BSA (Dade Behring), and 0.1% sodium azide. Cells were preincubated with anti-CD16/CD32 (2.4G2) to block Fc receptors. Antibodies used to identify mouse DC populations were anti-CD11c-allophycocyanin (APC; HL3) and anti-I-Ab-phycoerythrin (PE; AF6-120.1). The following mAbs were used to stain mouse T-cell subpopulations: anti-CD4-fluorescein isothiocyanate (FITC; GK1.5), anti-CD8-FITC (53-6.7), anti-CD3-APC (145-2C11) and anti-CD69-PE (H1.2F3). To identify granulocytes, anti-Gr-1-PE (RB6-8C5) and anti-CD11c-APC (HL3) were used. As a last step before analysis, cells were incubated with 7-aminoactinomycin D (or viaprobe; BD Pharmingen) for dead cell exclusion. All labeling reactions were performed on ice in FACS-EDTA buffer. Flow cytometry data acquisition was performed on a FACScalibur™ running CellQuest™ software (BD Biosciences, San Jose, CA, USA).

Measurement of Immunoglobulins

Retro-orbital blood was drawn for measurement of total IgE, IgG, IgM and IgA with ELISA. Commercially available ELISA kits were used to determine serum and BAL titers of IgG (ZeptoMetrix, Buffalo, NY, USA), IgM (ZeptoMetrix, Buffalo, NY, USA) and IgA (Alpha Diagnostic International, San Antonio, TX, USA). For the measurement of total IgE, a two-side in-house sandwich ELISA was used, with two monoclonal rat anti-mouse IgE antibodies reacting with different epitopes on the epsilon heavy chain (H. Bazin, Experimental

Immunology Unit, UCL, Brussels, Belgium). The second antibody was biotinylated and detected colorimetrically after adding horseradish peroxidase-streptavidine conjugate. Absorbance values, read at 492 nm (Labsystems Multiscan RC, Labsystems b.v., Brussels, Belgium) were converted to concentrations in serum and BAL fluid by comparison with a standard curve obtained with mouse IgE of known concentration (H. Bazin).

Goblet cell analysis

Left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Transversal sections of 3 µm were stained with periodic acid-Schiff (PAS) to identify goblet cells. Quantitative measurements of goblet cells were performed in the airways with a perimeter of basement membrane (Pbm) ranging from 800 to 2000 µm. Results are expressed as the number of goblet cells per millimeter of basement membrane.

Morphometric quantification of lymphoid neogenesis

To evaluate the presence of lymphoid infiltrates in lung tissues, sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to an immunohistological CD3/B220 double-staining as described previously [24]. Infiltrates in the proximity of airways and blood vessels were counted. Accumulations of ≥50 cells were defined as lymphoid aggregates. Counts were normalized for the number of bronchovascular bundles per lung section.

RT-PCR analysis

Total lung RNA was extracted with the Rneasy Mini kit (Qiagen, Hilden, Germany). Expression of CXCL-13, CCL19, IL-13 and MIP-3α mRNA relative to HPRT mRNA [25], were performed with Assay-on-demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA). Real-time RT PCR for CCL21-leucine and CCL21-serine started from 25 ng of cDNA. Primers and FAM/TAMRA probes were synthesized on demand (Sigma-Proligo). Primer/probe sequences and PCR conditions were performed as described previously [26,27].

Protein measurement in BAL

CXCL13 protein levels in BAL supernatant were determined using a commercially available ELISA (R&D Systems, Abingdon, UK). Cytometric Bead Array (BD Biosciences, San Jose, CA,

USA) was used to detect the cytokines KC, MCP-1, IL-17A and IFN-γ in the supernatant of BAL fluid.

Statistical analysis

Reported values are expressed as mean ± SEM. Statistical analysis was performed with SPSS software (version 18.0) using nonparametric tests. The different experimental groups were compared by a Kruskal-Wallis test for multiple comparisons. When a p-value ≤ 0.05 was obtained with the Kruskal-Wallis test, pairwise comparisons were made by means of a Mann-Whitney U test with Bonferroni corrections for multiple comparisons. A p-value $p \leq 0.05$ was considered significant.

4. Results

4.1. SEB aggravates the CS-induced pulmonary inflammation

To evaluate the effects of *Staphylococcus aureus* enterotoxin B (SEB) on cigarette smoke (CS)-induced pulmonary inflammation, C57Bl/6 mice were exposed to CS for 4 weeks, with a concomitant SEB exposure during the last 2 weeks (*Figure 1*).

In BAL fluid, sole endonasal SEB application and sole CS-exposure resulted in increased numbers of total cells, alveolar macrophages, dendritic cells (DCs), lymphocytes and neutrophils, compared to air/saline exposed animals (*Figure 2A-E*). However, these increases in cell numbers were much more pronounced upon SEB application compared to CS-exposure. Also a modest eosinophilic inflammation was observed in the SEB-exposed groups (*Figure 2F*).

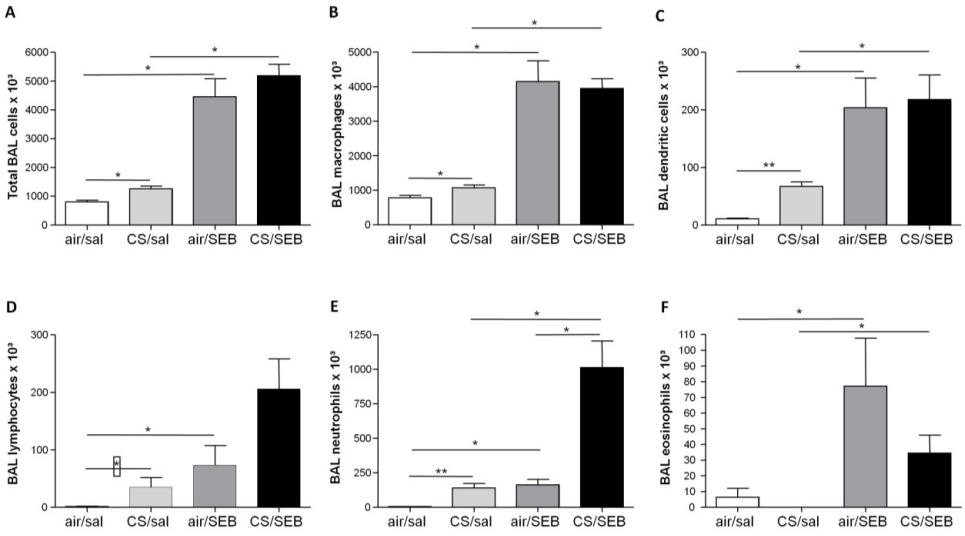


Figure 2: BAL fluid analysis. *Total BAL cells and cell differentiation in BAL fluid of mice exposed to saline or SEB, combined with air or CS. A) Total BAL cells, B) macrophages, C) dendritic cells, D) lymphocytes, E) neutrophils, F) eosinophils. Results are expressed as mean ± SEM, n = 8 animals/group, *p < 0.05, **p < 0.01.*

Interestingly, the combination of CS exposure and SEB significantly increased BAL neutrophil numbers compared to sole CS or SEB exposure (*Figure 2E*). Also BAL lymphocyte numbers in smoke-exposed mice were increased upon SEB application (*Figure 2D*).

In lung single cell suspensions, SEB solely induced an increase in DCs, $CD3^+$ T cells and macrophages, whereas CS exposure caused increased DCs and $CD3^+$ T cells in lung tissue (*Figure 3A,D,B*). Interestingly, combined CS and SEB exposure caused a further increase in $CD3^+$ T cells, and more specifically $CD8^+$ T-cells, compared to CS or SEB alone (*Figure 3D,F*). Also DC, $CD4^+$ T-cells and $GR1^+$ cells tended to be higher in the combined CS/SEB group versus sole CS or SEB application (*Figure 3A,E,C*).

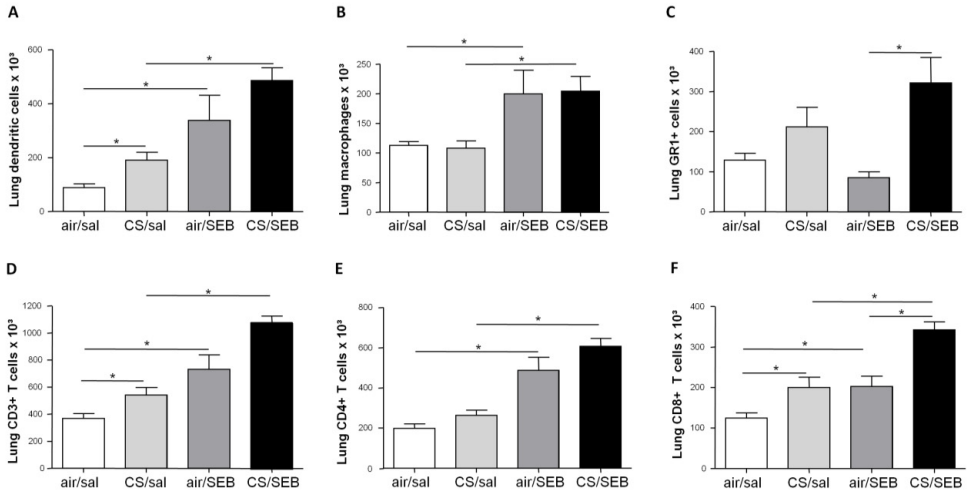


Figure 3: Lung cell differentiation. *Flow cytometric analysis of cells from lung digest: A) dendritic cells, B) macrophages, C) GR1+ cells, D) CD3+ T lymphocytes, E) CD4+ T lymphocytes and F) CD8+ T lymphocytes from mice exposed to saline or SEB, combined with air or CS. Results are expressed as mean ± SEM, n = 8 animals/group, *p < 0.05.*

4.2. Increased IL-17A in BAL upon combined SEB and CS exposure

As previously described [24], 4-wk CS-exposure clearly induced high levels of KC (mouse homolog for IL-8) and MCP-1 in BAL (*Figure 4A,B*). In contrast sole SEB application induced a modest increase in KC, and very low levels of IFN-γ and IL-17A (*Figure 4A,D,C*) . Whereas the CS-induced KC and MCP-1 levels in BAL were not affected by an additional SEB exposure, the combined CS and SEB exposure did induce IL-17A levels in BAL, compared to single CS or SEB exposure (*Figure 4C*). Also IFN-γ levels tended to be highest in the combined CS/SEB group (*Figure 4D*). mRNA levels of MIP-3α were increased after both CS or SEB exposure. Combined CS/SEB exposure did not cause a further MIP-3α increase (*Figure 4E*).

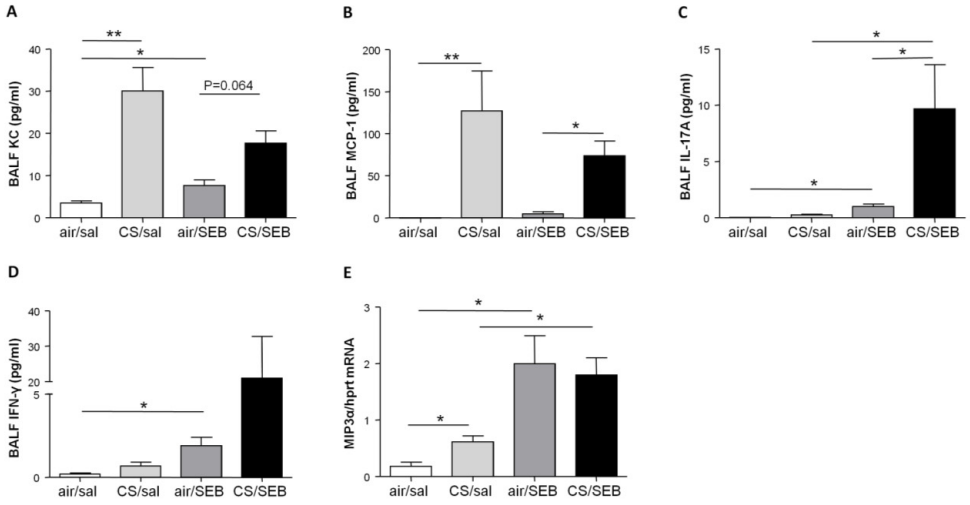


Figure 4: Protein measurements in BAL fluid. *Protein levels of A) KC, B) MCP-1, C) IL-17A, D) IFN-γ in BAL fluid of mice exposed to saline or SEB, combined with air or CS, as measured with ELISA. E) mRNA expression of MIP-3α in total lung tissue, measured by RT-PCR. The results are expressed as ratio with hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA. Results are expressed as mean ± SEM, n = 8 animals/group, *p < 0.05, **p < 0.01.*

4.3. SEB induces IgA and IgM levels in BAL

Systemic effects of either CS or SEB, or both were evaluated in serum, but no significant differences in total IgG, IgM, IgA or IgE levels were detected between the experimental groups. In BAL, CS exposure tended to increase IgA. Both IgA and IgM levels in BAL were significantly increased upon SEB-exposure (*Figure 5*). IgE in BAL was below the detection limit.

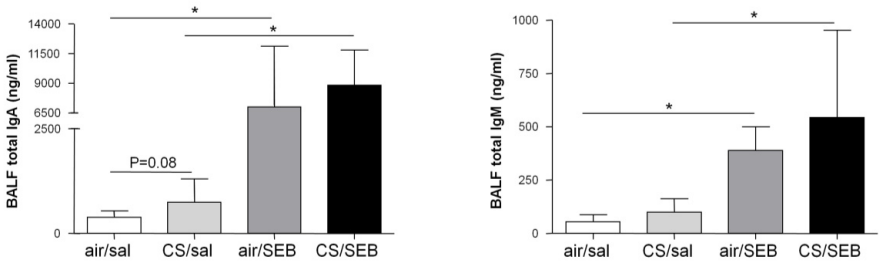


Figure 5: BAL fluid immunoglobulin levels. *A) Total IgA and B) total IgM in BAL fluid of mice exposed to saline or SEB, combined with air or CS. Results are expressed as mean ± SEM, n = 8 animals/group, *p < 0.05.*

4.4. Combined CS/SEB exposure affects epithelial remodeling

Epithelial remodeling was evaluated by counting the number of PAS-positive goblet cells per millimeter of basement membrane. A strong tendency towards increased numbers of goblet cells in the CS/SEB mice was observed, compared to all other conditions (*Figure 6A,B*). This finding correlated nicely with a significant increase in IL-13 mRNA expression in total lung in CS/SEB mice (*Figure 6C*).

