KU Leuven
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INFLAMMATORY PHENOTYPES OF CHEMICAL-INDUCED ASTHMA IN MICE

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Dissertation presented in partial fulfillment of the requirements for the degree of

Doctor in Biomedical Sciences

DANKWOORD

Graag wil ik Prof. Luc Sels, rector van de Katholieke Universiteit Leuven, Prof. Chris van Geet, vice-rector en Prof. Paul Herijgers, decaan van de Faculteit Geneeskunde bedanken om mij de kans te geven een doctoraat voor te bereiden aan deze universiteit.

Graag wil ik ook de Interuniversitaire attractiepolen (IUAP, P7/30) bedanken voor de financiële steun gedurende mijn doctoraatsperiode.

I am also grateful to the members of the jury, Prof. Lieven Dupont, Prof. Rik Schrijvers, Prof. Didier Cataldo and Prof. Armin Braun, to the chair of the examination committee, Prof. Robin Lemmens and to the chair of the public defense, Prof. Geert Verbeke for their constructive comments on this dissertation and their presence during the public defense. Their valuable suggestions have remarkably improved the content of my manuscript. I appreciate their expertise and interest in my work.

Mijn promotor, Prof. Jeroen Vanoirbeek en co-promotor, Prof. Peter Hoet en hoofd van het labo Prof. Ben Nemery wil ik graag bedanken om mij de kans te geven om een doctoraatsthesis te starten binnen de onderzoeksgroep 'Omgeving en Gezondheid'. Jeroen, bedankt voor al het vertrouwen en de nodige ondersteuning de afgelopen 4 jaar. Je slaagde erin om naast het harde werk een toffe sfeer te creëren waarin je altijd beschikbaar was om mij bij te staan. Peter, ook jou wil ik bedanken voor al jouw hulp. Je stond altijd klaar om experimenten te bespreken en mee te zoeken naar de juiste oplossingen. Hiernaast stond jouw deur ook altijd letterlijk open om mij bij te staan bij praktische/technische problemen in het labo. Ben, ook u wil ik bedanken om tijd vrij te maken om mijn werk na te lezen. Uw expertise heeft dit werk naar een hoger niveau getild. Bedankt voor alle suggesties en ideeën.

Verder wil ik Prof. Tania Maes en Dr. Sharen Provoost van de Dienst Longziekten in Gent bedanken om de nodige kennis door te geven en mij bij te staan tijdens het optimaliseren van het panel van de dendritische cellen. Jullie expertise heeft dit werk zeker en vast vooruitgeholpen.

Sven Seys en Jonathan Cremer ook jullie zijn bedankt om mij van het begin bij te staan met het optimaliseren van het panel van de dendritische cellen. Sven, bedankt om vol enthousiasme deze uitdaging aan te nemen en mee te zoeken naar de nodige oplossingen. Jonathan, zonder jouw FACS-expertise was dit panel nooit tot een goed einde gekomen, bedankt voor al jouw hulp! Ook wil ik Prof. Erik Verbeken bedanken voor het maken en het scoren van de histologische coupes in dit werk.

Zeker en vast wil ik ook een speciaal dankwoordje plaatsen voor het voltallige astma-team. Fien en Vanessa, bedankt om mij vanaf mijn thesisjaar onder jullie vleugels te nemen. Fien, dankjewel om mij de nodige ondersteuning te bieden gedurende al die jaren. Naast jouw praktische hulp en duizenden

Flexiventen zullen mij vooral de gezellige babbels en leuke momenten die we hebben meegemaakt bijblijven. Je bent ondertussen een andere weg ingeslagen waarin ik jou nog heel veel succes in wil wensen. Vanessa, ondertussen ligt de tijd in het labo al ver achter jou, maar enorm bedankt om mij zo dicht te begeleiden gedurende mijn thesisjaar en mij te motiveren om aan dit doctoraat te beginnen.

Uiteraard wil ik ook een speciaal dankwoordje plaatsen voor al mijn collega's binnen het pneumoteam. Eerst en vooral wil ik Sofie en Tatjana bedanken voor al hun harde werk dat ze gedurende hun thesisjaar bij ons hebben verwezenlijkt. Sofie, mede dankzij jou is er al een mooi 'MI' artikel gepubliceerd en Tatjana mede dankzij jou gaan er zeker nog volgen. Sofie, ik heb jou pas echt goed leren kennen vanaf het congres in San Francisco, de leuke babbels tot 's morgens vroeg, de cheesecake in de Cheesecake factory, allemaal momenten om nooit te vergeten. Recent vormen wij samen met ons drie een gezellig bureautje waar wij altijd paraat staan om elkaar te helpen en naast het harde werk ook af en toe een gezellig babbeltje plaatsen wat zorgt voor de nodige ontspanning tussendoor. Onze tijd samen was maar kort, maar ik ga er wel altijd met leuke herinneringen naar kunnen terugblikken waarvoor veel dank! Hannelore…ook jij was zo goed als deel van onze bureau… de vele babbels, konijnenverhaaltjes en vooral ook de vele lachmomenten. Ik heb enorm veel steun gehad aan jou tijdens mijn doctoraat, maar ook daarbuiten…bedankt om er altijd voor mij te zijn. ELISEUH… jouw geweldige lach ga ik alvast nooit vergeten. Wij zijn samen onze thesis gestart in het labo en gaan nu ook samen ons doctoraat beëindigen. De vele babbels en lachmomenten zorgden er keer op keer voor dat ik de nodige moed vond om er weer tegenaan te gaan, waarvoor dank! Nog heel veel succes met het afronden van jouw doctoraat. Stephanie, ondertussen ben je een geweldige mama voor Suzie geworden. Bedankt voor de leuke gesprekken tussendoor. Ik wil je nog heel veel succes wensen in jouw doctoraat en in jouw verdere carrière als pneumoloog. En dan natuurlijk Sofie, al komen wij uit dezelfde stad en hebben wij samen op dezelfde school gezeten, jou heb ik pas leren kennen in het labo. Je bent op weg om een geweldige anesthesiste te worden en ik wil jou hierbij nog heel veel succes wensen. Uiteraard zijn er ook nog Jef en Annelore die ik wil bedanken voor de leuke babbels in het labo. Jef, u wil ik specifiek bedanken om mij te helpen bij mijn experimenten en uiteraard voor de traktaties met uw zelfgemaakte wijnen. Valerie, ook jou mag ik zeker niet in de lijst vergeten: jouw deur stond ook altijd letterlijk open…Bedankt om er altijd voor mij te zijn. Je zorgde er keer op keer voor dat ik de moed terug bijeenraapte om terug aan de slag te gaan! Ook wil ik Birger en Carolien bedanken om altijd naar mij te luisteren en mee naar oplossingen te zoeken. Jullie waren zeker en vast het 'fun' bureautje waar je altijd terecht kon voor de nodige steun, ontspanning (en voedsel)! Jullie hebben nog een paar jaartjes te gaan, maar ik ben ervan overtuigd dat jullie dit met succes zullen afronden!

Anke en Laurens, ook jullie wil ik bedanken voor de toffe babbels gedurende de korte tijd dat wij samen in dezelfde bureau zaten. Nog heel veel succes in jullie doctoraat! Kristina, ook jou wil ik bedanken voor

de leuke babbels voor de momenten dat je aanwezig was in het labo, je bent momenteel zelf aan je laatste jaar bezig en ik wil je hierbij nog heel veel succes wensen!

I also want to thank Deniz, Yang, Siva, Tom, Tobias, Neel, Manosij,Tracy, Marko, John, Stijn, Karen and Allard for the pleasant talks in the lab. Wish you all the best! Ook wil ik Hanne, Elly, Jana, An en Nele bedanken voor de leuke praatjes in het labo, jullie zijn ondertussen allemaal jullie eigen weg ingeslagen waarin ik jullie nog veel succes in wil wensen. Ook een dikke dankjewel aan Anita en Elien voor het praktische geregel en al de bestellingen.

Mama en papa, zonder jullie steun had ik hier nooit gestaan. Jullie hebben mij geleerd om nooit op te geven en er steeds voor te blijven gaan met het nodige relativeringsvermogen. Bedankt voor alles wat jullie voor mij gedaan hebben en bedankt om steeds in mij te blijven geloven. Lien, ook jou wil ik bedanken voor jouw steun tijdens mijn doctoraat en de vele telefoongesprekken. Je stond als grote zus altijd voor mij klaar. Max, ook jou wil ik bedanken voor de vele hartelijke verwelkomingen in Genk en de fijne, ontspannende wandelingen in het bos. Ook wil ik mijn schoonouders bedanken voor de steun tijdens de drukke periodes.

Lieve Bryan, ondertussen mag ik jou mijn verloofde noemen. Wij kennen elkaar al van onze masterjaren tijdens onze studies en zijn sindsdien onafscheidelijk geweest. Zelfs tijdens ons doctoraat hebben we het klaargespeeld om toch samen een klein projectje uit te voeren en heb ik de eer om mee op jouw paper te staan. Ik heb al zoveel aan jou te danken en kan mij geen leven meer zonder jou inbeelden. Jij hebt mij altijd door dik en dun gesteund, stond altijd voor mij klaar en gaf mij het nodige zelfvertrouwen. Mede door jou is dit doctoraat tot een goed einde gekomen, bedankt voor alles! Ik kijk al uit naar de volgende stap in ons leven.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACGIH: American conference of governmental industrial hygienists

AHR: Airway hyperreactivity

AOO: Acetone olive oil

APC: Antigen presenting cell

AUC: Area under the curve

BAL: Broncho-alveolar lavage

CCL: Chemokine ligand

CCR: Chemokine receptor

CD: Clusters of differentiation

CGRP: Calcitonin-gene related peptide

ConA: Concanavalin A.

Da: Dalton

DAMPs: Damage-associated molecular patterns

DC: dendritic cells

EAR: Early asthmatic response

Est: Static elastance

FEV: Forced expiratory volume

FEF: Forced expiratory flow

FOT: Forced oscillations technique

FVC: Forced vital capacity

G: Tissue damping/resistance

GM-CSF: Granulocyte-macrophage colony-stimulating factor

H: Tissue elasticity

HDI: Hexamethylene diisocyanate

HMW: High molecular weight

IC: Inspiratory capactity

IFN-γ: Interferon-gamma

Ig: Immunoglobulin

IIA: Irritant-induced asthma

IL: Interleukin

KO: Knock-out

LAR: Late asthmatic response

LLNA: Local lymph node assay

LMW: Low molecular weight

MCI: Methylchloroisothiazolinone

MDI: Methylene diphenyl diisocyanate

MHC: Major histocompability complex

MI: Methylisothiazolinone

MMPs: Matrix metalloproteinases

NANC: Non-adrenergic non-cholinergic

NK: Neurokinin

NK1R: Neurokinin 1 receptor

NIOSH: National Institute for Occupational Safety and Health

OA: Occupational asthma

OVA: Ovalbumin

PAMPs: Pathogen-associated molecular pattern

PEF: Peak expiratory flow

ppm: Parts per million

RADS: Reactive airways dysfunction syndrome

R_n: Airway resistance

SD: Standard deviation

SI: Stimulation index

SIC: Specific inhalation challenge

SP: Substance P

STEL: Short term exposure limit

TDI: Toluene diisocyanate

TGF-β: Transforming growth factor beta

Th1: T helper lymphocyte type 1

Th2: T helper lymphocyte type 2

TLV: Threshold limit value

TLR: Toll-like receptor

TRP: Transient receptor potential

TRPA1: Transient receptor potential ankyrin-1

TRPV1: Transient receptor potential vanilloid-1

TSLP: Thymic stromal lymphopoietin

Veh: Vehicle

WT: Wild type

Chapter 1

Introduction

1. Asthma

Asthma is defined as a heterogeneous chronic airway disease, characterized by chronic airway inflammation, variable remodeling, reversible airway obstruction and non-specific airway hyper reactivity (AHR) [1,2]. The respiratory symptoms include wheezing, shortness of breath, chest tightness and cough [2]. Often, these symptoms are accompanied by co-morbidities such as multi-organ allergies like allergic rhinitis, conjunctivitis, atopic dermatitis and food allergy. Furthermore it can be accompanied by non-allergic disorders such as obesity, gastro-oesophageal reflux and psychiatric conditions [2].

Worldwide, more than 300 million people are affected by asthma, making it one of the most common chronic diseases. Although the disease is recognized worldwide, the prevalence of asthma depends on geographic location. In developed western countries the prevalence of asthma is estimated at 10% of the total populations, while in low-income and rural countries it tends to be less than 1% [2].

1.1 Influence of genetic and environmental factors

The origin and clinical phenotype of asthma are driven by genetic and environmental factors making asthma a heterogeneous disease. People can be genetically predisposed to develop allergic reactions to substances that do not generally elicit immune responses. Genome-wide association studies (GWAS) revealed genes susceptible for asthma. Most of these genes are related to the epithelium and innate immune pathways including ARMDL3, IL-33 and SMAD3. Less commonly, genes are found associated with atopy and serum IgE such as HLA-DQ locus, FCER1A, STAT6 and IL-13 [3–5].

Environmental risk factors have a prominent role in the development and exacerbations of asthma. They also modulate the expression of asthma susceptibility genes, making asthma a dynamic disease [6]. Children sensitized to environmental allergens are more likely to experience persistent asthma later in life. During childhood, viral infections such as rhinovirus and respiratory syncytial virus (RSV) are associated with the development of asthma and are the most common cause of asthma exacerbations. Indoor and outdoor air pollution are important contributing factors to the development and exacerbation of asthma [2,7]. Exposure to tobacco smoke boosts the Th2 type response and worsens existing asthma, probably through an increase of neutrophilic inflammation and oxidative stress [8]. Occupational exposures can initiate asthma or aggravate existing asthma [8]. Furthermore, obesity during childhood is strongly associated with the incidence and severity of asthma. Maternal obesity and high gestational weight gain are associated with an increased risk of childhood asthma, especially in non-asthmatic mothers [2,9]. Symptoms of asthma can also be experienced after exercise, named exercise-induced asthma (EIA). Aspirin, an anti-inflammatory drug, is also an important trigger inducing asthma in some subjects, named aspirin-exacerbated asthma [10]. In addition, according to the hygiene

hypothesis, increasing use of antibiotics, improved hygiene, and urbanization result in a decreased exposure to infections and environmental microorganisms during childhood. This results in a less Th1 directed response to infections early in life, leading to a persistent imbalanced Th2 response and consequently asthma [6].

1.2 Phenotypes and endotypes of asthma

For years, asthma has been considered as a single disease. Recently, studies have focused on its heterogeneity as it is presented with diverse symptom profiles and variable responses to medications [11]. As already mentioned, the origin and severity of asthma are driven by genetic and environmental factors. All these factors result in different clinical outcomes also called phenotypes of asthma [12,13]. A phenotype is defined as "observable properties of an organism that are produced by the interactions of the genotype and the environment" [13,14]. Nowadays, it is recognized that asthma consists of multiple phenotypes. Phenotypes are described based on clinical characteristics such as the age of onset, severity (based on lung function, symptoms and exacerbations), inflammatory characteristics (predominantly eosinophilic, predominantly neutrophilic, paucigranulocytic, or mixed granulocytic), and response to standard and new therapies. Phenotypes can also be distinguished by differences in immunological mechanisms (Th1 or Th2 cell mediated response), biomarkers (blood/sputum levels of eosinophils, exhaled nitric oxide (FeNO), serum periostin and immunoglobulins) and imaging (Computed Tomography) [11,13–16]. Additionally, phenotypes may be defined based on the causal agent or the trigger for worsening of symptoms such as occupational exposure, air pollution, cigarette smoke, diesel exhaust particles, aspirin, obesity, or exercise [13,14,17,18]. Thus, Sally Wenzel grouped asthma phenotypes based on the distinction between T_h2-high asthma and non-T_h2 asthma (fig 1.1) [14]. Nevertheless, substantial overlap exists among the different phenotypes [13].

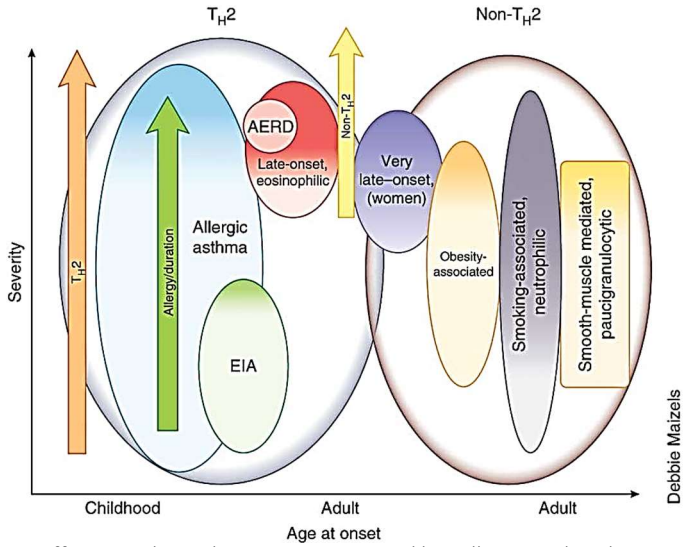


Fig. 1.1: Different asthma phenotypes suggested by Sally Wenzel[14]. The intensity of the colors represents the severity of the specific phenotype. The relative size of the subcircles represents the relative proportions of affected individuals.

To clarify and further distinguish different phenotypes, research has focused on the molecular pathways involved in the asthmatic response, thus leading to the notion of endotypes. An 'endotype' is defined as "a specific biological pathway which can explain the observable properties of a phenotype" [14]. Until now, there is no universal system to define asthma phenotypes, this results in researcher dependent categorizing of asthma patients [14].

2. Pathogenesis of allergic asthma

Traditionally, two forms of asthma have been distinguished in clinical practice: allergic and non-allergic asthma [14,16]. Most children and roughly 50% of adults have allergic asthma. These people are atopic (they have a genetic predisposition to develop immunoglobulin E (IgE)), have identifiable allergic triggers (serum IgE antibodies and/or a positive skin-prick test), other allergic diseases (such as rhinitis or eczema), or a family history of allergic disease [14,16]. This phenotype of asthma is characterized by humoral and T helper 2 cell (Th2) immune responses, resulting in infiltration of eosinophils, formation of allergen specific IgE antibodies and the release of Th2 cytokines.

Non-allergic asthma often develops later in life (after 40 years age) and often has a more severe clinical course [19]. This phenotype shows negative skin tests and has no evidence for specific IgE antibodies directed against common allergens, and has no clinical or family history of allergy [20].

2.1 Immune mechanisms

The pathogenesis of allergic asthma is the result of a complex interplay between innate and adaptive immunity. It comprise two phases, the induction (sensitization) phase and the elicitation (asthmatic) phase. The latter phase includes both early (EAR) and late (LAR) asthmatic reactions [21].

2.1.1 Close interplay between epithelial barrier and dendritic cells

Inhaled allergens, which passed the filtering of the upper airways, and escaped the mucociliary clearance, encounter the first line defense of the airways, namely the airway epithelial cells. Asthmatic epithelium has incomplete formation of tight junctions, which results in a defective barrier function. This can be the result of defective genes associated with the formation of the epithelium, but also proteolytic allergens (such as house dust mite, cockroach, animal and fungal) and environmental stimuli such as respiratory viruses and air pollutants (such as ozone, tobacco smoke and diesel particles). As a consequence, inhaled allergens can penetrate more easily into the airway tissue [22].

When exposed to allergens, epithelial cells express many pattern recognition receptors (PRRs), such as toll like receptors (TLR), to rapidly detect and respond to pathogen-associated molecular patterns (PAMPs) (present on microbes) to damage-associated molecular patterns (DAMPs) (released upon

tissue damage, cell death or cellular stress). Upon activation of PRR, epithelial cells release specific cytokines (thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-25 and IL-33), chemokines (chemokine ligand (CCL)2, CCL5, CCL20, CCL27, CCL19) and danger signals such as uric acid and ATP, that direct monocytes and immature dendritic cells (DC) from the bone marrow towards the epithelium of the lung (fig 1.2) [5,23–25]. Furthermore, IL-33, IL-25 and TSLP lead to an accumulation of type 2 innate lymphoid cells (ILC2) by acting on their receptors ST2, IL-17RB, and TSLPR respectively [26,27].