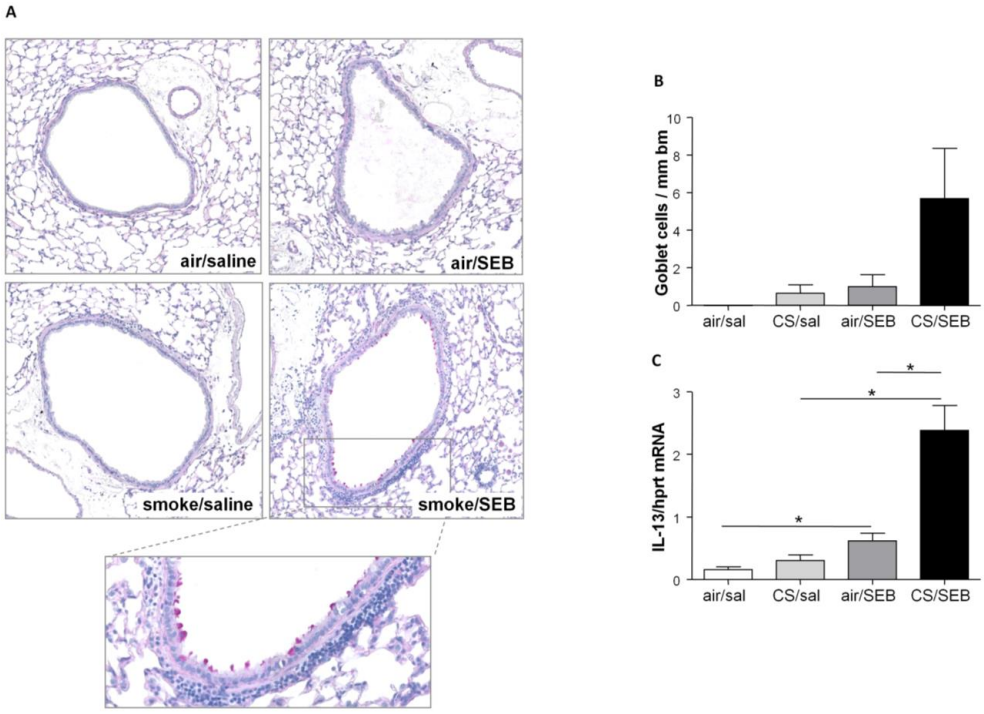


Figure 6: Epithelial remodeling. *A) Histological evaluation of goblet cell hyperplasia on Periodic Acid Schiff (PAS) stained lung tissue sections of mice exposed to saline or SEB, combined with air or CS. B) Quantification of goblet cells. C) mRNA expression of IL-13, relative to a housekeeping gene (HPRT) was measured on total lung homogenates by RT-PCR. Results are expressed as mean ± SEM, n = 8 animals/group, *p < 0.05.*

4.5. Combined CS/SEB induces the formation of dense lymphoid aggregates in lung tissue

Previously, our group has demonstrated increased lymphoid neogenesis after 6 months of CS-exposure [25]. As earlier shown in the CS-model, subacute CS-exposure as such did not result in lymphoid neogenesis. Interestingly however, already after 4-wk CS-exposure, dense, organized lymphoid aggregates could be demonstrated in the combined CS/SEB group whereas air/SEB mice displayed mainly loose, non-organized lymphoid aggregates (*Figure 7*).

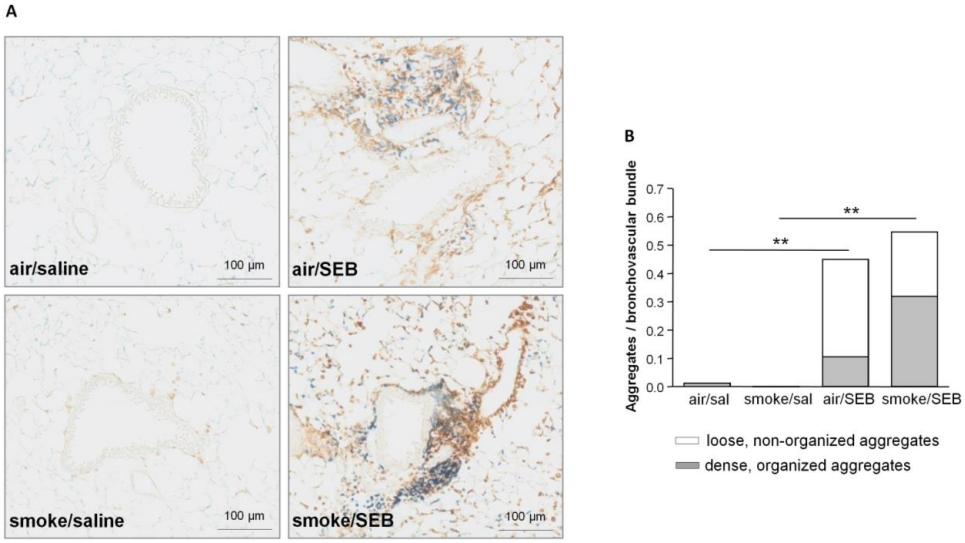


Figure 7: Evaluation of lymphoid aggregates in lung tissue. *A) Photomicrographs of lymphoid aggregates in CD3/B220 immuno-stained lung tissue of mice exposed to saline or SEB, combined with air or CS (brown: CD3 positive cells; blue: B220 positive cells). B) Quantification of loose and dense lymphoid aggregates located in the bronchovascular area. Results are expressed as mean, n = 8 animals/group, *p < 0.05, **p < 0.01.*

Since CXCL13, CCL19 and CCL21 are chemokines involved in the homeostatic trafficking of leukocytes, mainly lymphocytes, to the secondary and tertiary lymphoid tissues, their expression was also evaluated in this model. The increase in dense lymphoid aggregates in CS/SEB mice correlated nicely with significant increases in CXCL13 (protein levels in BAL fluid, mRNA levels in total lung) (*Figure 8A,B*) and CCL19 (mRNA levels) expression in CS/SEB mice compared to all other groups (*Figure 8E*). CCL21 mRNA levels (both isoforms CCL21-Ser and CCL21-Leu) decreased upon CS exposure, confirming previous findings of CCL21 downregulation upon subacute CS exposure [26] and decreased even further in the CS/SEB group. Intriguingly, the CCL21 mRNA levels of both isoforms tended to increase upon sole SEB exposure (*Figure 8C,D*).

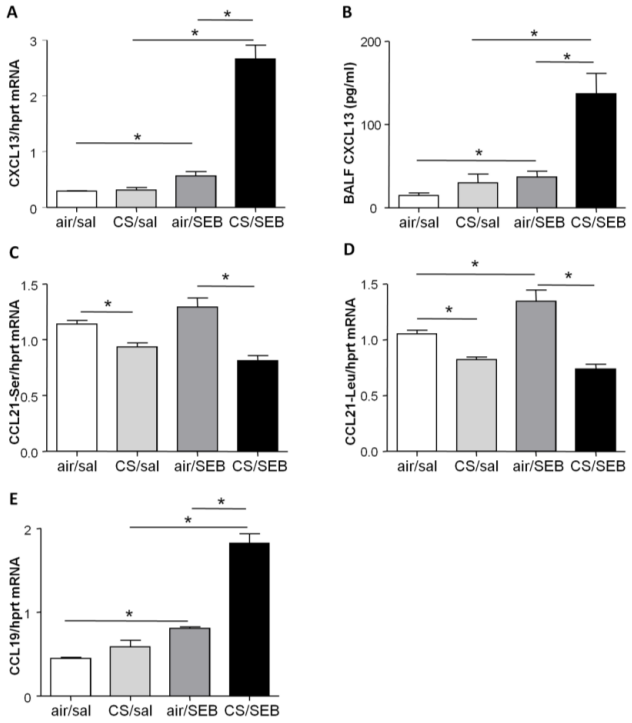


Figure 8: Chemokines involved in the homeostatic trafficking of leukocytes. *Measurements of lymphoid chemokines in lung tissue and BAL fluid. mRNA expression of A) CXCL-13, C) CCL21-Ser, D) CCL21-Leu and E) CCL-19 in total lung tissue of mice exposed to saline or SEB, combined with air or CS, measured by RT-PCR. The results are expressed relative to HPRT mRNA. B) Protein levels of CXCL-13 in BAL fluid as measured by ELISA. Results are expressed as mean ± SEM, n = 8 animals/group, *p < 0.05.*

5. Discussion

We hereby describe a novel mouse model of combined *Staphylococcus aureus* enterotoxin B (SEB) application and cigarette smoke exposure, which results in a significant aggravation of key features of cigarette smoke (CS)-induced pulmonary inflammation, such as neutrophils and $CD8^+$ T cells in BAL and lung. Furthermore, levels of IL-17A in BAL were significantly increased upon concomitant SEB and CS exposure, compared to sole exposures of SEB or CS. In addition, tendencies of increased goblet cell hyperplasia, IL-13 mRNA expression and lymphoid neogenesis in smoke/SEB mice have been demonstrated, as well as increased expression of the relevant chemokines CXCL13 and CCL19. Altogether, these findings point to a possible disease-modifying role for SEB in CS-induced inflammation in this mouse model of subacute CS exposure.

Increasing evidence from human and murine research suggests that SEB is able to aggravate underlying disease. Moreover, SEB itself is also able to induce inflammation, depending on the dosage and timing of the experimental protocol {892}{895}. Interestingly, these findings are not confined to SEB, as other staphylococcal superantigens demonstrate similar effects upon mucosal contact [28,29]. In line with previously reported findings, in our model sole endonasal SEB application caused an increase in total BAL cell number, lymphocytes and neutrophils [16]. Moreover, we could demonstrate raised numbers of macrophages and dendritic cells, a finding previously reported after *S. aureus* enterotoxin A exposure [28,29]. In the latter studies however, the authors could not demonstrate increased eosinophils, which was the case in our model. The superantigen effect of SEB caused the expected lymphocyte accumulation in BAL, which appeared to be non-specific, as both $CD4^+$ and $CD8^+$ T cells were increased. These data stress the potency of staphylococcal superantigens of initiating a massive immune response.

Concomitant CS/SEB exposure lead to a remarkable increase in neutrophil number, compared to CS or SEB exposure alone. Although the findings for neutrophils in lung (measured with granulocyte marker GR-1) were less convincing than in BAL, the combined CS/SEB group showed the highest number of $GR\text{-}1^+$ cells. Interestingly, also the $CD8^+$ T cell fraction in lung single cell suspensions, was significantly upregulated when smoke and SEB were combined. The potential clinical relevance of increased neutrophil and $CD8^+$ T-cell numbers lays in the fact that neutrophilic inflammation in the airways in smokers correlates

with an accelerated decline in lung function [30], and increased T-cell numbers correlate with the amount of alveolar destruction and the severity of airflow obstruction [31].

We confirm an increased MIP-3α expression in lungs after CS exposure leading to an accumulation of dendritic cells in this model [24]. Interestingly, this increase in MIP-3α is also seen after SEB exposure, with raised DCs in BAL and airway parenchyma in these groups.

As previously demonstrated in the subacute CS-model, we have observed an increase in levels of KC and MCP-1 after 4-wk CS exposure [24], explaining the accumulation of inflammatory cells in BAL and lung. Sole SEB application on the other hand resulted in raised levels of KC, IFN-γ and IL-17A, but not MCP-1. Interestingly, the combined exposure of smoke and SEB further increased the IL-17A levels, which might explain the exacerbated BAL neutrophilia in CS/SEB mice. Indeed, IL-17 is known to be important in neutrophil maturation, migration and function in the lung tissue and airways. Furthermore, IL-17 induction of neutrophil activation and migration is important in defense against organisms infecting the lung [32]. Interestingly, IL-17 can also induce eosinophilic accumulation, in particular circumstances [33].

IL-17 is normally produced by $CD4^+$ T cells, although it might also arise from $CD8^+$ T cells and in some cases even from macrophages, neutrophils or eosinophils [34], as a necessary step in the normal immunity against bacterial infections in the airways. However, IL-17 has been linked to unfavorable outcome to infection, in particular in the presence of IFN-γ [35], resulting a high inflammatory pathology and tissue destruction. Increasing evidence dedicates a role to exaggerated recruitment and activation of neutrophils in the clinical course of airway diseases like COPD. Therefore, it is tempting to speculate on a role for SEB in the induction of IL-17 release, leading to the aggravation of cigarette smoke-induced inflammation, with increased number and activation of neutrophils, which causes amplification of tissue destruction and subsequent disease progression.

In addition, we could observe already after 4-wks an increase in the number of dense lymphoid aggregates in CS/SEB mice, linked to increased levels of CXCL13 and CCL19, which are attractants for B- and T-cells respectively. Moreover, it has been described that the respective receptors for these chemokines – CXCR5 and CCR7 – are also expressed on Th17 cells migrating into inflamed tissue [36], indicating a potential contribution of IL17-producing Th17 cells in this model of early COPD. The finding that lymphoid aggregates and the chemokines responsible for their neogenesis and organization [25] are already upregulated

after 4-wk CS/SEB exposure, stresses the clinical relevance of this novel model of combined CS and enterotoxin exposure.

Staphylococcal superantigens are able to cause massive polyclonal T and B cell proliferation. Upon local application, as is done in this model, this leads to the mucosal synthesis of immunoglobulins, explaining the observed increase in BAL IgA and IgM. In humans, it is thought that continuous microbial stimulation leads to B cell turnover and plasma cell formation in nasal polyp disease, leading to an overproduction of immunoglobulins [37].

In this mouse model of early stage COPD with goblet cell hyperplasia and increased number of lymphoid follicles, endonasal SEB application has resulted in augmented CS-induced lower airway inflammation. CS and subsequent bacterial colonization are, amongst others, factors believed to determine both progression of COPD, as well as the frequency and severity of COPD exacerbations [38]. Therefore, mouse models of CS and bacterial co-exposure have been used in the past, mainly using *Haemophilus influenza* [39]. Bacterial colonization and infection is rare in lower airways, but not in upper airways. Local carriage of enterotoxin-producing *S. aureus* in the nasal cavity is common, although multiple sites can be colonized (e.g. skin, pharynx and perineum) [40]. These toxins, like toxic shock syndrome toxin-1 (TSST-1), are known superantigens causing systemic diseases like food poisoning and toxic shock syndrome [4]. In nasal polyp disease, these toxins are believed to drive the local immunoglobulin production in response to enterotoxin-producing *S. aureus.*

The use of a single toxin instead of *S. aureus* in this model is both a strength and a limitation, since it simplifies the interpretation on one hand, but is not the real life situation on the other hand. Another limitation is that we cannot rule out endotoxin related effects in our model, although the LPS content of our SEB was below detection limit. Also the potential differences between our mouse model and the human situation concerning exposure to bacterial toxins and its effects on the balance of cytokines and inflammation is a limitation of the study. In addition, SEB on itself has resulted in pronounced inflammation in BAL and lungs, as it is a known superantigen. Finally, another possible limitation of this model is the short term (4-wk) CS exposure, whereas COPD is a chronic disease. Despite these limitations, altogether our findings indicate the importance of bacterial toxins present in the upper airways, affecting lower airway inflammation.

6. Conclusion

The possible disease-modifying role for SAEs in COPD that has been described in humans [14], combined with our findings stress the potential role of airway colonizing and toxin-producing *Staphylococcus aureus*, in the pathophysiology of COPD [3].

7. Acknowledgements

The authors would like to thank Greet Barbier, Eliane Castrique, Indra De Borle, Philippe De Gryze, Katleen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, Ann Neessen, Christelle Snauwaert and Evelyn Spruyt for their technical assistance.
This project is supported by the Fund for Scientific Research – Flanders (FWO-Vlaanderen – Project G.0052.06), by a grant from the Ghent University (BOF/GOA 01251504), by the Interuniversity Attraction Poles program (IUAP) – Belgian state – Belgian Science Policy P6/35, and by grants to CB from the Fund for Scientific Research – Flanders, FWO, no. A12/5-HB-KH3 and G.0436.04, and to KB as a postdoctoral fellow of the Fund for Scientific Research Flanders (FWO).