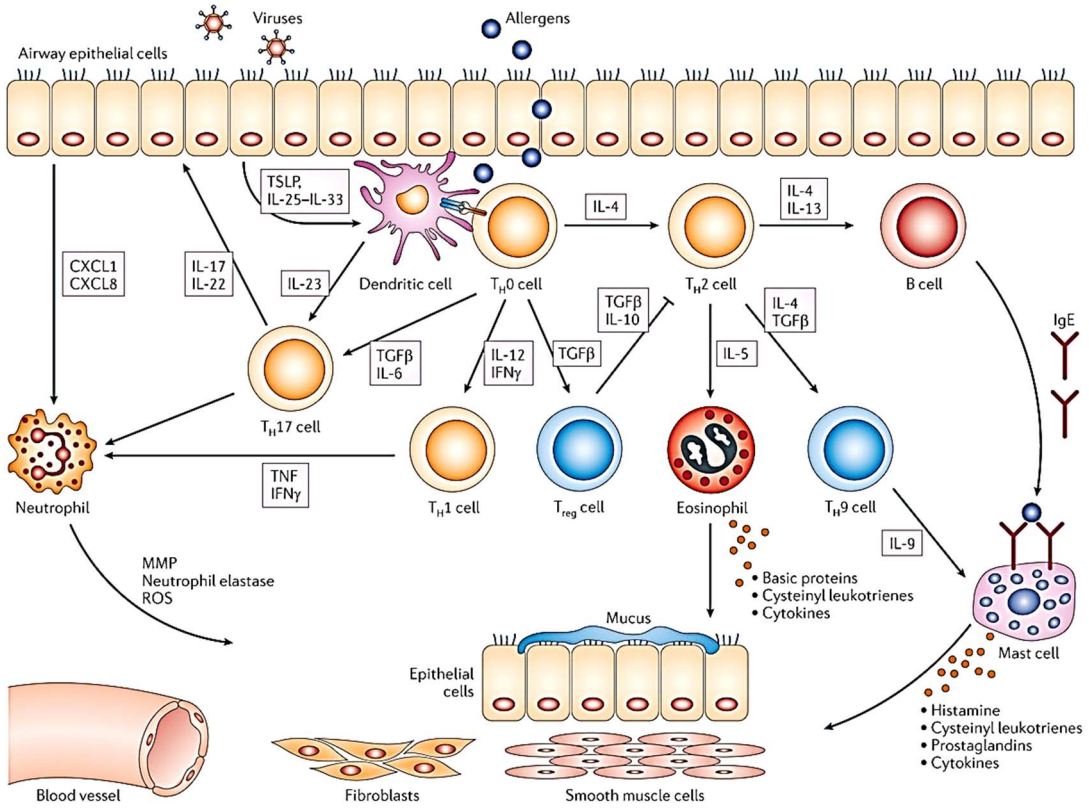


Figure 1.2: Mechanisms of allergic asthma, according to Pelaia *et al.*[30]. Abbreviations: CXCL1, chemokine CXC motif ligand 1; IFNγ, interferon-γ; IL, Interleukin; MMP, matrix metalloproteinase; ROS, reactive oxygen species; TNFα, tumour necrosis factor-α; TSLP, thymic stromal lymphopoietin, TGFβ, transforming growth factor-β; T_{reg}, regulatory T cells, T_H0, naive T helper cells.

Once arrived into the lung, DC extend their dendrites through the epithelial barrier to recognize and sample inhaled antigens from the airway lumen via PRR (such as TLR and NOD-like receptors) [22]. During antigen sampling, DC preserve the integrity of the epithelial barrier by forming tight junctions with the adjacent epithelial cells [28]. Upon signals secreted by the airway epithelial cells (IL-33, IL-25 and TSLP) DC become mature, activated and programmed to induce a specific T helper cell response [22]. After being internalized, the allergen is processed into small peptides and presented via major histocompability complex (MHC) I and MHC II on the cell surface [24]. Within a few hours of contact with the allergen, DC migrate towards the T cell area of the regional lymph node via a chemokine receptor (CCR)7 dependent manner in response to CCL7 [5,29]. The migration to the lymph nodes takes 12 hours and is accompanied by the expression of adhesion molecules and co-stimulatory molecules for naive T cells [24,29]. At the T cell

area of the LN, programmed DC present epitopes of the processed allergen to naive T lymphocytes via MHCII/MHCI complexes, resulting in the induction of specific T helper cells [5,16,22].

2.1.2 Different subsets and tasks of dendritic cells in mice

Lung

Originally, lung DC in mouse were described as a single population with a high degree of expression of CD11c and MHCII. To date, different DC subsets are described in the lung, each characterized by the expression of several cell-surface markers. The conventional dendritic cells (cDC) are characterized by a high expression of CD11c and are further divided into $CD11b^+$ and $CD103^+$ cDC. The monocyte-derived dendritic cells (moDC) are recognized by a high $CD64^+$ expression and the plasmacytoid dendritic cells (pDC) are characterized by a high siglec-H expression (table 1.1) [24].

Each of these DC subsets is geared to induce particular T cell responses. Nevertheless, this induction into a specific T cell seems to be context dependent [31].

- $CD103^+$ cDC are located between the epithelial cells of the conducting airways. They express the E-cadherin-binding integrin CD103 and langerin and have the capacity, through the formation of tight junctions with bronchial epithelial cells, to extend dendrites into the airway lumen (fig 1.3) [22,32]. They are specialized in cross-presentation of exogenous antigens to $CD8^+$ T cells, and have the capacity to induce tolerance [31,33,34]. *Ex vivo* they have also been shown to induce a type 1 T helper (Th1) or a Th2 cell response [31,34].
- $CD11b^+$ cDC are located in the lamina propria of the conducting airways (fig 1.3). In a HDM mouse model, they have been shown to be prone to induce a Th2 mediated immunity, while in a fungal model a Th17 mediated immunity was reported [31,33].

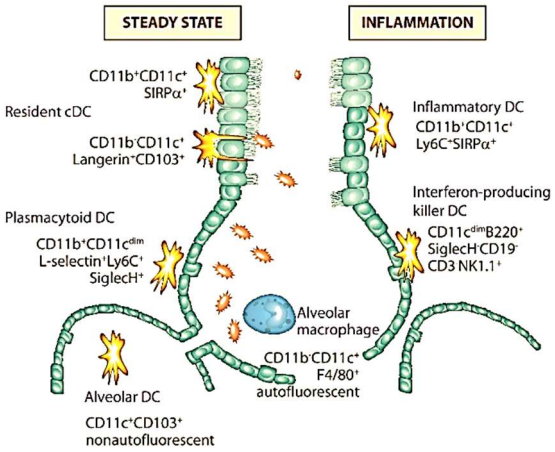


Fig 1.3: Lung dendritic cells subset from Lambrecht and Hammad [22].

- pDC are found in the alveolar septa and in the large conducting airways. Yet, their specific anatomical location is largely unknown [22]. pDC are recognized by the expression of siglec-H and are important for controlling overt airway inflammation through induction of regulatory T (T_{reg}) cells [33,35].
- Under inflammatory conditions, monocytes migrate to the local tissue and differentiate into moDC [33]. This subset is distinguishable from $CD11b^+$ cDC by using the markers CD64 and/or MAR1 [33,34]. moDC have been shown to be sufficient to induce a Th2 immunity. The main function of moDC is situated in the lung as they poorly migrate to the draining lymph nodes due to the lack of CCR7 expression. In the lung, they produce chemokines attracting Th2 cells to the lung [22,33,34].

Skin

In the skin, $CD11b^+$ cDC, are the most abundant type of DC. They have been shown to induce T_{regs} cells upon migration to the draining lymph nodes. Additionally, they are able to prime Th2 differentiation following skin sensitization in response to TSLP. Dermal ($CD11b^-$)$XCR1^+$cDC highly express CD207, and comprise both $CD103^+$ and $CD103^-$ cells. $XCR1^+CD103^+$ dermal cDC have been shown to induce Th1 cells during infections. Monocyte derived dendritic cells are $CD64^{low/+}$ and have a poor migratory capacity. Langerhans cells (LC) in the skin interact, via their expression of E-cadherin, closely with the surrounding keratinocytes. LC encompass both CD207 and CD11b expressing cells. Depending on the nature of the sensed threat, they can become immunogenic or tolerogenic. They have been shown to be responsible for the induction of naive T cells into T_h17 or T_{reg} cells. pDC are only identified in inflamed skin where they promote wound repair and mediate the systemic pro-inflammatory response (table 1.1) [36].

Table 1.1: Subsets of lung and skin dendritic cells [31,33,36,37].

	DC subsets	Surface molecule expression
Lung	$CD11b^+$cDC	$CD45^+CD11c^+MHCII^+CD11b^+CD103^-$
	$CD103^+$cDC	$CD45^+CD11c^+MHCII^+CD11b^-CD103^+$
	moDC	$CD45^+CD11c^+MHCII^+CD11b^+CD64^+$
	pDC	$CD45^+CD11c^{dim}MHCII^+$Siglec-H^+
Skin	$CD11b^+$cDC	$CD45^+CD11c^+MHCII^+CD64^-XCR1^-CD207^-CD11b^+$
	$CD103^+XCR1^+$cDC	$CD45^+CD11c^+MHCII^+CD64^-XCR1^+CD207^+CD11b^-CD103^+$
	$CD103^-XCR1^+$cDC	$CD45^+CD11c^+MHCII^+CD64^-XCR1^+CD207^+CD11b^-CD103^-$
	DNcDC	$CD45^+CD11c^+MHCII^+CD64^-XCR1^-CD207^-CD11b^{low}$
	moDC	$CD45^+CD11c^+MHCII^+CD64^+$
	LC	$CD45^+CD11c^+MHCII^+CD64^-XCR1^-CD207^+CD11b^+$

2.1.3 Induction of an immune response

Once arrived at the T cell area of the local lymph node, DC will polarize naive T cells towards specific T helper cells. After being programmed by the epithelial cell derived cytokines IL-33, IL-25 and TSLP combined with IL-4 (derived from basophils, eosinophils, natural killer T cells and T cells), DC polarize naive T cells towards Th2 cells [22,38]. Th2 cells may be important in mild to moderate asthma and are important producers of IL-4, IL-5, IL-6, IL-9, IL-13 and GM-CSF (fig 1.2) [38]. Additionally, innate lymphoid type 2 cells (ILC2) are also important sources of IL-5, IL-9 and IL-13 and, to a lesser extent IL-4. IL-5 is responsible for recruitment and activation of eosinophils to the lung. IL-9 attracts mast cells and triggers their differentiation, while IL-13 is involved in different aspects of airway inflammation, remodeling and AHR. IL-4 and IL-13 act on B-lymphocytes by driving immunoglobulin class switching towards the production of antigen-specific IgE (fig 1.2) [39].

After the production of antigen-specific IgE, they become systemically distributed where they bind to high affinity receptors (FcεRI) expressed on the surfaces of mast cells. Re-exposure to the same allergen leads to binding of the allergen on IgE antibodies that are attached to the mast cell FcεRI receptors. This causes coss-linking of the FcεRI receptors, which activate mast cells to release preformed mediators (histamine, serotonin, proteases and proteoglycans), newly synthesized lipid mediators (prostaglandins and leukotrienes and platelet-activating factor (PAF)) and increase the synthesis of many cytokines, chemokines and growth factors (TNF-α, IL-4, IL-5, IL-3, GM-CSF, TGF-β, vascular endothelial growth factor (VEGF)) (fig 1.2). The rapidly secreted preformed mediators contribute to the symptoms associated with the EAR that develop within 20-30 minutes. These symptoms include vasodilation, increased vascular permeability, increased mucus production and contraction of the bronchial smooth muscle. Meanwhile, mast cells and Th2-lymphocytes produce and secrete IL-5, IL-13 IL-17 and GM-CSF, resulting in recruitment of inflammatory cells (basophils, eosinophils and neutrophils) which are responsible for the LAR 2-6 hours later (fig 1.2) [21].

The inflammatory cells secrete products (cytokines, enzymes, metabolites and growth factors (IL-5, IL-13, TGF-β and matrix metalloprotease 9 (MMP9)), that damage and act on the surrounding tissue such as fibroblasts, smooth muscle cells, neuronal cells and epithelial cells [40]. Once established, repetitive cycles of tissue damage and inflammatory-cell recruitment cause persisting chronic airway inflammation, resulting in repetitive phases of tissue destruction and tissue repair, leading to structural changes of the airway, named airway remodeling. Airway remodeling is characterized by airway wall thickening, subepithelial thickening from increased deposition of extracellular matrix proteins (ECMs) such as collagens, proteoglycans and glycoproteins, increased vascularity, goblet cell hyperplasia, airway smooth muscle cell hyperplasia and hypertrophy and epithelial hypertrophy resulting in a lower baseline lung function [41–44].

Airway remodeling is traditionally recognized as a marker of a long-standing chronic disease, yet there are some suggestions that it can be an early feature of the disease. Airway remodeling was for instance found in children without the evidence of Th2 inflammation, suggesting that airway remodeling can occur independently of Th2 inflammation. This finding was strengthened by the observation that epithelial damage, thickening of basement membrane, angiogenesis already was found in 4 years old asthmatic children [42]. These observations are supported by the fact that, IL-33, activin A, endothelin-1 and IL-25, secreted by epithelium cells, may drive airway remodeling and AHR [43,45,46]. Although in certain cases airway remodeling can be dissociated from airway inflammation, there is a close link between airway remodeling and the loss in lung function seen in asthma [43,44].

The described Th2 mediated allergic immune response of asthma can be inhibited by the Treg cells. Treg cells play an important role in maintaining a balanced adaptive immune response by the production of IL-10 and TGF-β. A defect that effects the Treg cell number and/or function disrupts this balance, leading to a lower number and impaired suppressive activity of Treg cells, which will finally stimulate ongoing infiltration of immunological/inflammatory cells, thereby contributing to the pathogenesis of allergic asthma [16,47].

In addition to the Th2 response, DC polarize $CD4^+$ T (Th) cells into Th1 cells via the production of IL-12. Together with innate lymphoid cells type 1 (ILC1), Th1 cells secrete tumor necrosis factor (TNF)-α, IL-2 and interferon (IFN)-γ (fig 1.2). Th1 cells are involved in patients having a disease onset in adulthood, which is associated with a predominant neutrophilic lung inflammation and a severe asthmatic outcome [38,48].

2.2 Neural mechanisms

Besides the interplay between innate and adaptive immune system, the nervous system also regulates asthmatic responses. Upon irritant signals (cigarette smoke, vehicle exhaust and air pollutants), sensory nerves become activated via transient receptor potential (TRP) channels, resulting in coughing, mucus production, bronchoconstriction, vasodilatation and dyspnea [49]. In addition, stimulated sensory nerves will release tachykinins (substance P (SP), neurokinin (NK) A and NKB) and calcitonin gene-related peptide (CGRP) from sensory nerves terminals. These neuropeptides exert many inflammatory and immune-modulatory effects such as smooth muscle contraction, submucosal gland secretion, vasodilatation, increase vascular permeability, stimulation of mast cells, T- and B- lymphocytes and macrophages, chemo-attraction of eosinophils and neutrophils and vascular adhesion of neutrophils, summarized as neurogenic inflammation [50,51].

3. Work related asthma

Work related asthma encompasses both occupational asthma (OA) and work exacerbated asthma (WEA) (fig 1.4). Work exacerbated asthma is characterized by worsening of pre-existing asthma due to workplace related exposures, while occupational asthma is due to exposure to a specific causal agent present in a particular work environment and not to stimuli encountered outside the workplace [52]. Work related asthma is increasingly recognized as the most common cause of occupational lung diseases and is believed to cause up to 15-25% of the cases of adult onset asthma [53]. The clinical manifestations are similar to those found in non-occupational asthma, with varying degrees of disease severity. Mild cases are recognized by episodic dry cough, chest tightness, and increased breathing effort at the workplace, while severe cases are recognized by wheezing, cough, chest tightness, shortness of breath, and dyspnea that persist away from the work environment [52,54].

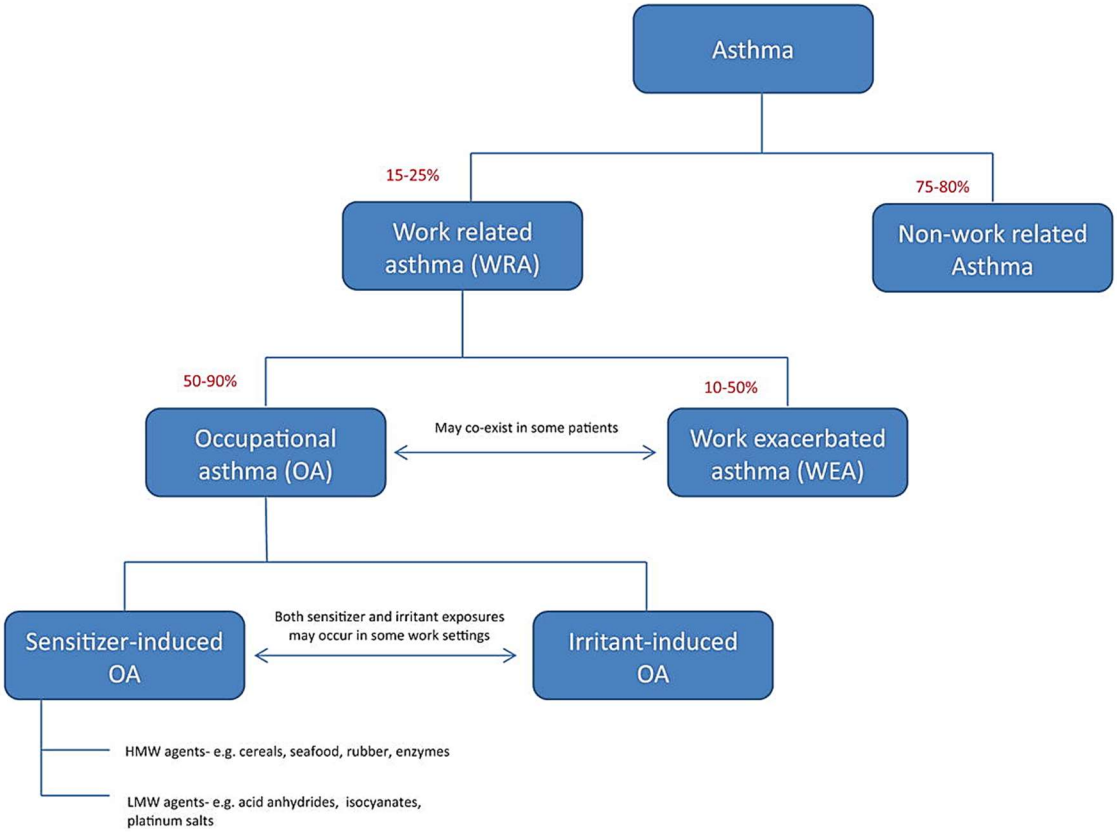


Fig 1.4: Subdivision of work related asthma [53].

Occupational asthma can be classified, based on the underlying pathogenesis and presence of a latency period, into two subtypes namely irritant-induced asthma and immune-mediated asthma [52]. Irritant-induced asthma occurs after exposure to irritants (such as ammonia or chlorine) at the workplace in the absence of sensitization. This subtype can be subdivided into a severe form which can occur within 24h after a single exposure to high concentrations of an irritant, named 'reactive airway dysfunction syndrome' (RADS). On the other hand, there is also a delayed onset of irritant-induced occupational

asthma, which may occur after multiple exposures to smaller doses of irritants over days, weeks, or months [53]. Immune-mediated occupational asthma develops after an asymptomatic latent period (weeks to years), during which allergic sensitization develops (fig 1.4).

To date, more than 400 agents are recognized as trigger of immune-mediated OA and the list continues to grow [53]. Agents capable of inducing immune-mediated OA can be classified according to their molecular weight: high molecular weight (HMW) (>10KD) proteins or low molecular weight (LMW) chemicals (<2KD) (Table 1.2) [53]. HMW agents include compounds in flour (cereals), seafood, latex, enzymes (derived from plant, animal and microbial), house dust mite and allergens of laboratory animals and shellfish, which induce immune-sensitization via classical IgE-mediated mechanisms. LMW compounds are typically small highly reactive chemical molecules. These small molecules are non-immunogenic in their native state, yet when they bind to endogenous proteins (such as albumin, keratin or tubulin) stable 'hapten-protein' conjugates are formed. Similar to HMW, some LMW chemicals (such as platinum salts, reactive dyes, acid anhydrides and some wood dusts) are associated with the production of specific IgE antibodies; however, several chemical sensitizers (such as isocyanates, persulphate salts and aldehydes) induce OA with an unclear role for IgE. The immunological mechanisms of this phenotype of asthma are still poorly understood [55].

Table 1.2: Cross-sectional studies of workers exposed to occupational asthmogens, from Jaakkola *et al*. [56].

Exposure/ Industry/Occupation	Exposure Before Diagnosis (months)	Prevalence of Work-Related Asthma	Number Studied
High-molecular-weight agents			
Enzymes/detergent	Intermittent	50	98
Guar gum/carpet industry	Up to 108	23	162
Snow-crab/food processing	Several months	21	303
Laboratory animal workers	26	7	238
Flour/bakery workers	12	6	264
Latex/hospital workers	120	2	289
Low-molecular-weight agents			
Platinum refinery	12-24	54	91
Colophony/ electronic plants	24	22	924
Isocyanates/ secondary industry	54	20	51
Spiramycin/ pharmaceutical	n/a	9	51
Plicatic acid/ forestry workers	n/a	4	625

3.1 Diagnosis

Occupational asthma has significant health consequences for affected patients, but also social-economic impacts for workers, their employers and wider society. Therefore, an accurate diagnosis is very important. Missing diagnosis of OA leads to continued exposure to a causative agent and worsening of the disease outcome. Various tests are described to make a valid diagnosis for OA.

Depending on the clinical and occupational history of the patient, the physician can determine whether the symptoms improve outside the workplace. However, to confirm that the patient has asthma, a bronchial provocation test is performed to determine AHR to non-specific stimuli by measuring lung function after inhalation of bronchoconstrictive agents, such as histamine or the non-selective muscarinic receptor agonist methacholine. Taking into account that sometimes AHR rapidly improves and even becomes absent a few days after cessation of exposure to the causative agent, this test is insufficient to diagnose OA.

To increase the specificity of the assessment of AHR, this measurement is complemented with an immunological skin prick test and assessment of serum-specific IgE antibodies to demonstrate IgE mediated sensitization. Since several LMW agents induce OA in an IgE independent manner, this test is inconclusive [57]. Consequently, specifically for LMW compounds, there is a strong need for developing additional reliable markers. Several studies focused on identifying environmentally responsive genes to recognize susceptible subpopulations. Specifically for diisocyanate-induced asthma, genetic susceptibility markers placed in the human leukocyte antigen (HLA)-region and CTNNA3 (a single nucleotide polymorphism of the alpha-T-catenin) are identified. HLA class 2 molecules are crucial in antigen recognition during Th2 inflammation. Therefore, HLA can be plausible candidate genes involving specific immunological responses and contributing to individual susceptibility in OA. Several studies suggested that HLA alleles (HLA-DQB1*0503, HLA-DQB1*0201-0301, HLA DQA1*0104, DRB1*15-DPB1*05, HLA-DRB1*1501, HLA-DQB1*0602, HLA-DPB1*0501) are associated with an increased risk of TDI-OA. On the other hand, HLA alleles HLA-DQB1*050, HLA-DQA1*0101 and HLA-DQB1*0501 seems to have a protective effect against TDI-OA [58].