Reference List

1. Drannik AG, Pouladi MA, Robbins CS, Goncharova SI, Kianpour S, Stampfli MR. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *Am. J. Respir. Crit Care Med.* 2004; 170: 1164-71.
2. Soler N, Ewig S, Torres A, Filella X, Gonzalez J, Zaubet A. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur. Respir. J.* 1999; 14: 1015-22.
3. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2008; 359: 2355-65.
4. Fraser JD, Proft T. The bacterial superantigen and superantigen-like proteins. *Immunol. Rev.* 2008; 225: 226-43.
5. Sundberg EJ, Deng L, Mariuzza RA. TCR recognition of peptide/MHC class II complexes and superantigens. *Semin. Immunol.* 2007; 19: 262-71.
6. Chau TA, McCully ML, Brintnell W, An G, Kasper KJ, Vines ED, Kubes P, Haeryfar SM, McCormick JK, Cairns E, Heinrichs DE, Madrenas J. Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nat. Med.* 2009; 15: 641-8.
7. Bachert C, Gevaert P, Zhang N, van Zele T, Perez-Novo C. Role of staphylococcal superantigens in airway disease. *Chem. Immunol. Allergy* 2007; 93: 214-36.
8. Rossi RE, Monasterolo G. Prevalence of serum IgE antibodies to the Staphylococcus aureus enterotoxins (SAE, SEB, SEC, SED, TSST-1) in patients with persistent allergic rhinitis. *Int. Arch. Allergy Immunol.* 2004; 133: 261-6.
9. Bachert C, Zhang N, Patou J, van Zele T, Gevaert P. Role of staphylococcal superantigens in upper airway disease. *Curr. Opin. Allergy Clin. Immunol.* 2008; 8: 34-8.
10. Heaton T, Mallon D, Venaille T, Holt P. Staphylococcal enterotoxin induced IL-5 stimulation as a cofactor in the pathogenesis of atopic disease: the hygiene hypothesis in reverse? *Allergy* 2003; 58: 252-6.
11. Breuer K, Kapp A, Werfel T. Bacterial infections and atopic dermatitis. *Allergy* 2001; 56: 1034-41.
12. Carayol N, Crampette L, Mainprice B, Ben-Soussen P, Verrecchia M, Bousquet J, Lebel B. Inhibition of mediator and cytokine release from dispersed nasal polyp cells by mizolastine. *Allergy* 2002; 57: 1067-70.
13. Bachert C, Gevaert P, Holtappels G, Johansson SG, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. *J. Allergy Clin. Immunol.* 2001; 107: 607-14.
14. Rohde G, Gevaert P, Holtappels G, Borg I, Wiethege A, Arinir U, Schultze-Werninghaus G, Bachert C. Increased IgE-antibodies to Staphylococcus aureus enterotoxins in patients with COPD. *Respir. Med.* 2004; 98: 858-64.
15. Huvenne W, Callebaut I, Reekmans K, Hens G, Bobic S, Jorissen M, Bullens DM, Ceuppens JL, Bachert C, Hellings PW. Staphylococcus aureus enterotoxin B augments granulocyte migration and survival via airway epithelial cell activation. *Allergy* 2010; 65: 1013-20.
16. Herz U, Ruckert R, Wollenhaupt K, Tschernig T, Neuhaus-Steinmetz U, Pabst R, Renz H. Airway exposure to bacterial superantigen (SEB) induces lymphocyte-dependent

airway inflammation associated with increased airway responsiveness--a model for non-allergic asthma. *Eur. J. Immunol.* 1999; 29: 1021-31.
17. Huvenne W, Callebaut I, Plantinga M, Vanoirbeek JA, Krysko O, Bullens DM, Gevaert P, van CP, Lambrecht BN, Ceuppens JL, Bachert C, Hellings PW. Staphylococcus aureus enterotoxin B facilitates allergic sensitization in experimental asthma. *Clin. Exp. Allergy* 2010; 40: 1079-90.
18. Hellings PW, Hens G, Meyts I, Bullens D, Vanoirbeek J, Gevaert P, Jorissen M, Ceuppens JL, Bachert C. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to Staphylococcus aureus enterotoxin B. *Clin. Exp. Allergy* 2006; 36: 1063-71.
19. Laouini D, Kawamoto S, Yalcindag A, Bryce P, Mizoguchi E, Oettgen H, Geha RS. Epicutaneous sensitization with superantigen induces allergic skin inflammation. *J. Allergy Clin. Immunol.* 2003; 112: 981-7.
20. Ganeshan K, Neilsen CV, Hadsaitong A, Schleimer RP, Luo X, Bryce PJ. Impairing oral tolerance promotes allergy and anaphylaxis: a new murine food allergy model. *J. Allergy Clin. Immunol.* 2009; 123: 231-8.
21. Marone G, Rossi FW, Detoraki A, Granata F, Marone G, Genovese A, Spadaro G. Role of superallergens in allergic disorders. *Chem. Immunol. Allergy* 2007; 93: 195-213.
22. Fletcher C, Peto R. The natural history of chronic airflow obstruction. *Br. Med. J.* 1977; 1: 1645-8.
23. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur. Respir. J.* 2005; 26: 204-13.
24. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, Brusselle GG. Cigarette Smoke-Induced Pulmonary Inflammation and Emphysema Are Attenuated in CCR6-Deficient Mice. *J Immunol.* 2006; 177: 4350-9.
25. Demoor T, Bracke KR, Maes T, Vandooren B, Elewaut D, Pilette C, Joos GF, Brusselle GG. Role of lymphotoxin-alpha in cigarette smoke-induced inflammation and lymphoid neogenesis. *Eur. Respir. J.* 2009; 34: 405-16.
26. Demoor T, Bracke KR, Vermaelen KY, Dupont L, Joos GF, Brusselle GG. CCR7 modulates pulmonary and lymph node inflammatory responses in cigarette smoke-exposed mice. *J. Immunol.* 2009; 183: 8186-94.
27. Chen SC, Vassileva G, Kinsley D, Holzmann S, Manfra D, Wiekowski MT, Romani N, Lira SA. Ectopic expression of the murine chemokines CCL21a and CCL21b induces the formation of lymph node-like structures in pancreas, but not skin, of transgenic mice. *J. Immunol.* 2002; 168: 1001-8.
28. Muralimohan G, Rossi RJ, Guernsey LA, Thrall RS, Vella AT. Inhalation of Staphylococcus aureus enterotoxin A induces IFN-gamma and CD8 T cell-dependent airway and interstitial lung pathology in mice. *J. Immunol.* 2008; 181: 3698-705.
29. Muralimohan G, Rossi RJ, Vella AT. Recruitment and in situ renewal regulate rapid accumulation of CD11c+ cells in the lung following intranasal superantigen challenge. *Int. Arch. Allergy Immunol.* 2008; 147: 59-73.
30. Stanescu D, Sanna A, Veriter C, Kostianev S, Calcagni PG, Fabbri LM, Maestrelli P. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 1996; 51: 267-71.
31. Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur. Respir. J.* 2003; 22: 672-88.

32. Linden A, Laan M, Anderson GP. Neutrophils, interleukin-17A and lung disease. *Eur. Respir. J.* 2005; 25: 159-72.
33. Wakashin H, Hirose K, Maezawa Y, Kagami S, Suto A, Watanabe N, Saito Y, Hatano M, Tokuhisa T, Iwakura Y, Puccetti P, Iwamoto I, Nakajima H. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am. J. Respir. Crit Care Med.* 2008; 178: 1023-32.
34. Mucida D, Salek-Ardakani S. Regulation of TH17 cells in the mucosal surfaces. *J. Allergy Clin. Immunol.* 2009; 123: 997-1003.
35. Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, Belladonna ML, Vacca C, Conte C, Mosci P, Bistoni F, Puccetti P, Kastelein RA, Kopf M, Romani L. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* 2007; 37: 2695-706.
36. Kim CH. Migration and function of Th17 cells. *Inflamm. Allergy Drug Targets.* 2009; 8: 221-8.
37. van ZT, Gevaert P, Holtappels G, van CP, Bachert C. Local immunoglobulin production in nasal polyposis is modulated by superantigens. *Clin. Exp. Allergy* 2007; 37: 1840-7.
38. Gaschler GJ, Bauer CM, Zavitz CC, Stampfli MR. Animal models of chronic obstructive pulmonary disease exacerbations. *Contrib. Microbiol.* 2007; 14: 126-41.
39. Gaschler GJ, Skrtic M, Zavitz CC, Lindahl M, Onnervik PO, Murphy TF, Sethi S, Stampfli MR. Bacteria challenge in smoke-exposed mice exacerbates inflammation and skews the inflammatory profile. *Am. J. Respir. Crit Care Med.* 2009; 179: 666-75.
40. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL. The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect. Dis.* 2005; 5: 751-62.

PART III: REVIEW

7

CHAPTER 7:

A NEW DANGER IN THE AIR: HOW PULMONARY INNATE IMMUNITY COPES WITH MAN-MADE AIRBORNE XENOBIOTICS

Ellen A. Lanckacker, Lander J. Robays, Guy F. Joos, MD, PhD, Karim Y. Vermaelen, MD, PhD

Journal of Innate Immunity. 2010;2(2):96-106.

Abstract

The pulmonary innate immune system has evolved over millions of years to provide swift detection of inhaled microbial agents and trigger well-balanced protective responses. Much more recent on the evolutionary scale is human activity, which has resulted in the release of a new class of potentially harmful, non-microbial compounds into the air. These xenobiotics include combustion by-products such as reactive oxygen species and polycyclic aromatic hydrocarbons. This review will summarize evidence showing how airborne xenobiotics can engage pulmonary innate immunity components at many levels. We will focus on potential effects of xenobiotics on airway dendritic cells, as these constitute key innate immune sensors in the lung, with the unique ability to initiate adaptive immunity. We propose that the aberrant processing of inhaled xenobiotics by an innate immune system that is now evolutionarily maladapted underlies the increase in chronic inflammatory lung diseases in modern times.

Ancient meets new: pulmonary innate immunity and the rise of anthropogenic airborne compounds

The innate immune system of the lung is one of the most critical homeostatic systems in our body. Life-threatening damage to the delicate gas-exchange structures can either occur by a failure to rapidly detect and clear inhaled airborne pathogens, or can result from an unbridled inflammatory response. Pulmonary innate immune defenses consist of several interacting components [1]. The integrity of the epithelial layer, combined with the mucociliary transport form a first mechanical barrier in the conductive airways. Surfactant proteins, synthesized by the epithelium of the deeper alveolar zones, bear structural analogy to serum complement and constitute an additional immediate defense mechanism [2]. Phagocytic cells such as macrophages and neutrophils complete the picture by neutralizing persistent inhaled pathogens. Invariant NK-T-cells and dendritic cells are also important innate immune sensors of the lung, with the latter being unique in their ability to initiate primary adaptive immune responses. Both structural cells (epithelium) and leukocyte components of the pulmonary innate immune system are equipped with pathogen recognition receptors (PRRs), i.e. Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-like receptors (RLRs) and provide immediate sensing of pathogen-associated molecular patterns (PAMPs) from inhaled bacteria, viruses or fungi [3].

It can be assumed that the pulmonary innate immune system had to co-evolve with the development of lungs in the first terrestrial vertebrates, i.e. during the Devonian era some 350 million years ago. The explosive colonization of the land by a myriad of organisms that charactarized that period must have brought a constant selection pressure on the very first airway innate defense mechanisms. This would have forced a repertoire diversification of pathogen recognition receptors, which were in part structural variations on a theme that also evolved in land-based arthropods (e.g. Toll-molecules in insects). Seen in this time-scale, human evolution is an extremely recent event. Since prehistoric times, and boosted by the first industrial revolution, human activity has resulted in the ever increasing release of airborne products largely derived from the combustion of biomasses and fossil fuels. Inhalational exposure to carbonaceous fine particles such as diesel exhaust particles (DEPs), as well as to a broad array of volatile molecules such as carbon monoxide, nitrogen oxides, sulfur dioxides and the potentially carcinogenic polycyclic aromatic hydrocarbons (PAHs) [4]

has increased proportionally with the growth of human communities (***Table 1***). Even higher levels of exposure to combustion products have been reached since the widespread use of tobacco.

The purpose of this review is to examine how the immune system of the lung reacts to these evolutionary recent anthropogenic stimuli. Among the different cellular actors of pulmonary innate immunity, we will particularly focus on the network of airway dendritic cells, given their unique role as both innate immune sensors and controllers of adaptive immune responses.

Air pollutant	Natural source	Anthropogenic source
Primary Pollutants		
SO_x (e.g. SO_2); precursors of acid rain and secondary fine sulfate particles	volcanic activities	industrial processes; fossil fuel combustion (mostly coal, petroleum) in power plants and other stationary sources; indoor gas burners (stoves and heaters)
NO_x (e.g. NO_2); precursors of acid rain and visibility reducing fine nitrate particles	lightning; biological processes in soil	high-temperature combustion processes in automobiles and power plants; indoor gas burners (stoves and heaters)
CO	wildfires	incomplete combustion of fuel (natural gas, coal, wood); motor vehicle exhaust; fuel combustion unrelated to transport; industrial processes (e.g. metal processing); indoor gas burners (stoves and heaters)
CO_2	natural gas in the atmosphere	combustion
NH_3		agricultural processes
Total suspended particulate matter	volcanic activity; dust storms; grassland fires; living vegetation; sea spray	fossil fuel combustion in vehicles, power plants and industrial processes; wood stoves
VOCs (CH_4, benzene, toluene, xylene, petrol vapour emissions, etc.)	food digestion (CH_4)	industrial uses; waste deposition in landfills which generate methane; petrol stations; fumes from paint, hair sprays, varnish, aerosol sprays, solvents utilization
Toxic metals (lead, cadmium, copper, mercury)		industrial processes from metal smelters; combustion of leaded gasoline, aviation fuels, etc.
Organic pollutants (dioxins, PAHs such as benzo(a)pyrene)	forest fires; volcanic activity	incomplete combustion of carbon-containing fuels (wood, coal, diesel, fat, tobacco); combustion processes and industrial processes (e.g. processing of coal, crude oil, creosote, coal tar, bitumen and asphalt industries); combustion of kerosene (liquid mixture of hydrocarbons)
Radioactive pollutants	radioactive decay of radon	nuclear weapons, war explosives
Secondary Pollutants		
Ground level O_3 (constituent of 'photochemical' smog)		formed from NO_x + VOCs
Peroxyacetyl nitrate (constituent of 'photochemical' smog)		formed from NO_x + VOCs

Primary pollutants constitute direct emissions, whereas secondary pollutants are formed as a result of primary pollutants undergoing chemical reactions. An important example of a secondary pollutant is ground level ozone, one of the pollutants that make up 'photochemical' smog and a rich source of reactive oxygen species. VOC = Volatile organic compounds.