To demonstrate that the symptoms are related to the workplace, lung function is measured on both workdays and free days. Serial measurements of peak expiratory flow (PEF) are an effective manner of monitoring changes in pulmonary functions during the day. These self-measurements can be complemented with serial measurements of non-specific AHR and airway inflammation by measuring exhaled nitric oxide and evaluation of sputum eosinophils [57,59].

To identify the causal agent responsible for OA, a specific inhalation challenge (SIC) test is used. During a SIC, the patient is exposed to the suspected agent in a controlled exposure room to investigate reactivity of the airways to the specific agent [57]. A drop of at least 15% in FEV1 after a specific challenge

is considered as a positive diagnostic SIC test. Although the SIC test is the gold standard for the diagnosis of OA it has several limitations namely, it is not easy to perform, it is time consuming, costly, carries some risk for the patient and should only be performed in specialized centers [60,61].

3.2 Prevention and management

Often, decisions made in the management/prevention of OA have a significant social, financial, and psychological impact on workers and their families [61].

Generally, there are three main preventive steps. The first step includes education regarding occupational asthma, safe work practices and avoidance of known sensitizer agents. This can be achieved by replacing sensitizing agents with non-sensitizers: e.g. replacing rubber latex gloves with nitrile gloves or volatile chemicals with less volatile chemicals. When replacement is not possible, reducing/eliminating exposure to the sensitizer is recommended (personal protective equipment and hygiene measures). As early diagnosis results in a better disease outcome, the secondary prevention strategy is the early detection of OA. This can be obtained by periodic monitoring using respiratory questionnaires, spirometry, immunologic tests and continued education regarding occupational asthma symptoms and signs. The third step is prevention/appropriate treatment of occupational asthma [53,55]. The main treatment for OA is identical as treatment of allergic asthma: long-acting β2 agonists and inhaled corticosteroids. However, as the underlying mechanisms of OA is not yet fully clarified, especially for LMW compounds, it is still a challenge to recommend the appropriate treatment. As a consequence, long-term deterioration cannot be prevented [39,41,46,47].

The management of OA is not different from the management of non-work related asthma, except the reduction/elimination of the causal exposure. As persistent exposure to a causative agent after diagnosis of OA leads further to a worse clinical outcome including fatal asthma exacerbations, complete cessation of further exposure to the causative agent results in the best clinical outcome [60,61]. This can be achieved by relocation of the patient to a new job in the same plant or in another site where there is no exposure to the causative agent or early retirement. However, only 32% recover completely from asthma symptoms, even after cessation of exposure to the causative agent [53,55,62].

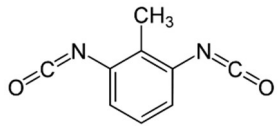
In the case of job change, it is important that the physicians are aware of the impact of the decisions made for the patient suffering from OA. Almost 50 % of the patients have depression and anxiety after leaving their job and are concerned about the economic consequences after being identified with OA. 42% to 78% of the workers diagnosed with OA have significant loss in income after changing their job [61]. Because of these concerns, certain workers choose to be remained exposed. For some patients, it is sometimes even not possible to be relocated to a job free from exposure to the causative agent. In this case a worker's compensation claim should be initiated to cover lost wages and medical expenses [53]. In

cases when patients are unable or unwilling to change their jobs, it is important to minimalize the exposure to the causative agent by personal protective equipment such as proper respiratory and skin protection along with hygiene measures such as lowering the dose below the limit values by improving ventilation. However, these measures are only effective for OA caused by HMW sensitizers. For most of the LMW agents, such as isocyanates, these measures are still inadequate in preventing new asthma cases [61].

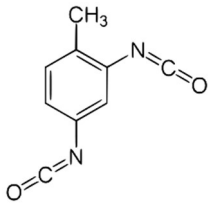
3.3 Diisocyanates

As shown in table 1.2 diiscocyanates are a commonly identified cause of LMW-induced OA worldwide. 5-30% of the workers exposed to isocyanates are at risk to develop the disease [63,64]. Diisocyanates are LMW chemicals and are characterized by two -N=C=O groups which exhibit strong chemical reactivity, making isocyanates highly reactive chemicals [65]. These highly reactive -N=C=O groups allow them to undergo direct polymerization reactions with alcohols (such as polyols) to form polyurethanes [66]. The most important commercial aromatic diisocyanates are toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI), and the most important commercial aliphatic isocyanate is hexamethylene diisocyanate (HDI) (fig 1.5). MDI and TDI together account for more than 90% of the global isoscyanate market, and their annual production exceeds 4 million tons [64,67]. TDI and MDI are used to manufacture products such as flexible and rigid foams, adhesives and sealants, such as foam rubber cushions, dashboards and finished coatings. Furthermore, MDI is increasingly used in the building industry, foam mattresses and footwear [68]. The aliphatic isocyanate HDI is primarily used in coatings and paints. Due to its unique physical properties, MDI has become the most abundantly produced and consumed diisocyanate.

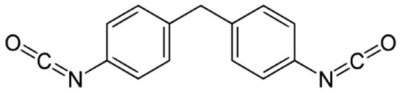
TDI, commonly used as a mixture of 2,4 and 2,6 isomers (80:20), is a colorless mixture and is highly volatile at room temperature, while MDI is a solid and needs to be heated before vapors are released [68]. Despite variable physical and chemical properties, including vapor pressure, solubility, and size, all of



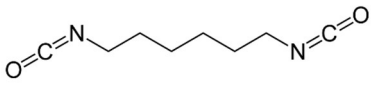
2,6 Toluene diisocyanate



2,4 Toluene diisocyanate



4,4' Methylene diphenyl diisocyanate



Hexamethylene diisocyanate

Fig 1.5: Structures of diisocyanates.

the major monomeric, prepolymeric and polydiisocyanate species are capable of inducing asthma in exposed workers [69].

High acute irritant exposure to diisocyanates is known to cause RADS and irritant-induced asthma. However, besides the acute toxicity, isocyanates are potent immune sensitizers. After sensitization, low airborne concentrations can elicit an asthmatic reaction.

3.3.1 Immune sensitization

To induce an immune response, LMW compounds can act as haptens that react with alcohols, phenols, amines and thiols of endogenous proteins. Diisocyanates mainly conjugate in the lung with proteins such as tubulin and actin. Other skin and lung proteins and peptides are glutathione-S-transferase (GSH), keratin and actin. After binding with endogenous proteins, diisocyanates are recognizable by the immune system [63,67]. MDI and TDI can first bind to the tri-peptide, γ-glutamyl-cysteinyl-glycine, also known as GSH, in the exposed tissue, which is followed by a GSH-mediated transfer of MDI or TDI into the blood stream where it can bind to specific lysines (Lys) residues of the highly present protein human serum albumin (HSA) [70,71]. In a liquid phase, TDI binds to 34 of the 59 lysine residues on HSA, while MDI only binds to 19 lysine residues on HSA (fig 1.6) [67]. However, using TDI in a vapor phase, less binding sites are found on HSA [64]. Additionally, different binding sites on HSA are detected between isomers 2,4- and 2,6-TDI. The 2,4-isomer has a higher reactivity to albumin resulting in more bindings compared to the 2,6-isomer [72]. The increasing binding capacity of TDI compared to MDI can be attributable to the increased reactivity of the functional isocyanate groups: the electron withdrawing character of the second -N=C=O group on the aromatic ring of TDI significantly increases the reactivity of the first isocyanate [67]. It has been observed that TDI can conjugate to serum albumin in two different forms. The first results from hydrolysis of one isocyanate to a primary amine, whereas the second isocyanate moiety undergoes nucleophilic addition to the protein. The second is the result of both isocyanates undergoing nucleophilic addition to the protein, resulting in an intramolecular crosslinking. This is often seen when two lysines are located in close proximity, like the four dilysine motifs in HSA. Rarely, intermolecular cross-linking of two protein molecules can be caused by one TDI. [64]. The binding of isocyanates on HSA changes its conformation and charges, resulting in an antigenic product [73].

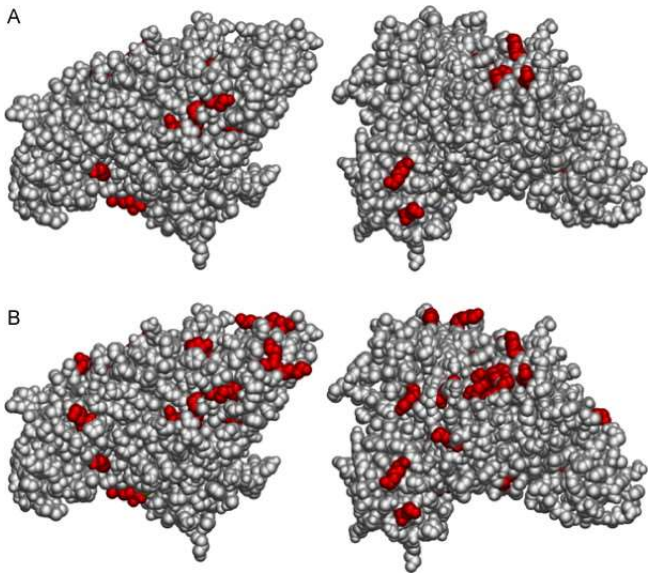


Fig. 1.6: Front (left) and rear (right) projections of human albumin. Conjugation sites for MDI (A) and TDI (B) are highlighted in red. From Justin M. Hettick and Paul D. Siegel [67].

As HSA is the major diisocyanate adduct in the blood, diisocyanate-albumin reaction products in the blood stream may serve as biomarkers. Artificially prepared diisocyanate protein conjugates have been used to develop *in vivo* monoclonal antibodies toward isocyanates, with the aim to identify different diisocyanate-protein adducts in the blood serum of isocyanate exposed workers [63,74–76]. In addition, to confirm immune sensitization, diisocyanate-protein conjugates have been developed with the aim to detect isocyanate specific immunoglobulins in serum of exposed workers. These studies mainly focused on HSA, identified as the major target for inhaled isocyanates.

It needs to be taken into account that the antigenic product resulting from artificial isocyanate-protein conjugation are influenced by the production method used (liquid phase or vapor phase) and molar ratio [64,73,75,77–80]. Preparing biologically relevant hapten-protein conjugates is technically difficult and by using inappropriate conjugates, specific antibodies will be missed. Therefore, it is necessary to be able to mimic the real life *in vivo* micro environment [81].