Table 1: *Overview of the major primary and secondary air pollutants and their natural vs anthropogenic sources. Primary pollutants constitute direct emissions, whereas secondary pollutants are formed as a result of primary pollutants undergoing chemical reactions. An important example of a secondary pollutant is ground level ozone, one of the pollutants that make up "photochemical" smog and a rich source of reactive oxygen species.*

Airway dendritic cells: specialized immune sensors at the forefront of the lung-air interface

Dendritic cells (DCs) are professional antigen presenting cells (APCs) capable of priming and sustaining the expansion of naïve T cells. They are present in tissues in close contact to the external environment, such as the skin (called Langerhans cells), and mucosal surfaces of upper and lower airways, gastro-intestinal and urogenital tract. In the lung, DCs are located as a network immediately above and beneath the basement membrane of conductive airways, as wells as within the interalveolar septa of the deeper lung parenchyma [5]. In the steady state, airway DCs can project their dendrites in between epithelial cells without breaking the epithelial layer integrity and sample the epithelial lining fluid which is in direct contact with the inhaled air [6]. DCs are equipped with specialized receptors (e.g. C-type lectin receptors, TLRs) for antigen capture and for sensing PAMPs and danger-associated molecular patterns (DAMPs), the latter being released as a result of tissue damage (whether as result of microbial invasion or not) [7]. Inhalational exposure to PAMPs unveils the innate character of airway DC dynamics, as reflected by a fast and massive recruitment of these cells into the airways, being at least as rapid as the prototypical neutrophilic influx [8]. Capture of inhaled antigen in the presence of PAMPs and/or DAMPs leads to DC activation, which initiates a series of events leading to the adaptive immune response: stimulated migration to the T-cell zones of draining thoracic lymph nodes, strong upregulation of processed antigen on surface major histocompatibility molecules (MHC) and upregulation of T-cell costimulatory molecules such as CD40 and B7-1, B7-2. An emerging paradigm is that the resulting T-cell polarization (T-helper 1, 2 or 17) is likely "programmed" as result of DC exposure to specific molecular patterns present at the time and place of antigen encounter in the periphery. This includes both direct stimulation of DCs by pathogen-derived factors, or indirect conditioning by innate factors released by surrounding cells. As an illustration of the latter, stimulation of TLR4 on airway epithelial cells results in the production of innate cytokines (including TSLP, GM-CSF and IL-33) and secondary activation of the DC network, which in this case is conditioned to induce a T-helper 2 polarized immune response [6].

By contrast, aero-antigen sampling in the absence of innate stimuli is followed by steady state airway DC migration and antigen presentation in the draining LN [9], resulting in the induction of regulatory T-cells and establishment of inhalational tolerance [10].

With these concepts in mind, the question arises whether airborne xenobiotics can act directly as innate stimuli on airway dendritic cells, and if so, how this could affect the outcome of pulmonary immune responses. In the next sections, we will highlight the effects of two main active components in inhaled xenobiotics on DC biology: reactive oxygen species (ROS) and polycyclic aromatic hydrocarbons (PAHs). It should be stressed that insights into these mechanisms have mostly been derived from *in vitro* models. Hence, extrapolation to *in vivo* situations must be made with caution: in a more realistic tissue or whole organism context, effects on DCs can also be indirect, as mentioned earlier. Co-culture models can provide additional useful information, as shown in a study involving dendritic cells incubated with bronchial epithelial cells. In this system, addition of DEPs triggers, in an oxidant-dependent manner, epithelial release of TSLP which then activates DCs and induces Th2 polarization [11]. *In vivo* animal studies aim to be more relevant to clinical observations, but are not without drawbacks either: animal exposure models such as inhalation of tobacco smoke or diesel exhaust particles involve multiple potential mediators acting simultaneously on several receptor systems on a broad range of cells, making it difficult to clearly delineate relevant pathogenetic molecular mechanisms. For instance, both diesel exhaust particles and tobacco smoke are complex vectors for ROS, PAHs and a myriad of other bioactive compounds. Moreover, DEPs and tobacco smoke are often administered to animals in ways that do not reflect real-world situations, i.e. use of excessive doses, and/or instillation of soluble extracts rather than inhalation of airborne forms [12].

How oxidative stress can affect pulmonary dendritic cells

Reactive oxygen species are now recognized as important elements in the pathogenesis of several inflammatory disorders. ROS, which include oxygen ions, free radicals and peroxides, are highly unstable molecules with unpaired electrons, capable of initiating oxidation of cellular components. Endogenous ROS are generated by biochemical redox reactions of the natural cell metabolism including mitochondrial respiration or the oxidative burst of phagocytic cells, whereas exposure to air pollutants is the main source of exogenous ROS [13]. Carbonacous fine particulate matter (including diesel exhaust particles), gases such as ozone, nitrogen dioxide and sulphur dioxide are all potential sources of ROS-generation at the level of airway surfaces. Not surprisingly, the lung has evolved elaborate anti-oxidant mechanisms

to limit damage caused by ROS. ROS can affect cells at multiple levels. ROS generation in close proximity to the cell membrane oxidizes membrane phospholipids, resulting in the formation of highly reactive aldehydes such as acrolein, 4-hydroxy-2-nonenal (4-HNE) and related 4-hydroxy-2,3-alkenals (HAKs) [14] (*Figure 1*). HNE and HAKs possess high affinity towards specific amino-acid residues of intracellular proteins involved in several aspects of cell signalling, chromatin remodelling and gene transcription. 4-HNE for instance, is capable of inducing adenylate cyclase activation, increase cAMP levels and thus affect cellular function by activating cAMP-dependent protein kinases (PKAs) [15]. Reactive aldehydes can form stable adducts with JNK1/2 leading to subsequent activation of the transcription factor activator protein 1 (AP-1) and suggesting a possible role for 4-HNE in the modulation of cell proliferation and differentiation [16]. Interestingly, 4-HNE and HAKs both possess chemotactic activity towards neutrophils and monocytes, an important aspect of innate immunity [17]. Isoprostanes are alternative by-products of membrane lipid oxidation, which are further processed into bioactive molecules, such as 1-palmitoyl-2-(5)oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), 1-palmitoyl-2-epoxyiso-prostane-sn-glycero-3-phosphorylcho-line (PGPC) and (1-palmitoyl-2-epoxyisoprostane-sn-glycero-3-phosphorylcholine (PEIPC). These molecules possess innate pro-inflammatory effects as they can activate monocyte and neutrophil adhesion to the endothelium and increase their cytokine release [18].

Protein nitration is another product of ROS-induced tissue damage. A reaction between NO en O_2^- radical results in the formation of peroxynitrite anions (ONOO-). These are highly reactive radicals, able to nitrosylate tyrosine residues to produce the stable product nitrotyrosine. Tyrosine nitration affects the function of important intracellular proteins such as NFkB [19], MAPK [20] and HDACs [21]. In addition, oxidation of cystein residues in the DNA binding domain of so-called redox sensitive transcription factors (NFkB, AP-1) can have profound effects on inflammatory gene expression and cell differentiation [22]. An important redox-sensitive protein is Nuclear erythroid 2 p45-related factor 2 (Nrf2). Oxidative stress interferes with the default cytoplasmic anchoring of Nrf2 to the cytoskeleton-associated protein Keap1. Thus freed, Nrf2 translocates to the nucleus and activates transcription of several genes which have been shown to have anti-oxidant and anti-inflammatory activity in the lung [23]. Also, in homeostatic conditions Nrf2 activity is low as a consequence of a Keap1-dependent proteosomal degradation [24].

Finally, ROS can also mediate the activation of the NALP3 inflammasome and trigger the secretion of the pro-inflammatory cytokine IL-1β, as was shown in a model of asbestos fiber inhalation [25]. Like IL-1β, IL-18 is produced following cleavage of a pro-form by the NALP3-activated caspase 1. Interestingly, Nrf2-deficient DCs challenged with ambient particulate matter show enhanced production of IL-18 compared to wildtype DCs [26], suggesting a possible interaction between inflammasome-dependent IL-18 generation and Nrf2 redox sensing mechanisms.

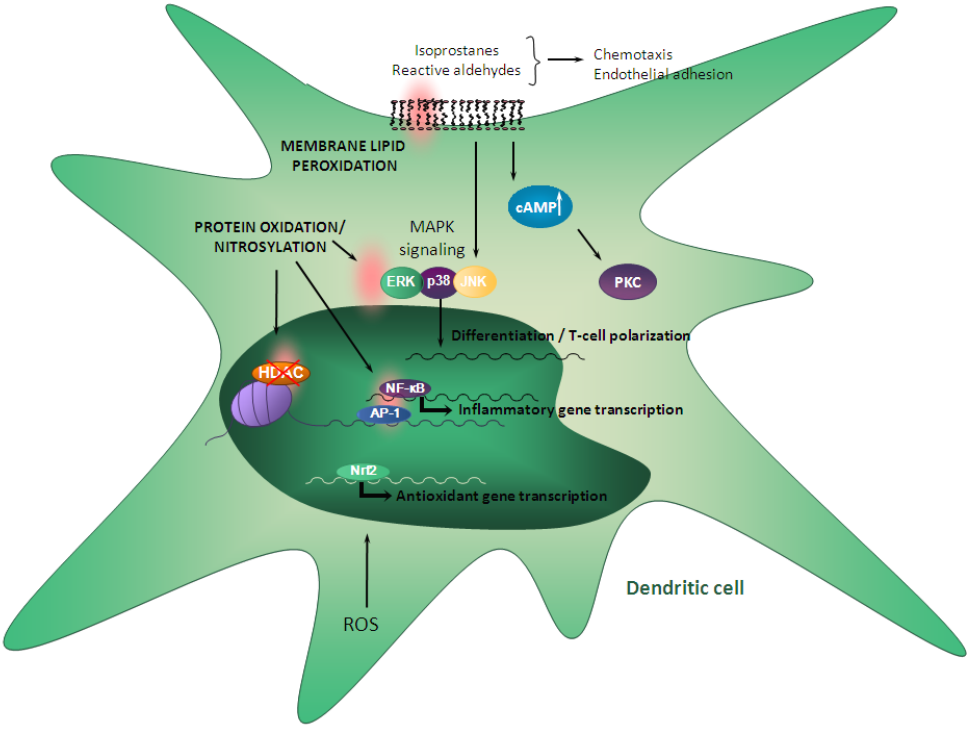


Figure 1. *Schematic overview on the cellular-molecular effects of reactive oxygen species, with a special emphasis on pathways that are relevant to DC biology, such activation of NFkB, ERK/MAPK and AP-1 signalling cascades. See main text for details.*

With that knowledge in mind, several groups have used in vitro systems to closely dissect the effects of ROS on key features of dendritic cell biology. Preynat-Seauve *et al* analyzed the effects of oxidative stress on antigen processing and T cell presentation and indicated that oxidative stress inhibits the capacity of APCs to process antigens and to initiate a primary T cell response [27]. By contrast, a study by Kantengwa *et al* demonstrated a ROS-induced early

maturation of human monocyte-derived DCs, characterized by a clear up-regulation of co-stimulatory molecules (CD80, CD83 and CD86) and a decrease in endocytic activity [28]. Likewise, it was shown that exposure of DCs to H_2O_2 induces up-regulation MHC II and costimulatory molecules (CD40, B7-2). Accordingly, H_2O_2 conditioned DCs were more efficient in promoting T cell proliferation than non-treated DCs [29]. These findings link ROS production and subsequent tissue damage to the innate activation of DCs and the initiation of adaptive immune responses. The involvement of oxidative stress mechanisms in DC activation and cytokine secretion includes a significant increase in protein oxidation, measured by the formation of carbonyl radicals, and triggers p38 and extracellular signal-regulated mitogen-activated protein kinase (ERK/MAPK), resulting in the upregulation of CD40 [30]. Thus, MAPK, which has been described earlier as an important mediator of DC maturation following innate immune stimuli [31], appears to be an important target of ROS attack in itself. The exquisite sensitivity of DCs to oxidative influences is further illustrated by a study showing how treatment with anti-oxidants can virtually paralyze the NFkB, MAPK, and PKC response following innate inflammatory stimuli such as LPS or IL-1β. Interestingly, this led to the preferential induction of regulatory T-cells by the DCs [32]. In the same line, Vassallo and coworkers showed that the high production of the neutrophil chemoattractant CXCL8 by *in vitro* tobacco smoke-conditioned DCs and *ex vivo* DCs isolated from tobacco smoke-exposed mice can be suppressed by the use of the antioxidant N-acetyl-cysteine (NAC) [33]. In contrast to these observations, Kroening *et al* demonstrated that activation of ERK-dependent pathways in DCs by oxidative stress from soluble cigarette smoke components, potently inhibits the production of IL-12 and IL-23 by mature DCs [34].

Further reinforcing the notion that ROS may promote DC-dependent pro-allergic responses, Williams et al showed exacerbated DC maturation, inflammatory cytokine secretion and Th2-polarization in Nrf2-deficient dendritic cells [26] exposed to particulate matter-associated ROS. This was consistent with a study by the same group showing that Nrf2-deficient mice develop more severe features of allergic airway inflammation in an ovalbumin sensitization/challenge model than wildtype controls [35].

The contrasting results of ROS and DC response investigations are an unevitable consequence of the reductionist nature of the experimental models and reflect the complex nature of oxidative stress effects on living cells. ROS-sensitive epigenetic modulation of gene expression adds another layer of complexity to the impact of oxidative stress on DCs. An

important element of epigenetic regulation is post-translational modification of histones by histone acetylases (HATs) and deacetylases (HDACs), whereby histone deacetylation results in compact winding of chromatin, obstructing access to transcription factors and suppressing gene transcription [36]. As mentioned earlier, HDACs are a target of ROS attack by means of lipid peroxidation products or tyrosine residue nitrosylation. ROS can inactivate certain histone deacetylases (HDAC-2, -5 and -8) to promote the expression of pro-inflammatory genes in a number of pulmonary cells [37]. Interestingly, HDACs are increasingly recognized as important regulators of innate immune responses and of DC function in particular, as illustrated by several studies. For instance, pharmacological HDAC inhibition (by the synthetic compound LAQ824) can alter TLR-4 dependent activation of macrophages and DCs and suppress Th1 but not Th2-cell activation and migration [38]. In a different study, it was shown that HDAC inhibition decreases TLR- mediated activation of proinflammatory gene expression, due to impaired transcription factor recruitment [39]. HDAC inhibition also changes DC differentiation by affecting the expression of costimulatory and adhesional molecules. The HDAC inhibitor butyrate inhibits the expression of CD1 molecules, but not CD83, CD86 and MHCII molecules and butyrate-treated immature DCs showed lower production of IL12 and IL-6 [40]. The observed defects in DC function after HDAC inhibition seem to rely on impaired nuclear translocation of NFκB, IRF-3 and IRF-8 [41]. Thus, ROS-induced defects in HDAC activity could have profound effects on normal innate responses of pulmonary DC, just as it does on other cells in the lung.

Recently, an intriguing report was published pointing to a fundamental role of Toll-like receptors as alternative sensors for exogenous oxidants in the lung. The study by Paul-Clarck *et al* clearly identified TLR2, but not TLR4, as an important receptor mediating the production of CXCL8 after exposure to soluble oxidants [42]. This may shed a new light on the finding by our group that tobacco smoke-induced airway inflammation, pulmonary DC recruitment and DC activation occur in a TLR4-dependent fashion [43]. In an *in vivo* mouse model of tobacco smoke inhalation, we observed a marked upregulation of MHC II and the costimulatory molecules CD40 and B7-2 on airway DCs, and this effect was profoundly impaired in TLR4-deficient mice. *In vivo* studies from a different group have raised doubts whether the TLR4-dependent effects of tobacco smoke are due to the well-documented presence of endotoxin [44]. Consequently, the link between ROS-exposure and TLR-signalling may offer an attractive alternative explanation.