3.3.2 Skin-lung interaction

In the past, inhalation has long been considered as the primary route of isocyanate exposure, induction of sensitization and asthma [82]. Consequently, many measures were taken to decrease the airway exposures to isocyanates, such as increased use of the less-volatile isocyanate MDI, improved hygiene practices such as personal projective equipment and reducing TDI airborne concentration. In 2016, the American Conference of Governmental Industrial Hygienists (ACGIH) proposed a threshold limit value (TLV, 8h time weighted average) of 0.001 ppm and 0.005 ppm for the short time exposure limit (STEL, 15 min timed weighted average) [83]. In contrast, in Belgium the TLV and STEL are still 0.005 ppm and

0.037 ppm, respectively. Yet, despite the low airborne isocyanate concentrations, asthma continues to occur [82].

To date, a growing body of evidence exists that skin exposure is an efficient route to induce sensitization, with subsequent inhalation challenge resulting in an asthmatic response [82]. Depending on the type of isocyanate or application method, isocyanate exposure can occur in different forms (vapors, aerosols or liquids). The introduction of less volatile forms of isocyanates, such as MDI, reduced the risk of respiratory exposure but not necessarily skin exposure. Skin exposure can result from deposition of aerosols and/or absorption of vapors of isocyanate end uses such as spraying and application of foams and adhesives. Furthermore, workers can come into contact via the skin trough accidental spills, cleanup and contact with contaminated equipment [82]. In addition, Isocyanates are commonly mixed with other compounds such as polyols, catalysts and blowing agents which affect their reactivity and skin absorption. Impairment of the skin barrier, caused by mutations in the skin protein filaggrin, overexpression of TSLP, frequent use of soaps and cleaners, additional exposure to other skin irritants, physical trauma, heat and low humidity and glove use, can facilitate allergen penetration and systemic sensitization to chemicals [84].

Biomarkers such as urinary metabolites (toluenediamine, methylenediamine and hexa-methylenediamine) can be useful indicators for isocyanate exposure, but are not able to distinguish between skin and respiratory exposure. Specific isocyanate skin exposure can be identified by skin tape strips [84,85]. Tape-strip sampling is a removal technique in which the stratum corneum of the skin is collected using specific tape, after exposure in the work place. Subsequently, chemical agents present on the tape are analyzed and quantified using liquid chromatography/mass spectrometry (LC-MS), and are used as a proxy to assess possible skin exposure [86].

3.4 Animal models of chemical-induced asthma

The pathology of asthma is complex, heterogeneous and in many instances poorly understood [87]. Unraveling the cellular and molecular mechanisms of asthma is not fully possible in human [88]. As 99% of the genes are shared between mice and human, the mouse has become the most widely used species to study the pathophysiology of asthma [89,90]. Mice are easy to breed, maintain and handle. Additionally, a wide array of specific reagents to analyze cellular and mediator responses are available together with the availability of genetically engineered transgenic mice for modelling airway disease [91].

Due to the complexity of asthma, it is unlikely that a single animal model can mimic all of the morphology and functional features of the chronic human disease [92]. Therefore, mouse models need to be refined so that they are able to reflect clinical findings of disease characteristics, pathways and specific phenotypes [93]. As mice do not spontaneously develop asthma, artificial asthmatic-like reactions have to

be induced to investigate the disease [92]. To mimic asthma-like reactions, mice first need to be sensitized to an antigen of interest (via intra peritoneal injections (i.p), skin exposure or airway exposure) followed subsequently by airway challenging (intranasal, oropharyngeal, intratracheal instillations or aerosols) [88]. The disease outcome of asthma models is influenced by the compound, dosing, route and schedules of sensitization and challenge, and the choice of mouse strain [92].

Compounds

Asthma research primarily focused on HMW allergens, which are frequently associated with allergic asthma such as HDM, ragweed extracts, aspergillus, ovalbumin (OVA) and cockroach antigens [93]. Less commonly, despite being also an important cause of OA, research focused on animal models of asthma induced by LMW compounds. In the case of LMW compounds, most animal research focused on diisocyanates, more specifically TDI, MDI and HDI [94–101].

Treatment protocols

To mimic the wide variety of characteristics of asthma, both acute and chronic mouse models were introduced. After sensitization, the acute challenge mouse models reproduce many key features of asthma such as early ventilatory response, AHR, airway inflammation and elevated levels of IgE. Nevertheless, because of the short-term nature of acute models, many of the characteristics observed in chronic human asthma such as chronic inflammation of the airways and airway remodeling remain absent. Furthermore, in acute models, the key features such as airway inflammation and AHR appear to resolve within days/few weeks after the final allergen challenge. To develop airway remodeling and persistent inflammation and AHR, chronic mouse models were introduced [92]. Yet, also inflammation and AHR can disappear during chronic challenge protocols [102].

Inhalation, intra-tracheal, oropharyngeal or intranasal instillations are widely used to administer agents to the respiratory tract. Inhalation is the most natural way of exposure, but it is technically demanding, expensive and time-consuming. Intranasal applications are easy to perform and can be repeatedly used in a short time period; however the instilled volume, degree of anesthesia and vehicle (aqueous or non-aqueous) heavily influence the distribution between the upper and lower airways. Intra-tracheal instillation bypasses the nose by placing a catheter into the trachea, which allows administration of exact amounts of the agent into the lung. However, it requires general anesthesia and can cause injury to the trachea, certainly after repeated use. Therefore, alternatively, an oropharyngeal challenge has been introduced, in which the liquid is pipetted onto the back of the tongue. Both intra-tracheal and oropharyngeal challenge techniques have been shown to uniformly distribute agents into the lung [103–106]. However, by placing the pipette on the tongue during the oropharyngeal challenge, the compound goes not consistently to the lung, resulting sometimes in gastro-intestinal exposure. Therefore, an

endotracheal instillation technique is introduced as an alternative method. During this instillation, mice are held vertically and the pipette is placed just above their vocal cords preventing swallowing of the agent.

In an attempt to develop mouse models of chemical-induced asthma, different routes of sensitization have been described. Airway exposure (inhalation and intranasal application) has been successfully used in the sensitization and subsequently elicitation phase in isocyanate-induced asthma [95,107,108]. Nevertheless, as already discussed in isocyanate-induced asthma, skin exposure is also an important route of sensitization. Therefore, in many models the sensitization phase was based on skin exposure (topically or subcutaneously), subsequently followed by an elicitation reaction using airway exposure [94–96,101,103,107,109–113]. Ban *et al.*, evaluated several sensitization protocols (inhalation exposure, intra-tracheal instillation and skin application) and demonstrated that skin exposure followed by airway exposure resulted in the most pronounced systemic Th2-dominated response [98]. However, it should be taken into account that the used sensitization concentrations influence the lung response. It has been shown that high skin exposure abolish the asthmatic features [96,101,114].

Choice of mouse strain

The genetic background of different mouse strains has a large influence on the phenotypical outcome of TDI-induced asthma. In a well-defined acute mouse model of chemical-induced asthma, De Vooght *et al.* showed that in seven different mouse strains, of which three were Th2-prone strains (BALB/c, BP2 and A/J) and four were Th1-prone strains (C57Bl/6, DBA/2, AKR and CBA), there were different responses in AHR, BAL cellular inflammation, cytokine profile, and serum antibody levels. The AHR differed even in baseline level, which can be due to the differences in alveolar size, lung volume, elastic recoil properties and airway structure between the different mouse strains [115–117]. In general, the features of human OA are best mimicked in BALB/c mice [118]. Using different treatment protocols and mouse strains, several phenotypes of occupational asthma can be developed.

3.4.1 TDI-induced mouse models of asthma

Our lab developed an acute mouse model of chemical-induced asthma using TDI as model agent. In this mouse model, mice are dermally sensitized on the dorsum of both ears with TDI on days one and eight resulting in an increased number of T- and B- cells in the auricular draining lymph nodes. The increase of T cells is associated with an increased release of Th2 cytokines (IL-4, IL-13, IL-10) and Th1 (IFN-γ) cytokine, along with changes in proteome [119]. A subsequent TDI airway challenge on day 15 results in a reproduction of important features of chemical-induced asthma such as early ventilatory response, non-specific airway hyperresponsiveness and airway inflammation, characterized by mainly neutrophils but also eosinophils 24h after the challenge [114].

This mouse model showed the ability to distinguish respiratory sensitizers (trimellitic anhydride (TMA)) from dermal sensitizers (dinitrochlorobenzene (DNCB)), along with differentiating chemicals for their sensitization and asthmogenic potentcy [120]. Ammonium persulfate showed a positive asthma-like response, which was not the case for methylisothiazolinone [121,122]. Furthermore, this mouse model was used to test cross-reactions between two related chemicals, toluene diamine (TDA) and TDI. TDA was not able to induce an asthmatic response and there was no cross-reaction with TDI [123].

Besides validation of chemicals, our and other mouse models are used to study the underlying pathophysiology of chemical-induced asthma. Many features of diisocyanate asthma are similar to atopic asthma such as airway inflammation characterized by activated $CD4^+$ T cells, eosinophils, mast cells, airway remodeling and increased levels of Th2 cytokines IL-4 and IL-5. Nevertheless, there are some differences. Diisocyanate-induced asthma is characterized by a prominent airway neutrophilia, increased IL-8, TNF-α, monocyte chemoattractant protein (MCP)-1 production and a mixed Th1/Th2 cytokine response. Furthermore, $CD8^+$ T cells seems to be more involved than $CD4^+$ T cells. Additionally, the prevalence of diisocyanate specific IgE antibodies are low and the disease is not associated with atopy [124]. However, as the exact role of these immunological responses are not yet fully delineated, mouse models have been developed to study the underlying mechanisms.

Lymphocytes and associated cytokines in chemical-induced asthma

Both animal and human studies showed an increase of both $CD4^+$ and $CD8^+$ lymphocytes in diisocyanate-induced asthma [125]. To assess the role of lymphocytes in chemical-induced asthma, various mechanistic studies have been performed in different diisocyanate-induced mouse models. Already from 1996, Scheerens *et al.* performed an adoptive transfer experiment where lymphocytes were transferred from TDI treated mice into naive mice resulting in AHR, without airway inflammation, after a TDI challenge [126]. Matheson *et al*. demonstrated that in both transgenic $CD4^+$ or $CD8^+$ knockout (KO) mice, the AHR and airway inflammation were reduced after sub chronic TDI exposure [110]. Furthermore, they showed that adoptive transfer of purified T-, or B- cells from sub-chronically exposed mice into naive mice resulted in AHR after TDI challenge [95]. More recently, our lab demonstrated an abolished asthmatic response in severe combined immunodeficient (SCID) mice, who lack mature lymphocytes, demonstrating the crucial role of these lymphocytes [111]. To establish the role of these lymphocytes, naive mice became passively sensitized via an adoptive transfer technique using, compared to previous described adoptive transfer studies, very low numbers of lymph node cells from TDI-sensitized mice, resulting in AHR and airway inflammation after an airway challenge. More specifically, transferring B-lymphocytes from lymph nodes of TDI-sensitized mice in to SCID mice, resulted in airway hyperreactivity and airway inflammation after a TDI challenge, demonstrating that B-lymphocytes may play an important role in asthma without the presence of T lymphocytes [127]. Additionally, proteomic studies

performed by Haenen S *et al.* demonstrated that sensitization changed the expression of several B cell proteins involved in the recruitment of neutrophils, antigen presenting capacities and the production of immunoglobulins [128].