Polycyclic aromatic hydrocarbons as a separate class of innate stimuli for dendritic cells

Airborne PAHs are mainly byproducts of industrial activity as well as domestic combustion processes such as woodfire heating, tobacco smoking and cooking. Because PAHs are highly resistant to metabolic breakdown, they tend to accumulate in the body, hence further extending exposure time and increasing the risk for a broad range of adverse health effects including carcinogenesis, immunotoxicity and endocrine dysregulation. The toxic effects of PAHs, among which dioxin-like compounds, are mediated by the aryl hydrocarbon receptor (AhR) [45]. This is a ligand-dependent basic helix-loop-helix transcription factor involved in the regulation of xenobiotic metabolism and detoxification. The evolutionary highly conserved structure of the AhR suggests interactions with endogenous ligands. This is illustrated by functions which are unrelated to detoxification of modern environmental xenobiotics [46], including vascular development, regulation of circadian rhythm and modulation of immune responses, as discussed below. In the non-activated form, the AhR is a soluble cytosolic protein, forming a complex with chaperone proteins hsp90 (heat shock protein 90) and an immunophilin-like protein called XAP2 (*Figure 2*). Both chaperone proteins bind to the AhR nuclear localization sequence (NLS), preventing the inappropriate trafficking of the receptor into the nucleus. Upon ligand activation, the XAP2 protein disocciates from the AhR, resulting in the exposure of NLS and subsequent translocation to the nucleus. Once in the nucleus, the AhR dissociates from hsp90 and dimerizes with ARNT (AhR nuclear translocator) to reconstitute an active transcription factor. The AhR-ARNT complex binds to specific DNA response elements known as dioxin-responsive elements (DREs) or xenobiotic responsive elements (XREs). DREs are present in many promotors and drive transcription of a wide range of genes, not only for xenobiotic metabolism (i.e. induction of cytochrome family of proteins), but also genes involved in the regulation of cell differentiation, proliferation and activation [47].

There is growing evidence pointing to an active role of the AhR signaling pathway in immune responses [46]. Engagement of the AhR leads to diverse, sometimes opposing immunological effects depending on ligand binding characteristics as well as the broader cellular and molecular environmental context. Exposure to TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), a prototype of PAH with high affinity to the AhR, can lead to both pro-inflammatory effects with the induction of TNF-α, IL-1β, COX-2 and IL-8 [48,49,50] or suppression of adaptive

immunity. TCDD-dependent activation of the AhR can induce suppression of B and T cell-dependent responses and result in increased susceptibility to infection [51]. A series of *in vivo* studies have highlighted the complex effects of AhR-triggering in the setting of influenza infection, a good model to illustrate the shift from innate immunity to adaptive response in the lung (reviewed in [52]). The innate response characterized by neutrophilia and interferon production is amplified in TCDD-treated, infected mice, while the clonal expansion of virus-specific CD8 T-cells is profoundly suppressed. Interestingly, the latter phenomenon appears indirect, as CD8 T-cells from Ahr -/- animals are still prone to suppression when transferred into Ahr +/+ hosts [53]. This suggests that the defect in adaptive immune response after AhR triggering occurs at the level of the accessory cell, i.e. most probably targeting the dendritic cell. Several *in vitro* studies have further highlighted the immunosuppressive effects of AhR ligands. Benzo(a)pyrene (BP) has been reported to impair antigen presentation by mouse macrophage and alters the T cell-macrophage interaction [54]. Similarly, PAHs can impair differentiation of blood-derived monocytes into functional macrophages [55].

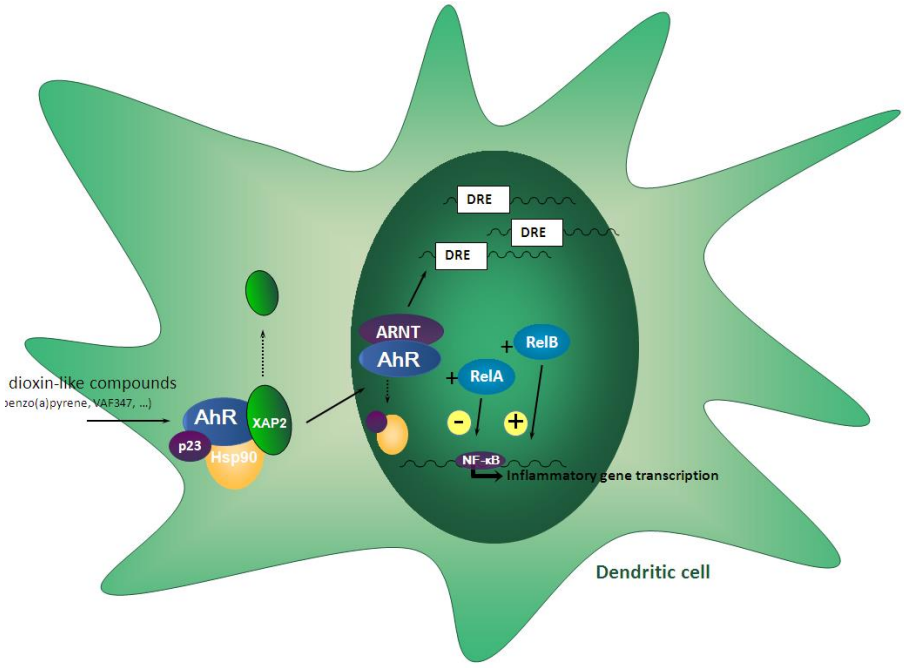


Figure 2. *Hypothetical diagram illustrating possible effects of aryl hydrocarbon receptor signalling in DCs. A central element is the dual nature of interactions of AhR with components of the NFkB signalling cascade, the latter being essential for DC activation following innate stimuli and the acquisition of T-cell immunostimulatory power. Probably depending on the ligand, AhR engagement can engage different subunit of NFkB complexes and lead to both suppression or induction of inflammatory gene expression.*

When it comes to dendritic cells specifically, direct effects of PAHs are similarly complex, often producing contradicting reports. Jux *et al* provided evidence for a physiological role of the AhR in Langerhans cells (LCs), the predominant DC population of the epidermis. They observed an impaired upregulation of costimulatory molecules in TCDD-stimulated, AhR-deficient murine LCs compared to AhR-competent LCs, and consequently an impaired induction of contact hypersensitivity. Interestingly, the AhR was also necessary for the upregulation of Indoleamine-2,3-dioxygenase (IDO) expression in DCs. IDO enzymatic activity in DCs is known result in T-cell anergy or the generation of regulatory T-cells [56].

Laupeze *et al* studied the effects of PAHs on the differentiation, maturation and biological function of human monocyte-derived DCs in vitro. Exposure of monocytes to BP during their development into DCs resulted in impaired upregulation of DC-specific differentiation and maturation markers such as CD1a, CD80 and CD40. Moreover, DCs generated in the presence of BP displayed decreased endocytic activity and showed impaired IL-12 secretion. BP-exposed DCs poorly stimulated T cell proliferation compared to their untreated counterparts [57]. In a recent report, Platzer *et al* further highlight the suppressive effects of AhR triggering on DC biology. Using the synthetic AhR ligand VAF347, they reveal the involvement of the AhR in suppressing commitment of human myeloid progenitors towards the monocyte/DC lineage [58]. In a different report, VAF347 also appears capable of inhibiting the upregulation of MHCII and B7-2 on DCs [59]. In the same line, Hwang (and Lee) et al. showed that the AhR ligand benzopyrene inhibits the growth and functional differentiation of mouse bone marrow-derived DCs. BP induced little alterations in CD11c, MHCII and CD86 surface expression, but clearly impaired production of IL-12, IL-10 and TNF-α as well as allogeneic T cell stimulating ability. This was accompanied by a reduced expression of the RelB NFkB protein family member, which is known to be pivotal for DC differentiation and function [60]. Even though BP and the dioxin-like TCDD are both ligands of the AhR, their effects on the development and function of DCs can be divergent. This is illustrated by a study in which TCDD-treated DCs expressed significantly higher levels of DC differentiation markers such as MHCII and the co-stimulatory molecule CD86, impaired IL-10 and intact IL-12 production. Accordingly, T-cell stimulatory capacity was increased in TCDD-treated compared to control DCs [61]. Also Vogel *et al* showed increased maturation of human monocyte-derived DCs after AhR activation by TCDD, as manifested by characteristic morphological changes and upregulation of costimulatory molecules. Again, AhR triggering

by TCDD in DCs led to induction of IDO expression and enzymatic activity. A similar effect of AhR-triggering was observed *in vivo*, with increased IDO expression in lung and spleen of TCDD-treated mice, resulting in the induction of FoxP3 transcripts (pointing to an increased differentiation of regulatory T-cells) [62].

Insights into the divergent effects of PAHs on DCs came with the discovery that the AhR is involved in a complex cross-talk with several members of the NFkB pathway. Specifically, AhR can dimerize with RelA and antagonize the canonical NFkB pathway, leading to suppression of inflammatory gene expression. At the same time, the AhR can form a complex with RelB and in some cases enhance transcription of inflammatory cytokines and chemokines [63], or presumably affect functional features of DCs which rely on non-canonical NFkB activation (eg antigen cross-presentation) [64]. Which downstream signalling pathway will be activated following binding of a specific AhR ligand will likely depend on concomitant innate stimuli, as well as the differentiation/maturation state of the DC target.

From airborne xenobiotic exposure to pulmonary immunopathology: the dendritic cell link?

Given their anatomical distribution, airway DCs constitute unevitable targets of inhaled xenobiotics. Because innate receptors of DCs have not evolved under selective pressure of anthropogenic compounds, it can be assumed that confrontation of airway DCs with these agents will initiate aberrant pulmonary immune responses, or modulate the outcome of classical anti-microbial defenses. This working hypothesis may shed new light on the pathogenesis of several chronic inflammatory pulmonary diseases. A good illustration is the implication of tobacco smoke (TS) exposure in the development of asthma. Tobacco smoke inhalation activates multiple mechanisms that can potentially program DCs to polarize pro-allergic T-helper 2 type responses towards co-inhaled antigen. TS is a known vector for endotoxin-like compounds, the latter having been implicated in the induction of Th2 responses [65]. Despite this fact, our group has recently shown unimpaired development TS-induced allergic sensitization in TLR4 and Myd88 gene deficient mice (Robays et al, in press). This suggests alternative Th2-promoting effects of TS on DCs, such as ROS-induced activation of the MAPK/ERK pathway [34], or the secondary release of Th2-promoting innate factors such as TSLP, ATP or IL-33 [66]. Another illustration of pulmonary immunopathology with a strong link to human activity is COPD, a chronic pulmonary disease associated with the inhalation of

tobacco smoke and presumably biomass combustion products in general. Among the many xenobiotics present in smoke, the dual effects of PAHs as both promoting chronic inflammation, while simultaneously suppressing adaptive immunity, may contribute to the relentless progression of pulmonary damage, coexisting with the enhanced sensitivity to infection that charactizes COPD. This is reflected in the specific changes in airway DC populations observed in COPD patients, i.e. increased recruitment of airway epithelial DCs (a sign of innate pro-inflammatory response), which however display an immature phenotype [67]. COPD is clearly a disease of aberrant immunostimulation in the lung and it is very likely that the reprogramming of pulmonary DCs by smoke-derived xenobiotics plays a central role in perpetuating the chronicity of the inflammation, even after smoking cessation. Epigenetic reprogramming of DCs is an interesting hypothesis to explain the hard-wiring of this aberrant response. In support of this, extensive studies have unveiled progressive reduction in HDAC activity in COPD bronchial biopsies, a phenomenon that was proportional to COPD severity. Specifically, HDAC activity was suppressed in alveolar macrophages from COPD-patients, resulting in enhanced and glucocorticoisteroid-resistant production of innate pro-inflammatory cytokines [37]. It is very likely that airway DCs are subject to similar epigenetic modulations, probably under influence of ROS, as detailed above. In contrast to the innate scavenger function of alveolar macrophages, the impact of these effects on DCs is more far-reaching, as DC function can shift from innate immune sensor to initiator of adaptive immnunity, with the potential to instruct tolerance vs active immune response. Accordingly, reports have recently emerged suggesting the presence of an aberrant adaptive response in COPD, including reactivity against self-antigens within the lung tissue [68]. Provided the necessary stimuli are present, DCs are probably the only antigen-presenting cells powerfull enough to override this self-tolerance.

Besides asthma and COPD, the lung is also the stage for other chronic, often debilitating immunopathologies such as interstitial pneumonitis/fibrosis, vasculitis (e.g. Churg-Strauss, Wegener disease) and granulomatous inflammation (e.g sarcoidosis). The etiology of these diseases is still obscure, and it remains to be examined whether airborne anthropogenic xenobiotics, by acting on pulmonary DCs, could provide the necessary spark to initiate an aberrant immune response in predisposed individuals.

Conclusion

In summary, it appears increasingly evident that the pulmonary innate immune system is a critical target of airborne xenobiotics. The large amount of experimental data available reflects the complex nature of these man-made compounds in terms of triggering specific innate molecular responses. Diverging effects are observed depending on co-existing stimuli and the cellular and tissue context. Nevertheless, it is worth noting that classical pathogen-associated molecules and human-made xenobiotics can use common sets of innate receptors such as TLRs and presumably inflammasome components. Even more intriguing is the way the aryl hydrocarbon receptor system and the cellular response to oxidative stress interconnect with immunological pathways. In this review, we provided evidence that molecular responses to xenobiotics can operate at the level of the dendritic cell. Given the extensive network of DCs in the airways, and the pivotal role of these cells in linking innate and adaptive immunity, we believe intensive preclinical and translational research in this field may produce unexpected insights in many pulmonary immunopathologies.

Acknowledgements

E. Lanckacker and L. Robays are supported by grants from the Concerted Research Initiative Ghent University (BOF/GOA 12050698 and BOF/GOA 12.515.04), Interuniversity Attraction Poles Program (Belgian State, Belgian Science Policy, Project P6/35) and Research Foundation - Flanders (Project 3G.0052.06).