Both animal and human studies of diisocyanate-induced asthma, demonstrated the presence of both Th2 and Th1 associated cytokines [95,125]. To evaluate the contribution of those cytokines in diisocyanate-induced asthma, several animal studies with exposure to HDI have been performed. Mice deficient in cytokine IL-4, IL-13, IL-4/IL-13 or IFN-γ showed a reduced AHR; moreover, the least effect on AHR was found in the IL-4 deficient mice. BAL cell infiltration was decreased in mice deficient in IL-4 or IL-13 and particularly in the combined IL-4/IL-13 deficient mice. Recently, our lab showed that by systemically blocking IL-13, AHR is diminished in a paucigranulocytic phenotype of TDI-induced asthma. IFN-γ KO mice showed only a slight decrease in BAL cell numbers [110]. TNF-α receptor (TNFR) knockout and anti–TNF-α antibody–treated mice showed that TNF-α played an important role in AHR, airway inflammation and dendritic cell migration to the draining lymph nodes [113].

Immunoglobulins in chemical-induced asthma

In the majority of people with diisocyanate-induced asthma, allergen-specific IgE cannot be identified [129]. Additionally, atopy is not considered as a risk factor for diisocyanate-induced asthma [130]. Nevertheless, Lavaud *et al*. described an improvement of FEV_1 after treatment with omalizumab (anti-IgE therapy) in two atopic diisocyanate-asthmatic patients having very high levels of total IgE [131]. Furthermore, IgG are found in diisocyanate workers without asthmatic symptoms, suggesting that they are an indicator of exposure rather than disease [95]. Although IgE is only found in very low proportions of patients of diisocyanate asthma, total IgE and IgG are easily detected in several mouse models of diisocyanate-induced asthma [95,96,103,111,112,114,132]. In mice, IgE and IgG1 antibodies are associated with Th2 cytokine response, while IgG2a antibodies are associated with Th1 cytokine response [110].

To examine the role of humoral immunity in TDI-induced asthma, several experiments have been performed. Serum from sub-chronically exposed mice passively transferred into naive mice resulted in an increase of airway inflammation, and AHR 24 post TDI challenge [95]. In an acute mouse model, De Vooght *et al.* transferred serum from TDI treated mice into naive mice which also resulted in airway inflammation and AHR [127]. On the other hand, there are indirect evidences showing a weak association between IgE and asthma. By decreasing the sensitization concentration, and consequently lowering serum IgE, AHR and airway inflammation increased after an airway challenge [96,101,114]. Vanoirbeek *et al.* showed that by prolonging the time between sensitization and a single challenge, the AHR and airway inflammation decreased despite that the IgE, IgG1 and IgG2a concentrations remained unchanged [133].

The role of IgE in isocyanate asthma remains an important but controversial topic. There are several explanations why isocyanate specific IgE are only found in a very low amount of patients. It is possible that isocyanate specific IgE are not detectable because of wrong forms of 'isocyanate antigen' in the immunoassay. Isocyanate-self protein reaction products are extremely diverse, and their antigenicity may differ significantly depending upon the reaction conditions under which they are formed. Furthermore, 30 days away from exposure, IgE serum levels may decrease and become undetectable [129].

Eosinophils and neutrophils in chemical-induced asthma

Diisocyanate-induced asthma is a phenotype of asthma characterized by a predominantly neutrophilic inflammation, and to a less extent by an eosinophilic inflammation [103,124,134]. Predominantly neutrophilic airway inflammation is often associated with severe asthma and has typically an onset of disease in adulthood and is less corticosteroids responsive [48]. Moreover, neutrophils are the first cells that migrate toward the site of inflammation [135]. De Vooght *et al*, studied the role of both neutrophils and eosinophils during the effector phase in an acute mouse model of chemical-induced asthma. In that study, depletion of both cells abolished AHR and lung epithelial damage, suggesting that both neutrophils and eosinophils are key cellular players in inducing asthma in a mouse model of chemical-induced asthma [136]. Furthermore, it has been shown that toluene diisocyanate exposure induces airway inflammation via the activation of transient receptor potential melastatin 8 (TRPM8) [137].

Dendritic cells in chemical-induced asthma

Dendritic cells play a role in both the sensitization and effector phases of asthma. Several studies suggest an involvement of dendritic cells in the pathophysiology of diisocyanate-induced asthma. Twenty years ago, Ban *et al.* already showed an accumulation of DC in lung associated lymph nodes after inhalational TDI exposure [138]. In 2002 Matheson showed, using TNF-α KO mice, that dendritic cells migrate within 24h to the cervical lymph nodes in a TNF-α dependent manner after intranasal instillation of TDI in subcutaneously sensitized mice [113]. Recently, Nayak *et al.* showed that during TDI skin sensitization, TDI-haptenated proteins are co-localized with antigen presenting cells in the skin and local draining lymph nodes [139]. Yet, the involvement of the different DC subpopulations has not yet been studied in chemical-induced asthma; this in contrast to HMW compounds HDM and OVA.

Sensory nerves and neuropeptides in chemical-induced asthma

Several studies concerning the role of sensory nerves and neuropeptides in chemical-induced asthma have been performed. In the past Scheerens *et al*. showed the involvement of tachykinin neuropeptides (including substance P (SP), neurokinin A (NKA) and calcitonin-gene-related peptide) and their receptor neurokinin 1 receptor (NK1R), during the effector phase of TDI-induced OA [140]. Recently, Devos *et al*,

showed that TDI directly and indirectly activates TRPA1 and TRPV1, respectively, resulting in the release of the tachykinin neuropeptide substance P. Moreover, Devos *et al.* showed that in TRPA1, TRPV1, substance P receptor NK1R and mast cells are crucial in inducing AHR [141]. Tachykinins released after activation of TRPA1 and TRPV1, do not act directly on the tracheal smooth muscle cells. In fact, tachykinins probably induce indirectly tracheal hyperreactivity by the induction of mast cell degranulation, as their NK1 receptor is upregulated on mast cells during inflammation, leading to the release of histamine and other inflammatory mediators [140,142]. These inflammatory mediators can directly activate nerve cells in the airways and thereby induce nerve depolarization. This hypothesis was strengthened by Devos *et al.*, who showed an abolished AHR response in mast cell deficient mice [141].

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Chapter 2

Rationale and Aims

Rationale:

Occupational asthma (AO) is one of the most common work-related respiratory diseases in industrialized countries. Low molecular weight (LMW) agents like diisocyanates are a major cause of OA. The mechanisms by which these chemicals induce OA are still not fully understood. They differ from high molecular weight (HMW) allergens since they first need to bind endogenous proteins before they are recognizable by the immune system. Moreover, in several cases no specific immunoglobulins (Ig)E's can be identified. Although HMW and LMW seems to be phenotypically alike, the mechanisms do differ. In this thesis, we focused on the mechanistic insight of LMW induced asthma

Aims:

1. Study the cross-reactivity between the two most prominent used diisocyanates (chapter 3)

We evaluated the cross-reactivity between the most prominent used diisocyanates toluene diisocyanate (TDI) and methylene diphenyl diisocyanate (MDI). Human and animal studies showed that both diisocyanates affect the respiratory system and have common bindings to lysine residues on human serum albumin (HSA). Moreover, both diisocyanates are often used in the same plants and sometimes even together in the same production process. Therefore, it seems likely that cross-reactivity will occur between both chemicals in exposed workers. To investigate the cross-reactivity between TDI and MDI, we dermally sensitized mice with TDI and gave them an airway challenge with MDI and vice versa.

2. Study dendritic cells in different phenotypes of chemical-induced asthma (chapter 4 and 5)

Asthma is driven by genetic and environmental factors, making it a complex and heterogeneous disease resulting in different clinical outcomes, also called phenotypes. Due to the complexity and heterogeneity of asthma, it is unlikely that one single mouse model is capable to mimic all the features of human asthma. Until now, we mainly performed studies using an acute mouse model, consisting of a sensitization phase followed by only one single airway exposure. Yet, due to the short-term exposure, many of the features of chronic human asthma, such as chronic inflammation and airway remodeling could not be investigated in this model. To induce severe inflammation and airway remodeling we developed two different exposure models. Both exposure models represent two different phenotypes of chemical-induced asthma. As dendritic cells (DC) are crucial during the sensitization and elicitation phase of Th2 mediated allergic asthma, we investigated, for the first time, the presence of DC in the lungs of both exposure models.

2.1 Development of a chronic intranasal mouse model (chapter 4)

Based on a chronic house dust mite model (HDM), we developed a chronic mouse model using TDI as model agent. In this chronic model, we aimed to induce AHR, severe airway inflammation and airway remodeling. Furthermore, we wanted to investigate the presence of DC in the lung of this asthma phenotype. To achieve this aim, we dermally treated mice on the dorsum of both ears on days one and eight followed by five intranasal exposures each week, for five consecutive weeks.

2.2 Development of a sub-chronic oropharyngeal mouse model (chapter 5)

The previous described chronic-nasal mucosa exposure protocol showed pronounced AHR, but was not accompanied with airway inflammation nor airway remodeling. This chronic model reflects rather a paucigranulocytic phenotype of chemical-induced asthma, a phenotype where sputum cell counts are within the normal range. With the aim to induce pronounced airway inflammation and airway remodeling, and consequently investigate the presence of DC in an inflammatory phenotype of chemical-induced asthma, we decided to change the airway exposure to an oropharyngeal instillation. In this mouse model, mice were dermally treated on dorsum of both ears followed by five oropharyngeal instillations within two weeks.

Chapter 3

Toluene diisocyanate and methylene diphenyl diisocyanate: asthmatic response and cross-reactivity in a mouse model

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Archives of Toxicology, 2016 Jul;90(7):1709-17

Toluene diisocyanate and methylene diphenyl diisocyanate: asthmatic response and cross-reactivity in a mouse model

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Abstract

Both 2,4-toluene diisocyanate (TDI) and 4,4- methylene diphenyl diisocyanate (MDI) can cause occupational asthma. In this study, we optimized our mouse model of chemical-induced asthma in the C57Bl/6 mice strain using the model agent TDI. Furthermore, we validated MDI in this mouse model and investigated whether cross-reactivity between TDI and MDI is present. On days one and eight, C57Bl/6 mice were dermally treated (20 µl/ear) with 3 % MDI, 2 % TDI or the vehicle acetone olive oil (AOO) (3:2). On day 15, they received a single oropharyngeal challenge with 0.04 % MDI, 0.01 % TDI or the vehicle AOO (4:1). One day later, airway hyperreactivity (AHR) and pulmonary inflammation in the broncho-alveolar lavage (BAL) were assessed. Furthermore, total serum IgE levels, lymphocyte subpopulations in auricular lymph nodes and cytokine levels in supernatants of lymphocytes were measured. Both dermal sensitization with TDI or MDI resulted in increased total serum IgE levels along with T and B cell proliferation in the auricular lymph nodes. The auricular lymphocytes showed an increased release of both Th2 and Th1 cytokines. Mice sensitized and challenged with either TDI or MDI showed AHR, along with a predominant neutrophil lung inflammation. Mice sensitized with MDI and challenged with TDI or the other way around showed no AHR, nor BAL inflammation. Both TDI and MDI are able to induce an asthma-like response in this mouse model. However, cross-reactivity between both diisocyanates remained absent.