Reference List

1. Zaas AK, Schwartz DA. Innate immunity and the lung: defense at the interface between host and environment. *Trends Cardiovasc. Med.* 2005; 15: 195-202.
2. Voss T, Eistetter H, Schafer KP, Engel J. Macromolecular organization of natural and recombinant lung surfactant protein SP 28-36. Structural homology with the complement factor C1q. *J. Mol. Biol.* 1988; 201: 219-27.
3. Mizgerd JP. Acute lower respiratory tract infection. *N. Engl. J. Med.* 2008; 358: 716-27.
4. Lewtas J. Air pollution combustion emissions: characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. *Mutat. Res.* 2007; 636: 95-133.
5. Vermaelen K, Pauwels R. Pulmonary dendritic cells. *Am. J Respir Crit Care Med.* 2005; 172: 530-51.
6. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.
7. Willart MA, Lambrecht BN. The danger within: endogenous danger signals, atopy and asthma. *Clin. Exp. Allergy* 2009; 39: 12-9.
8. McWilliam AS, Nelson D, Thomas JA, Holt PG. Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces. *J. Exp. Med.* 1994; 179: 1331-6.
9. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J. Exp. Med.* 2001; 193: 51-60.
10. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2001; 2: 725-31.
11. Bleck B, Tse DB, Curotto de Lafaille MA, Zhang F, Reibman J. Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation and polarization via thymic stromal lymphopoietin. *J. Clin. Immunol.* 2008; 28: 147-56.
12. Sydbom A, Blomberg A, Parnia S, Stenfors N, Sandstrom T, Dahlen SE. Health effects of diesel exhaust emissions. *Eur. Respir. J.* 2001; 17: 733-46.
13. Rahman I. Oxidative stress, chromatin remodeling and gene transcription in inflammation and chronic lung diseases. *J. Biochem. Mol. Biol.* 2003; 36: 95-109.
14. Dianzani MU, Barrera G, Parola M. 4-Hydroxy-2,3-nonenal as a signal for cell function and differentiation. *Acta Biochim. Pol.* 1999; 46: 61-75.
15. Dianzani MU. 4-Hydroxynonenal and cell signalling. *Free Radic. Res.* 1998; 28: 553-60.
16. Parola M, Robino G, Marra F, Pinzani M, Bellomo G, Leonarduzzi G, Chiarugi P, Camandola S, Poli G, Waeg G, Gentilini P, Dianzani MU. HNE interacts directly with JNK isoforms in human hepatic stellate cells. *J. Clin. Invest* 1998; 102: 1942-50.
17. Schaur RJ, Dussing G, Kink E, Schauenstein E, Posch W, Kukovetz E, Egger G. The lipid peroxidation product 4-hydroxynonenal is formed by--and is able to attract--rat neutrophils in vivo. *Free Radic. Res.* 1994; 20: 365-73.
18. Lee H, Shi W, Tontonoz P, Wang S, Subbanagounder G, Hedrick CC, Hama S, Borromeo C, Evans RM, Berliner JA, Nagy L. Role for peroxisome proliferator-activated receptor alpha in oxidized phospholipid-induced synthesis of monocyte

chemotactic protein-1 and interleukin-8 by endothelial cells. *Circ. Res.* 2000; 87: 516-21.
19. Matata BM, Galinanes M. Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. *J. Biol. Chem.* 2002; 277: 2330-5.
20. Zhang P, Wang YZ, Kagan E, Bonner JC. Peroxynitrite targets the epidermal growth factor receptor, Raf-1, and MEK independently to activate MAPK. *J. Biol. Chem.* 2000; 275: 22479-86.
21. Ito K, Hanazawa T, Tomita K, Barnes PJ, Adcock IM. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration. *Biochem. Biophys. Res. Commun.* 2004; 315: 240-5.
22. Rahman I, Adcock IM. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur. Respir. J.* 2006; 28: 219-42.
23. Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, Kleeberger SR. Role of NRF2 in protection against hyperoxic lung injury in mice. *Am. J. Respir. Cell Mol. Biol.* 2002; 26: 175-82.
24. McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J. Biol. Chem.* 2003; 278: 21592-600.
25. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 2008; 320: 674-7.
26. Williams MA, Rangasamy T, Bauer SM, Killedar S, Karp M, Kensler TW, Yamamoto M, Breysse P, Biswal S, Georas SN. Disruption of the transcription factor Nrf2 promotes pro-oxidative dendritic cells that stimulate Th2-like immunoresponsiveness upon activation by ambient particulate matter. *J. Immunol.* 2008; 181: 4545-59.
27. Preynat-Seauve O, Coudurier S, Favier A, Marche PN, Villiers C. Oxidative stress impairs intracellular events involved in antigen processing and presentation to T cells. *Cell Stress. Chaperones.* 2003; 8: 162-71.
28. Kantengwa S, Jornot L, Devenoges C, Nicod LP. Superoxide anions induce the maturation of human dendritic cells. *Am. J. Respir. Crit Care Med.* 2003; 167: 431-7.
29. Rutault K, Alderman C, Chain BM, Katz DR. Reactive oxygen species activate human peripheral blood dendritic cells. *Free Radic. Biol. Med.* 1999; 26: 232-8.
30. Matos TJ, Duarte CB, Goncalo M, Lopes MC. Role of oxidative stress in ERK and p38 MAPK activation induced by the chemical sensitizer DNFB in a fetal skin dendritic cell line. *Immunol. Cell Biol.* 2005; 83: 607-14.
31. Arrighi JF, Rebsamen M, Rousset F, Kindler V, Hauser C. A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-alpha, and contact sensitizers. *J. Immunol.* 2001; 166: 3837-45.
32. Tan PH, Sagoo P, Chan C, Yates JB, Campbell J, Beutelspacher SC, Foxwell BM, Lombardi G, George AJ. Inhibition of NF-kappa B and oxidative pathways in human dendritic cells by antioxidative vitamins generates regulatory T cells. *J. Immunol.* 2005; 174: 7633-44.

33. Vassallo R, Kroening PR, Parambil J, Kita H. Nicotine and oxidative cigarette smoke constituents induce immune-modulatory and pro-inflammatory dendritic cell responses. *Mol. Immunol.* 2008; 45: 3321-9.
34. Kroening PR, Barnes TW, Pease L, Limper A, Kita H, Vassallo R. Cigarette smoke-induced oxidative stress suppresses generation of dendritic cell IL-12 and IL-23 through ERK-dependent pathways. *J. Immunol.* 2008; 181: 1536-47.
35. Rangasamy T, Guo J, Mitzner WA, Roman J, Singh A, Fryer AD, Yamamoto M, Kensler TW, Tuder RM, Georas SN, Biswal S. Disruption of Nrf2 enhances susceptibility to severe airway inflammation and asthma in mice. *J. Exp. Med.* 2005; 202: 47-59.
36. Kirkham P, Rahman I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol. Ther.* 2006; 111: 476-94.
37. Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC, Barnes PJ. Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2005; 352: 1967-76.
38. Brogdon JL, Xu Y, Szabo SJ, An S, Buxton F, Cohen D, Huang Q. Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. *Blood* 2007; 109: 1123-30.
39. Bode KA, Schroder K, Hume DA, Ravasi T, Heeg K, Sweet MJ, Dalpke AH. Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. *Immunology* 2007; 122: 596-606.
40. Wang B, Morinobu A, Horiuchi M, Liu J, Kumagai S. Butyrate inhibits functional differentiation of human monocyte-derived dendritic cells. *Cell Immunol.* 2008; 253: 54-8.
41. Dong X, Lutz W, Schroeder TM, Bachman LA, Westendorf JJ, Kumar R, Griffin MD. Regulation of relB in dendritic cells by means of modulated association of vitamin D receptor and histone deacetylase 3 with the promoter. *Proc. Natl. Acad. Sci. U. S. A* 2005; 102: 16007-12.
42. Paul-Clark MJ, McMaster SK, Sorrentino R, Sriskandan S, Bailey LK, Moreno L, Ryffel B, Quesniaux VF, Mitchell JA. Toll-like receptor 2 is essential for the sensing of oxidants during inflammation. *Am. J. Respir. Crit Care Med.* 2009; 179: 299-306.
43. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA, Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int. Arch. Allergy Immunol.* 2006; 141: 354-68.
44. Doz E, Noulin N, Boichot E, Guenon I, Fick L, Le Bert M, Lagente V, Ryffel B, Schnyder B, Quesniaux VF, Couillin I. Cigarette smoke-induced pulmonary inflammation is TLR4/MyD88 and IL-1R1/MyD88 signaling dependent. *J. Immunol.* 2008; 180: 1169-78.
45. Denison MS, Nagy SR. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* 2003; 43: 309-34.
46. Stevens EA, Mezrich JD, Bradfield CA. The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. *Immunology* 2009; 127: 299-311.
47. Schmidt JV, Bradfield CA. Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* 1996; 12: 55-89.

48. Taylor MJ, Lucier GW, Mahler JF, Thompson M, Lockhart AC, Clark GC. Inhibition of acute TCDD toxicity by treatment with anti-tumor necrosis factor antibody or dexamethasone. *Toxicol. Appl. Pharmacol.* 1992; 117: 126-32.
49. Fan F, Yan B, Wood G, Viluksela M, Rozman KK. Cytokines (IL-1beta and TNFalpha) in relation to biochemical and immunological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rats. *Toxicology* 1997; 116: 9-16.
50. Vogel C, Schuhmacher US, Degen GH, Bolt HM, Pineau T, Abel J. Modulation of prostaglandin H synthase-2 mRNA expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin in mice. *Arch. Biochem. Biophys.* 1998; 351: 265-71.
51. Kerkvliet NI. Immunological effects of chlorinated dibenzo-p-dioxins. *Environ. Health Perspect.* 1995; 103 Suppl 9: 47-53.
52. Head JL, Lawrence BP. The aryl hydrocarbon receptor is a modulator of anti-viral immunity. *Biochem. Pharmacol.* 2009; 77: 642-53.
53. Lawrence BP, Roberts AD, Neumiller JJ, Cundiff JA, Woodland DL. Aryl hydrocarbon receptor activation impairs the priming but not the recall of influenza virus-specific CD8+ T cells in the lung. *J. Immunol.* 2006; 177: 5819-28.
54. Myers MJ, Schook LB, Bick PH. Mechanisms of benzo(a)pyrene-induced modulation of antigen presentation. *J. Pharmacol. Exp. Ther.* 1987; 242: 399-404.
55. van GJ, Rion S, Le FE, Le VM, Amiot L, Fauchet R, Fardel O. Polycyclic aromatic hydrocarbons inhibit differentiation of human monocytes into macrophages. *J. Immunol.* 2003; 170: 2374-81.
56. Jux B, Kadow S, Esser C. Langerhans cell maturation and contact hypersensitivity are impaired in aryl hydrocarbon receptor-null mice. *J. Immunol.* 2009; 182: 6709-17.
57. Laupeze B, Amiot L, Sparfel L, Le Ferrec E, Fauchet R, Fardel O. Polycyclic aromatic hydrocarbons affect functional differentiation and maturation of human monocyte-derived dendritic cells. *J. Immunol.* 2002; 168: 2652-8.
58. Platzer B, Richter S, Kneidinger D, Waltenberger D, Woisetschlager M, Strobl H. Aryl Hydrocarbon Receptor Activation Inhibits In Vitro Differentiation of Human Monocytes and Langerhans Dendritic Cells. *J. Immunol.* 2009.
59. Lawrence BP, Denison MS, Novak H, Vorderstrasse BA, Harrer N, Neruda W, Reichel C, Woisetschlager M. Activation of the aryl hydrocarbon receptor is essential for mediating the anti-inflammatory effects of a novel low-molecular-weight compound. *Blood* 2008; 112: 1158-65.
60. Hwang JA, Lee JA, Cheong SW, Youn HJ, Park JH. Benzo(a)pyrene inhibits growth and functional differentiation of mouse bone marrow-derived dendritic cells. Downregulation of RelB and eIF3 p170 by benzo(a)pyrene. *Toxicol. Lett.* 2007; 169: 82-90.
61. Lee JA, Hwang JA, Sung HN, Jeon CH, Gill BC, Youn HJ, Park JH. 2,3,7,8-Tetrachlorodibenzo-p-dioxin modulates functional differentiation of mouse bone marrow-derived dendritic cells Downregulation of RelB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Lett.* 2007; 173: 31-40.
62. Vogel CF, Goth SR, Dong B, Pessah IN, Matsumura F. Aryl hydrocarbon receptor signaling mediates expression of indoleamine 2,3-dioxygenase. *Biochem. Biophys. Res. Commun.* 2008; 375: 331-5.
63. Vogel CF, Sciullo E, Li W, Wong P, Lazennec G, Matsumura F. RelB, a new partner of aryl hydrocarbon receptor-mediated transcription. *Mol. Endocrinol.* 2007; 21: 2941-55.

64. Lind EF, Ahonen CL, Wasiuk A, Kosaka Y, Becher B, Bennett KA, Noelle RJ. Dendritic cells require the NF-kappaB2 pathway for cross-presentation of soluble antigens. *J. Immunol.* 2008; 181: 354-63.
65. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 2002; 196: 1645-51.
66. Robays LJ, Maes T, Joos GF, Vermaelen KY. Between a cough and a wheeze: dendritic cells at the nexus of tobacco smoke-induced allergic airway sensitization. *Mucosal. Immunol.* 2009; 2: 206-19.
67. Tsoumakidou M, Demedts IK, Brusselle GG, Jeffery PK. Dendritic cells in chronic obstructive pulmonary disease: new players in an old game. *Am. J. Respir. Crit Care Med.* 2008; 177: 1180-6.
68. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, Green L, Hacken-Bitar J, Huh J, Bakaeen F, Coxson HO, Cogswell S, Storness-Bliss C, Corry DB, Kheradmand F. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat. Med.* 2007; 13: 567-9.

PART IV: GENERAL DISCUSSION

CHAPTER 8: DISCUSSION AND FUTURE PERSPECTIVES

8

Over the last decades, the incidence of asthma and chronic obstructive pulmonary disease (COPD) has increased continuously, especially in the industrialized Western countries. Although genetic predisposition is a fundamental factor underlying both diseases, the observed increase in prevalence has occurred too rapidly to be explained by genetic variations alone, implicating a role for environmental factors [1].

To unravel the impact of environmental insults on the induction, progression or aggravation of asthma and COPD, we moved towards a new generation of animal models, with combined exposures to natural and man-made environmental stimuli.

In the first part of the thesis we focused on allergic asthma. Epidemiological studies have demonstrated that cigarette smoke (CS) exposure is a considerable risk factor in the development or aggravation of asthma, predominantly early in life [2]. To better understand the impact of CS exposure on immunological responses towards allergens, ***Chapter 6.1, 6.2*** and ***6.3*** of the thesis focused on the role of CS as a risk factor for allergic sensitization and asthma development in mice.

In ***Chapter 6.1*** we studied the impact of CS inhalation on key aspects of airway dendritic cell (DC) biology. Airway DCs are generally accepted to drive mucosal sensitization towards inhaled aeroallergens [3]. *In vivo* depletion of lung $CD11c^+$ DCs abrogates Th2 sensitization in response to allergen exposure [4], whereas transfer of allergen-primed DCs into naïve mice induces Th2 immunity and features of asthma upon allergen rechallenge [5]. Using the previously established mouse model of Moerloose *et al*. – in which CS breaks inhalation tolerance to aerosolized ovalbumin (OVA) [6] – we demonstrated increased recruitment and activation of airway DCs in mice concomitantly exposed to OVA and CS. Moreover, prolonged CS inhalation markedly amplified the DC-mediated transport of OVA to the draining lymph nodes, probably in response to CS-induced up-regulation of the homing receptor CCR7 on airway DCs [7]. Although our data are consistent with previous results in CS-exposed mice [7-10], some groups rather suggest impaired DC maturation and migration in response to CS inhalation [11,12]. This discrepancy may be explained due to dose-dependent adjuvant or anti-inflammatory properties of CS [13,14] and differences in exposure protocol. To explain the mechanistic pathway through which CS affects DC biology and disrupts normal tolerogenic immunity against OVA, we hypothesized a role for the contaminating endotoxin (or LPS) content in CS [15]. Eisenbarth *et al.* reported that low doses of endotoxin, in

combination with OVA, are sufficient to break inhalation tolerance in a TLR4-MyD88 dependent way [16,17]. Unexpectedly, we showed Th2-oriented immunity in both TLR4 deficient mice and MyD88 knockout mice upon concomitant OVA/CS exposure, suggesting that other pathways are responsible for the enhanced sensitization towards OVA antigens.

In contrast to the "surrogate" allergen OVA, most clinically relevant allergens (e.g. house dust mite (HDM), ragweed or animal dander) possess intrinsic proteolytic adjuvant activity, directly activating DCs or epithelial cells to break inhalation tolerance and promote Th2 immunity [18]. Biochemical and immunogenic differences between real-life allergens and OVA may have a profound impact on the nature of the allergen exposure and may even affect the mechanisms behind the elicited allergic response. Moreover, as the stimulatory effect of CS on asthma development probably results from a fine interplay between CS compounds, environmental allergens, and innate and adaptive immune cells, the use of real-life allergens (e.g. HDM) to obtain relevant mechanistic insights, is therefore crucial.

In ***Chapter 6.2***, we established a clinically relevant mouse model, using low doses of HDM as real-life allergen, together with CS as indoor pollutant. In conformity with epidemiological data, we provided biological proof that CS indeed favours HDM-driven asthma development, as illustrated by massive inflammatory cell recruitment, elevated Th2 cytokine production and increased airway hyperresponsiveness. In addition, we demonstrated that CS inhalation during the sensitization phase is sufficient to induce asthma development in mice. We found that only a few days of CS inhalation during the initial allergen contact already enhanced DC recruitment, activation and HDM-driven DC migration to the lymph nodes, supporting the local induction of HDM-specific Th2 immunity.
From a medical point of view, these findings are of great clinical relevance. As allergic sensitization mainly occurs during early life, young children may become more vulnerable for future asthma development due to (short) CS inhalation.

As HDM is a complex mixture of various proteolytic enzymes, together with a number of non-protein compounds (endotoxins, β-glucans, chitin, …), these elements may act synergistically, or at least in combination with CS constituents (LPS, aluminium, ...), to modulate the disease phenotype. In ***Chapter 6.3*** we obtained preliminary mechanistic insights regarding

the role of CS during asthma development. HDM allergens have been reported to posses auto-adjuvant activity, in a protease dependent (e.g. *Der p 1*) [19] or independent [20] way and together with CS [21,22], these allergens may act synergistically to further decrease the epithelial barrier function and to increase transepithelial delivery to antigen-presenting cells. In our model of CS-induced facilitated early Th2 sensitization, we observed no significant difference in E-cadherin expression – a marker of epithelial integrity – between all 4 groups, although the individual values of the PBS/air control mice seemed to be higher. However, combined exposure to HDM and CS did result in elevated levels of the endogenous danger signal uric acid (UA) compared to HDM or CS alone. UA may act as a Th2 prone adjuvant [23] and may be released upon tissue injury [24], oxidative stress [25] or alum [26], which is present a high concentrations in CS [27]. Furthermore, UA may amplify the production of innate pro-Th2 cytokines, such as IL-1β [23]. In fact, acute HDM/CS exposure for 3 days induced additionally more IL-1β compared to control mice, suggesting a putative role for the IL-1β – IL1RI signalling pathway during facilitated allergic sensitization upon CS inhalation. Using IL1RI knockout mice, we confirmed the IL1RI pathway to be necessary to prime local Th2 immunity in the draining lymph nodes. To our knowledge, only one study already demonstrated the role of the IL1RI signalling during allergic sensitization, however in the absence of any environmental pollutant [28].

Moreover, many HDM components possess Toll-like receptor (TLR) agonist activity [29-31]. HDM can induce the release of endogenous danger signals or pro-allergic cytokines from airway epithelial cells, in a TLR4-dependent way [23,28,29,32]. Although the role of TLR4 in our combination model has not been investigated yet, prolonged HDM/CS exposure further increased UA and IL-1β, as well as IL-25 and IL-33, illustrating the immunostimulatory effect of CS on airway epithelial cells. ***Figure 14*** summarizes our findings from the HDM/CS exposure model and illustrates potential mechanisms.

Albeit our findings suggest a role for CS as an adjuvant during allergic sensitization and subsequent asthma development, additional experiments are needed to further unravel the mechanisms behind the facilitated allergic response. To further elucidate the role of the ***airway epithelium***, we should focus on the expression profile of other tight junction and adherens junction molecules. Together with UA, the role of other danger molecules (e.g. ATP, hsp70, S100 proteins) and innate pro-allergic cytokines can be unravelled, using

degradation enzymes, neutralization antibodies or knockout mice. Moreover, it may be interesting to analyse the long-term effect of HDM/CS exposure on the induction of epithelial-to-mesenchymal (EMT) transition and the development of airway wall remodeling. ***Mast cells*** are key effector cells of allergic inflammation, located in close proximity to sensory ***nerves*** [33]. Within this regard, the role of mast cells and their mediators, neurogenic inflammation and the involvement of transient receptor potential channels may be interesting to examine in HDM/CS exposed mice. Our model also offers the opportunity to investigate the role of epigenetic changes (***miRNA expression*** [34], ***histone modifications*** and ***DNA-methylation*** [35]), during CS-induced asthma development. As oxidative stress originating from HDM or CS, may compromise the protein folding capacity of the endoplasmic reticulum (ER) [36], it may be significant to unravel the role of ***ER stress*** during enhanced CS-induced HDM sensitization. Finally, it will be interesting to examine whether ***promising asthma genes***, identified from genome wide association studies (GWAS) (e.g. *ORMDL3* [37], *TLSP* [38], *PCDH1* [39]) are upregulated in our mouse model, as well as to examine their role in asthma development. Conversely, we need to check whether data obtained from mice can be confirmed in patients with allergic asthma.

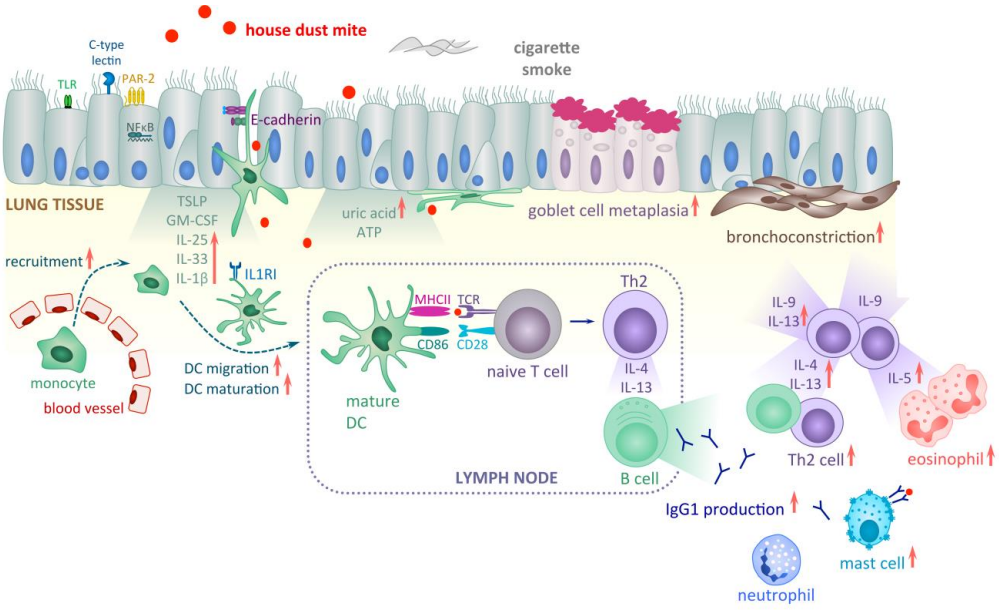


Figure 14: *Summary of the immunostimulatory effect of CS on house dust mite-driven allergic inflammation and an illustration of potential mechanisms (e.g. E-cadherin, uric acid, IL-1β – IL1RI pathway).*
Figure by Lanckacker EA

In the second part of the thesis, we focused on environmental aggravating factors in the pathogenesis of smoking-related diseases, such as COPD. More specifically, in ***Chapter 6.4*** we investigated the disease-modifying role of bacterial superantigens. *Staphylococcus aureus* (*S. aureus*) has been identified as one of the main pathogenic bacteria, capable of producing a variety of bacterial toxins with superantigenic capacity. Although bacterial *S. aureus* is often associated with asymptomatic colonization of the respiratory tract, repeated CS exposure may compromise the mucosal barrier function, thus providing the opportunity for the pathogen to break through. Moreover, the discovery of specific IgE antibodies directed against *Staphylococcus aureus* (*S. aureus*) enterotoxins in patients with COPD [40], provides indications that these antigens may act as potential aggravating factors of COPD pathophysiology. In order to identify whether *S. aureus* superantigens may alter the pulmonary inflammatory response upon CS, we designed a novel mouse model of concomitant *S. aureus* enterotoxin B (SEB) and CS inhalation. Interestingly, simultaneous exposure to both stimuli resulted in a significant aggravation of hallmark features of CS-induced pulmonary inflammation, such as a marked increase in $CD8^+$ T lymphocytes and neutrophils, associated with the increased release of IL-17A, and not KC, as neutrophil-attracting chemokine. Furthermore, combined SEB/CS exposure enhanced goblet cell hyperplasia, IL-13 mRNA expression and the formation of dense lymphoid aggregates in the lung, in an experimental protocol of 4 weeks. In COPD patients, the presence of pulmonary lymphoid follicles correlates with the progression and severity of the disease [41]. Similarly, using the standard murine COPD model within our lab, lymphoid follicle formation occurs after a chronic CS exposure period for 24 weeks [42]. The observed increase in lymphocytes and their organization into follicles after only 4 weeks of concomitant SEB/CS exposure, illustrates the disease-aggravating role of SEB in COPD pathology. SEB exposure also enhanced tissue-local production of IgM and protective mucosal IgA, which may offer local protection against the invading pathogens.

As we postulated to design clinically relevant mouse models, using complex environmental stimuli, the use of SEB within our combination model, instead of *S. aureus* itself, may be considered as a weakness of the study. However, because of its proven relevance in other respiratory diseases, the use of SEB solely may be justified [43,44]. Another limitation of the study may be the fact that SEB on itself already induced stimulatory effects, probably in a dose-dependent way.

Little is known about the mechanisms underlying the aggravating effect of SEB on immune activation and further research is needed to obtain mechanistic insights. Considering the stimulatory effect of SEB, it may be interesting to ***down-titrate the SEB dosage***, in order to further emphasize the immunomodulatory effect of SEB in combination with CS-induced inflammation. It may be interesting to focus on the ***airway epithelium*** and to identify pathogen recognition receptors on epithelial cells, DCs or other innate and adaptive immune cells which may be activated upon SEB and CS constituents. Moreover, the role of danger molecules and innate cytokines in response to SEB/CS exposure deserves further attention. As the enhanced production of lymphoid chemokines CXCL13 and CCL19 upon CS exposure are lymphotoxin ***(LT)$\alpha_1\beta_2$-LTβR dependent***, the role of this pathway may be significant in our model [45]. Finally, ***chronic SEB/CS exposure*** for 24 weeks may reveal additional information on the immunostimulatory effect of SEB on key aspects of COPD pathophysiology, such as emphysema or lung function.

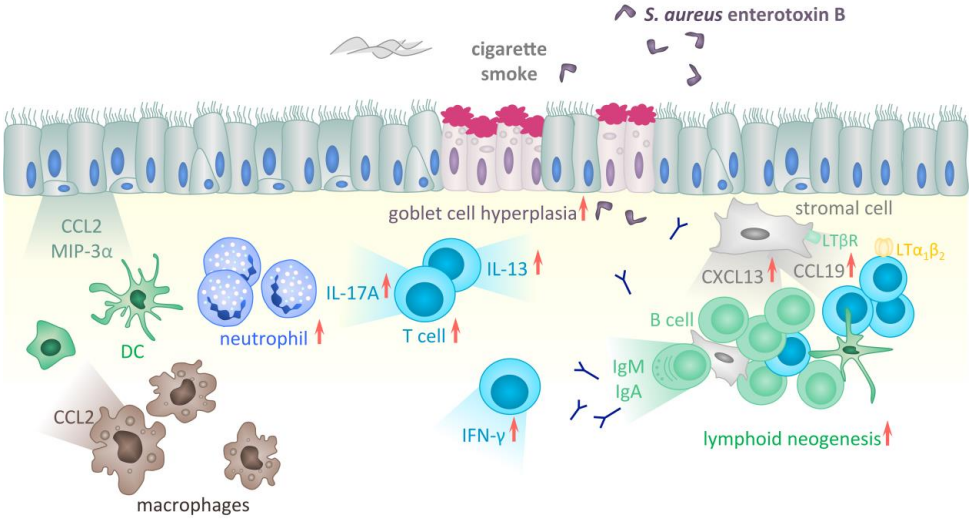


Figure 15: *Summary of the disease-modifying role of S. aureus enterotoxin B on CS-induced pulmonary inflammation.* *Figure by Lanckacker EA*

As a general conclusion, preclinical mouse models may be very useful to provide biological mechanistic data, supporting the hypothesis that environmental factors may be responsible for the increased prevalence and aggravation of asthma and COPD.

Reference List

1. von Mutius E. Gene-environment interactions in asthma. *J. Allergy Clin. Immunol.* 2009; 123: 3-11.
2. Pietinalho A, Pelkonen A, Rytila P. Linkage between smoking and asthma. *Allergy* 2009; 64: 1722-7.
3. Lambrecht BN, Hammad H. Biology of lung dendritic cells at the origin of asthma. *Immunity.* 2009; 31: 412-24.
4. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J. Exp. Med.* 2005; 201: 981-91.
5. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, Muskens F, Lambrecht BN. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 2010; 207: 2097-111.
6. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res.* 2006; 7: 49.
7. Demoor T, Bracke KR, Vermaelen KY, Dupont L, Joos GF, Brusselle GG. CCR7 modulates pulmonary and lymph node inflammatory responses in cigarette smoke-exposed mice. *J. Immunol.* 2009; 183: 8186-94.
8. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005; 26: 204-13.
9. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA, Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int. Arch. Allergy Immunol.* 2006; 141: 354-68.
10. Botelho FM, Gaschler GJ, Kianpour S, Zavitz CC, Trimble NJ, Nikota JK, Bauer CM, Stampfli MR. Innate immune processes are sufficient for driving cigarette smoke-induced inflammation in mice. *Am. J. Respir. Cell Mol. Biol.* 2010; 42: 394-403.
11. Robbins CS, Franco F, Mouded M, Cernadas M, Shapiro SD. Cigarette smoke exposure impairs dendritic cell maturation and T cell proliferation in thoracic lymph nodes of mice. *J. Immunol.* 2008; 180: 6623-8.
12. Robbins CS, Pouladi MA, Fattouh R, Dawe DE, Vujicic N, Richards CD, Jordana M, Inman MD, Stampfli MR. Mainstream cigarette smoke exposure attenuates airway immune inflammatory responses to surrogate and common environmental allergens in mice, despite evidence of increased systemic sensitization. *J Immunol.* 2005; 175: 2834-42.
13. Trimble NJ, Botelho FM, Bauer CM, Fattouh R, Stampfli MR. Adjuvant and anti-inflammatory properties of cigarette smoke in murine allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 2009; 40: 38-46.
14. Thatcher TH, Benson RP, Phipps RP, Sime PJ. High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function. *Am. J. Physiol Lung Cell Mol. Physiol* 2008; 295: L412-L421.
15. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999; 115: 829-35.

16. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* 2002; 196: 1645-51.
17. Piggott DA, Eisenbarth SC, Xu L, Constant SL, Huleatt JW, Herrick CA, Bottomly K. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *J. Clin. Invest* 2005; 115: 459-67.
18. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 2008; 8: 193-204.
19. Wan H, Winton HL, Soeller C, Tovey ER, Gruenert DC, Thompson PJ, Stewart GA, Taylor GW, Garrod DR, Cannell MB, Robinson C. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J. Clin. Invest* 1999; 104: 123-33.
20. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, Heijink IH. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax* 2011.
21. Heijink IH, Brandenburg SM, Postma DS, van Oosterhout AJ. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. *Eur. Respir. J.* 2012; 39: 419-28.
22. Olivera DS, Boggs SE, Beenhouwer C, Aden J, Knall C. Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro. *Inhal. Toxicol.* 2007; 19: 13-22.
23. Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, Rogers N, Osorio F, Reis e Sousa, Hammad H, Lambrecht BN. An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity.* 2011; 34: 527-40.
24. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* 2003; 425: 516-21.
25. Vorbach C, Harrison R, Capecchi MR. Xanthine oxidoreductase is central to the evolution and function of the innate immune system. *Trends Immunol.* 2003; 24: 512-7.
26. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, Hoogsteden HC, Hammad H, Lambrecht BN. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J. Exp. Med.* 2008; 205: 869-82.
27. Exley C, Begum A, Woolley MP, Bloor RN. Aluminum in tobacco and cannabis and smoking-related disease. *Am. J. Med.* 2006; 119: 276-11.
28. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* 2012; 209: 1505-17.
29. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15: 410-6.
30. Mueller GA, Edwards LL, Aloor JJ, Fessler MB, Glesner J, Pomes A, Chapman MD, London RE, Pedersen LC. The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins. *J Allergy Clin. Immunol.* 2010; 125: 909-17.
31. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, Karp CL. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009; 457: 585-8.

32. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J. Immunol.* 2004; 173: 6384-92.
33. Bienenstock J, MacQueen G, Sestini P, Marshall JS, Stead RH, Perdue MH. Mast cell/nerve interactions in vitro and in vivo. *Am. Rev. Respir. Dis.* 1991; 143: S55-S58.
34. Turner ML, Schnorfeil FM, Brocker T. MicroRNAs regulate dendritic cell differentiation and function. *J. Immunol.* 2011; 187: 3911-7.
35. Kabesch M, Adcock IM. Epigenetics in asthma and COPD. *Biochimie* 2012; 94: 2231-41.
36. Todd DJ, Lee AH, Glimcher LH. The endoplasmic reticulum stress response in immunity and autoimmunity. *Nat. Rev. Immunol.* 2008; 8: 663-74.
37. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, Depner M, von Berg A, Bufe A, Rietschel E, Heinzmann A, Simma B, Frischer T, Willis-Owen SA, Wong KC, Illig T, Vogelberg C, Weiland SK, von Mutius E, Abecasis GR, Farrall M, Gut IG, Lathrop GM, Cookson WO. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* 2007; 448: 470-3.
38. Harada M, Hirota T, Jodo AI, Hitomi Y, Sakashita M, Tsunoda T, Miyagawa T, Doi S, Kameda M, Fujita K, Miyatake A, Enomoto T, Noguchi E, Masuko H, Sakamoto T, Hizawa N, Suzuki Y, Yoshihara S, Adachi M, Ebisawa M, Saito H, Matsumoto K, Nakajima T, Mathias RA, Rafaels N, Barnes KC, Himes BE, Duan QL, Tantisira KG, Weiss ST, Nakamura Y, Ziegler SF, Tamari M. Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 2011; 44: 787-93.
39. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ, Xu J, Koning H, Bruinenberg M, Nolte IM, van Diemen CC, Boezen HM, Timens W, Whittaker PA, Stine OC, Barton SJ, Holloway JW, Holgate ST, Graves PE, Martinez FD, van Oosterhout AJ, Bleecker ER, Postma DS. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. *Am. J. Respir. Crit Care Med.* 2009; 180: 929-35.
40. Rohde G, Gevaert P, Holtappels G, Borg I, Wiethege A, Arinir U, Schultze-Werninghaus G, Bachert C. Increased IgE-antibodies to Staphylococcus aureus enterotoxins in patients with COPD. *Respir. Med.* 2004; 98: 858-64.
41. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N. Engl. J. Med.* 2004; 350: 2645-53.
42. D'hulst AI, Maes T, Bracke KR, Demedts IK, Tournoy KG, Joos GF, Brusselle GG. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respir. Res.* 2005; 6: 147.
43. Huvenne W, Callebaut I, Plantinga M, Vanoirbeek JA, Krysko O, Bullens DM, Gevaert P, van CP, Lambrecht BN, Ceuppens JL, Bachert C, Hellings PW. Staphylococcus aureus enterotoxin B facilitates allergic sensitization in experimental asthma. *Clin. Exp. Allergy* 2010; 40: 1079-90.
44. Hellings PW, Hens G, Meyts I, Bullens D, Vanoirbeek J, Gevaert P, Jorissen M, Ceuppens JL, Bachert C. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to Staphylococcus aureus enterotoxin B. *Clin. Exp. Allergy* 2006; 36: 1063-71.

45. Demoor T, Bracke KR, Maes T, Vandooren B, Elewaut D, Pilette C, Joos GF, Brusselle GG. Role of lymphotoxin-alpha in cigarette smoke-induced inflammation and lymphoid neogenesis. *Eur. Respir. J.* 2009; 34: 405-16.

Dankwoord

Vooraleer ik dit doctoraat aanvatte, wist ik niet wat een rollercoaster dit zou worden. Zonder deze steunpilaren was het me nooit gelukt! Ik wil dan ook enkele onder hen uitgebreid bedanken.

Vooreerst wil ik mijn promotor Professor Guy Joos hartelijk bedanken voor de uitzonderlijke kans en het vertrouwen bij het toewijzen van dit doctoraatsproject. Uw eeuwige optimisme en wetenschappelijke visie werkten aanstekelijk en wanneer de nood het hoogst was, kon ik altijd op uw steun rekenen! Bedankt voor deze mooie ervaring! U bent een fantastisch persoon, een voorbeeld voor velen! Ook Prof. Tournoy verdient een eervolle vermelding. Kurt, met jouw gedrevenheid en positiviteit hielp je me onvoorwaardelijk door de moeilijkste periode van mijn doctoraat. Hiervoor ben ik jou ontzettend dankbaar!

Beste Tania, co-promotor en post-doc. Niet alleen leidde je het labo in goede banen, ook ben jij de drijvende kracht achter dit doctoraat. Kritisch als je bent, las je alles nauwgezet na en gaf je de nodige commentaar. Ik heb veel van jou geleerd, meer dan je denkt! Veel succes nog in je verdere loopbaan, ze zal je zeker nog veel moois brengen!

Hierbij wil ik ook onze tweede post-doc, Ken, bedanken. Nadat we ERS in Amsterdam en Wenen samen onveilig maakten, kan ik jou ondertussen mijn ERS-maatje noemen. Dank je wel voor de onvergetelijke ervaring van de ‘ERS-speakers dinner’. Verder kon ik jou steeds lastig vallen met mijn niet-al-te-slimme pc-probleempjes en andere wetenschappelijke kwesties.

Mijn collega’s, lieve Tine, Nele, Sharen, Leen, Lisa, Fien, Lies, Smitha, Griet, Katrien, Costas en Chris. Heel hartelijk bedankt voor jullie morele support. Steeds kon ik bij jullie terecht voor een theetje, een toffe babbel, een lach en een traan. Dank je wel voor jullie interesse op wetenschappelijk en sociaal vlak. Tine, met jou de bureau delen heeft mij echt goed gedaan. We zaten vaak op dezelfde golflengte en begrepen elkaar als geen ander. Leen, hoewel je de nieuwste aanwinst bent, fleurde je de bureau al aardig op met je enthousiaste en frisse wind. Lisa, dank je wel dat ik ook in de eindspurt naar mijn verdediging steeds op

jou kon rekenen. Lies, ontzettend bedankt voor de leuke momenten in San Fransisco! Met jou een kamer delen, maakte dit congres pas echt compleet! Nog veel succes met jullie verdere loopbaan! Wat ga ik jullie missen!

Uiteraard wil ik ons schitterend team laboranten uitvoerig bedanken voor wat jullie stuk voor stuk hebben bijgedragen aan mijn doctoraat. Eliane, Christelle, Evelyn, Greet, Ann, Indra, Katleen, Anouck, Marie-Rose en buurman Philippe, even enthousiast als jullie zijn, maakten jullie me elke dag opnieuw wegwijs in de wondere wereld van de wetenschap en leerden me de kneepjes van het vak. De mooie resultaten die ik behaalde, zijn mede dankzij jullie professionalisme en toewijding. Jullie zijn stuk voor stuk prachtige mensen wie ik nooit zal vergeten. Het ga jullie goed!

Een welgemeende dank aan Prof. Lambrecht en Hamida voor de boeiende samenwerking. Bedankt Karim voor je bijzondere wetenschappelijke inbreng en waardering voor mijn werk. Prof. Brusselle, dank u voor uw interesse en opmerkingen tijdens de wekelijkse research staffs. Bedankt ook aan de overige stafleden en de medewerkers van het secretariaat. Een welgemeende dank aan Hilde, Bart, Annie en Chantal voor de uitstekende administratieve ondersteuning uit K12, alsook aan de mensen van het centraal animalarium voor de goede verzorging van de proefdieren.

In het bijzonder wil ik ook mijn vrienden Astrid, Carine en Dirk, Ann, Catherine en Rik bedanken die er reeds van bij het prille begin van mijn doctoraat bij waren en telkens voor mij klaar stonden op de meest onmogelijke uren. Bedankt voor jullie trouwe vriendschap, jullie interesse in mijn onderzoek, de fijne babbels, aangename etentjes en toffe ‘Rik-jokes’.

Een speciale dank gaat uit naar mijn lieve mama en papa. Het doet deugd te weten dat jullie er ALTIJD voor mij zijn, met jullie grenzeloze vertrouwen en onvoorwaardelijke steun. Jullie weten als geen ander hoe hard ik heb moeten werken om hier te kunnen staan. Dank je wel lieve mama voor al je telefoontjes, je goeie raad en je overredingskracht om me telkens opnieuw te overtuigen dat alles wel goed komt. Ik kan jullie niet genoeg bedanken voor ALLES!!!

Tot slot, liefste Bart, mijn steun en toeverlaat in mooie en moeilijke tijden. Dank je wel liefje voor jouw enorme geloof in mijn kunnen!! Even enthousiast als je bent, ben je er telkens om mij aan te moedigen en geef je me de nodige energie om door te zetten en te volharden in mijn kunnen. Bedankt om te zijn wie je bent! Mijn doctoraatsthesis heeft veel van mij gevraagd en ook dit begreep jij als geen ander.

En natuurlijk … onze bollies Charlie, Lumi en Foxy. Jullie stonden me elke dag weer even enthousiast op te wachten met een welkome miauw en toepasselijke cadeautjes ;-)

Hartelijk dank aan iedereen!
Ellen, april 2013

Curriculum Vitae

Personal details

Home
Ommegangstraat 51
B-9080 Lochristi
E-mail: ellen.lanckacker@yahoo.com
Phone: +32 (0) 472 32 53 47
Date, place of birth: Februari 4th 1982, Ghent
Nationality: Belgian

Office
Department of Respiratory Medicine
Ghent University Hospital
Heymansinstitute – Blok B, 4th floor
De Pintelaan 185
B-9000 Ghent
Phone: +32 (0) 9 332 59 68

Education – Work experience

2007- today: **PhD student in Medical Sciences**
Department of Respiratory Medicine, Ghent University Hospital
Supervisors: Prof. Dr. Guy Joos, Dr. Tania Maes

2005-2007: **Biomedical and Clinical Engineering**
Ghent University, Faculty of Engineering

2000-2005: **Master of Biomedical Sciences**, speciality Regenerative Medicine
Ghent University, Faculty of Life Sciences and Medicine

1994-2000 : **Mathematic and Sciences**
O.L.V. Visitatie, Sint-Amandsberg

Additional courses

2012: **Academisch Engels**: writing skills (Universiteit Gent, UCT)

2011: **SPSS basic course** (Ghent University)

2007-2008: **Master in Laboratory Animal Science**, FELASA category C (Experiment Leader)
Ghent University, Faculty of Veterinary Medicine

2006: **English level 5** (Ghent University, UCT)

List of International Peer - Reviewed Publications

Lanckacker EA, Tournoy KG, Holtappels G, Hammad H, Lambrecht BN, Joos GF, Maes T.
Short cigarette smoke exposure facilitates allergic sensitization and subsequent asthma development in mice. Eur Respir J. 2012 Aug 16. [E-pub ahead of print]

Huvenne W*, **Lanckacker EA***, Krysko O, Bracke KR, Demoor T, Hellings PW, Brusselle GG, Joos GF, Bachert C, Maes T. (*equal contribution)
Exacerbation of cigarette smoke-induced pulmonary inflammation by Staphylococcus aureus enterotoxin B in mice. Respir Res. 2011 May 27;12:69.

Robays LJ*, **Lanckacker EA***, Moerloose KB, Maes T, Bracke KR, Brusselle GG, Joos GF, Vermaelen KY. (*equal contribution)
Concomitant inhalation of cigarette smoke and aerosolized protein activates airway dendritic cells and induces allergic airway inflammation in a TLR-independent way. J Immunol. 2009 Aug 15;183(4):2758-66.

Lanckacker EA, Robays LJ, Joos GF, Vermaelen KY.
A new danger in the air: how pulmonary innate immunity copes with man-made airborne xenobiotics. J Innate Immun. 2010;2(2):96-106. (Review)

Maes T, Provoost S, **Lanckacker EA**, Cataldo DD, Vanoirbeek JA, Nemery B, Tournoy KG, Joos GF.
Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. Respir Res. 2010 Jan 21;11:7 (Review)

Vander Beken S, Al Dulayymi JR, Naessens T, Koza G, Maza-Iglesias M, Rowles R, Theunissen C, De Medts J, **Lanckacker E**, Baird MS, Grooten
Molecular structure of the Mycobacterium tuberculosis virulence factor, mycolic acid, determines the elicited inflammatory pattern. Eur J Immunol. 2011 Feb;41(2):450-60.

List of National and International Presentations

Belgian Thoracic Society, GSK Awards in Pneumology – Brussels (2009) *(oral presentation*)
Lungstorming – Aix-en-Provence (2010) *(oral presentation in French*)
Belgian Thoracic Society, GSK Awards in Pneumology – Brussels (2010) *(oral presentation*)
American Thoracic Society – New Orleans (2010) *(poster presentation*)
European Respiratory Society – Barcelona (2010) *(poster presentation*)
Belgian Thoracic Society, GSK Awards in Pneumology – Brussels (2011) *(oral presentation*)
Wetenschapsdag – Ghent (2011) *(poster presentation*)
American Thoracic Society – San Francisco (2012) *(poster presentation*)
European Respiratory Society – Vienna (2012) *(oral presentation*)

Awards/grants

Belgian Thoracic Society – GSK Basic Science Award in Pneumology (2011)
European Respiratory Society Annual Inflammatory Airway Diseases and Clinical Allergy Grant (2012)