Keywords: 4,4′-Methylene diphenyl diisocyanate · 2,4-Toluene diisocyanate · Cross-reactivity · Occupational asthma · C57Bl/6 mice

Introduction

Diisocyanates, low molecular weight agents containing two highly reactive isocyanate groups, are widely produced and consumed in industrialized countries [1]. Predominantly, 4,4-methylene diphenyl diisocyanate (MDI) and 2,4-toluene diisocyanate (TDI) are used and account together for more than 90 % of the global diisocyanate market. These chemicals are used for the production of polyurethanes, which are mainly present in products such as spray foam insulation, coatings, varnishes and paints [2]. Nowadays, it is recognized that diisocyanates can cause occupational asthma (OA), which is the most common occupational respiratory disease in the industrialized world [3].

The incidence of OA varies depending on the form and type of diisocyanate. Furthermore, it is influenced based on the level and duration of diisocyanate exposure [3]. To date, it is well known that diisocyanates are potent sensitizers, with dermal exposure as the leading cause of sensitization. Once immune sensitization to diisocyanates occurs, low airborne levels [below the Occupational Safety and Health Administration (OSHA) established permissible exposure levels] can trigger asthmatic reactions [4,5].

Since evidence showed that TDI is an asthmogen, industry started working on 'safer' compounds. It is known that the basic structure of the monomer attached to the –NCO group (aliphatic or aromatic), and the number of –NCO groups alters the vapor pressure and reactivity of the monomer [3]. The basic structure of TDI consists of one aromatic ring and is highly volatile at room temperature, while MDI is a solid and built upon 2 rings. Because of its lower vapor pressure and therefore safer use, MDI has become the most abundantly produced and consumed diisocyanate [6]. Although MDI is safer than TDI, human and animal studies show that it also affects the respiratory system [5–10].

The mechanism for diisocyanates to interact with the immune system is via protein binding, such as albumin [2,11,12]. Binding to the 'self' protein albumin results into formation of immunogenic haptens which can trigger an immune response, associated with airway inflammation and asthma [6,12]. At present, it is known that MDI and TDI react with 20 and 37 residues of albumin, respectively. All the residues of albumin to which MDI binds are analogous to those of TDI [2].

Cross-reactivity is a phenomenon that occurs in daily practice, via cross-reactive IgEs of homologous proteins, e.g., between mushrooms and airborne molds, or between pollen (bee and weed) and different types of legumes (lupine and peanut) [13–15]. Between chemicals cross-reactivity is also described. Patients sensitized to the chemical methylchloroisothiazolinone (MCI) can react to high levels of methylisothiazolinone (MI) and vice versa [16]. Since the chemicals MDI and TDI have structural and binding similarities, it is important to study possible cross-reactivity between these diisocyanates. In the past, there was already immunological and respiratory evidence concerning cross-reactivity between

MDI and TDI, although the results remain contradictory and the number of patients included in the studies was limited [7,17–22].

In light of the widely spread combinational use of both TDI and MDI, and to elucidate the contradictory results about cross-reactivity, we optimized our mouse model in the C57Bl/6 mice, tested whether MDI has the same potency as TDI to induce an asthmatic response and investigated possible cross-reactivity between TDI and MDI in our experimental mouse model of chemical-induced asthma.

Materials and methods

Reagents

4,4′-Methylenebis phenyl diisocyanate (MDI) (89 %, CAS101-68-8), 2,4-toluene diisocyanate (TDI) (98 %, CAS584-84-9), acetyl-β-methylcholine (methacholine) and acetone were obtained from Sigma-Aldrich (Bornem, Belgium). Sodium chloride (NaCl) 0.9 % was obtained from B. Braun (Melsungen, Germany). Pentobarbital (Nembutal®) was obtained from Sanofi Santé Animale (CEVA, Brussel, Belgium) and isoflurane (Forene®) from Abbott Laboratories (Louvain-la-Neuve, Belgium). The vehicle acetone olive oil (AOO) used to dissolve MDI and TDI consists of a mixture of 2 volumes of acetone and 3 volumes of olive oil (selection de Almazara, Carbonell, Madrid, Spain) for the dermal sensitization and 1 volume of acetone and 4 volumes of olive oil for the challenge. Concentration of MDI is given as (w/v), while TDI is given as percentage (v/v) in AOO.

Mice

Male C57Bl/6 mice (6–8 weeks old) were obtained from Harlan (Horst, The Netherlands). All mice were housed under a conventional animal house with 12-h dark/light cycles. They were housed in filter-top cages and received lightly acidified water and pelleted food ad libitum. All experimental procedures performed in mice were approved by the local ethical committee for animal experiments (P118-2011).

Experimental protocol

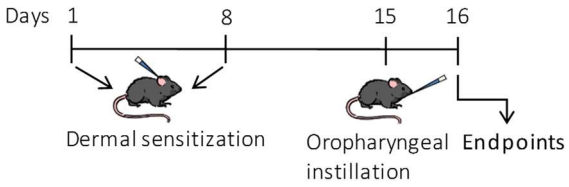

Illustration 1: acute exposure protocol.

On days one and eight, mice received dermal applications of 20 µl of 3 % MDI, 2 % TDI or the vehicle AOO (2:3). On day 15, mice received, under light isoflurane anesthesia, an oropharyngeal instillation of 20 µl 0.04 % MDI, 0.01 % TDI or the vehicle AOO (1:4). All experimental groups are indicated with 2 symbols: The first symbol indicates the dermal treatment on days one and eight, whereas the second symbol indicates the oropharyngeal challenge on day 15. Each group consists of 7–10 mice.

Airway and lung tissue hyperreactivity measurements

Twenty-four hours after the challenge, airway and lung tissue reactivity to methacholine was measured using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). Mice were anesthetized with pentobarbital (70 mg/ kg body weight, Nembutal®). Applying the 'quick prime 3' (QP3) perturbation, mice were exposed to increasing concentrations of methacholine (0, 1.25, 2.5, 5, 10, 20 mg/ml) dissolved in NaCl 0.9 % via an inline nebulizer to measure the airway resistance (Rn), tissue damping (G)

and tissue elasticity (H). After each concentration, the QP3 perturbation was performed 5 times spread over 2 min. If the coefficient of determination (COD) of a QP3 perturbation was lower than 0.90, the measurement was excluded and not used to calculate the average. For each mouse Rn, G and H were plotted against methacholine concentration and the area under the curve (AUC) was calculated to perform statistical analysis.

Broncho-alveolar lavage (BAL) and serum sampling

After measuring airway hyperreactivity to methacholine, mice were killed. Blood was sampled from the retro-orbital plexus and centrifuged (14,000*g*, 4 °C, 10 min), and serum samples were stored at −80 °C until analysis. The lungs were lavaged, in situ, three times with 0.7 ml sterile saline (0.9 % NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cell count), and the BAL fluid was centrifuged (1000*g*, 10 min). For differential cell counts, 250 µl of the resuspended cells (100,000 cells/ml) were spun (300 g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils and neutrophils.

Lymph node analysis

Retro-auricular lymph nodes, obtained from the same mice, were pooled and kept on ice in RPMI-1640 (1×) + GlutaMAX™-I (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 µm) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml tissue culture medium (RPMI-1640 (1×) + GlutaMAX™-I). After centrifugation (1000*g*, 10 min), cells were counted using a Bürker hemocytometer and resuspended (107 cells/ml) in complete tissue culture medium (RPMI-1640 (1×) + GlutaMAX™-I supplemented with 10 % heatinactivated fetal bovine serum, 10 mg/ml streptomycin/penicillin). Five hundred thousand cells were stained with anti-CD3+ (APC), anti-CD4+ (APC-Cy7), anti-CD8+ (PerCP-Cy5.5) and anti-CD25+ (PE) or received a single staining with anti-CD19+ (PE)-labeled antibodies, according to standard procedures (BD Biosciences, Erembodegem, Belgium). Percentages of labeled cells were determined by performing flow cytometry (FACSArray, BD Biosciences, Erembodegem, Belgium) on at least 105 cells. Cells were seeded into 48-well culture plates at a density of 106 cells/ml and incubated in complete RPMI-1640 (1X) + GlutaMAX™-I for 42 h with 2.5 µg/ml of concanavalin A (ConA) (Sigma-Aldrich, Bornem, Belgium). Cell suspensions were then centrifuged (1000 g, 10 min), and supernatant was stored at −80 °C. Concentrations of interleukin-2 (IL-2), IL-4, IL-10, IL-13 and interferon gamma (IFN-γ) were measured via cytometric bead array and analyzed with the FCAPArray version 3 software (BD Biosciences, Erembodegem, Belgium) on the LSR Fortessa (BD Biosciences, Erembodegem, Belgium). Lower detection limits were, respectively, 0.2, 0.3, 9.6, 2.4 and 0.5 pg/ml.

Total serum IgE

The OptEIA Mouse IgE set from Pharmingen (BD Biosciences) was used to measure total serum IgE (diluted 1/70). Measurements were performed according to the manufacturer's instructions.

Data analyses

The data are presented as means with standard error of the mean (SEM) or as individual mice and group means. Normality of distribution was assessed by the Kolmogorov–Smirnov test, followed by a one-way parametric ANOVA combined with a Bonferroni multiple comparison post hoc test (Graph Pad Prism 5.01. Graphpad Software Inc, San Diego, USA). A level of $p < 0.05$ (two tailed) was considered to be significant.

Results

Airway and lung tissue hyperreactivity

Airway hyperreactivity (AHR), using a methacholine provocation test, was assessed 24 h after the challenge. Figure 1a, c, e shows the mean dose–response curves of airway and tissue hyperreactivity response to methacholine provocation, whereas fig. 1b, d, f shows the area under the curve (AUC) of the individual mice and the group average. Mice fully treated with MDI (MDI/MDI) or TDI (TDI/TDI) show a significant AHR, indicated by significantly increased Rn after methacholine challenge (fig. 1a, b), compared to the vehicle-treated control mice (AOO/AOO). The TDI/TDI group was also significantly increased compared to their control group AOO/TDI. Both cross-reactivity groups (TDI/MDI and MDI/TDI) show no AHR compared to all control groups (AOO/AOO, AOO/ MDI and AOO/TDI) (fig. 1a, b).

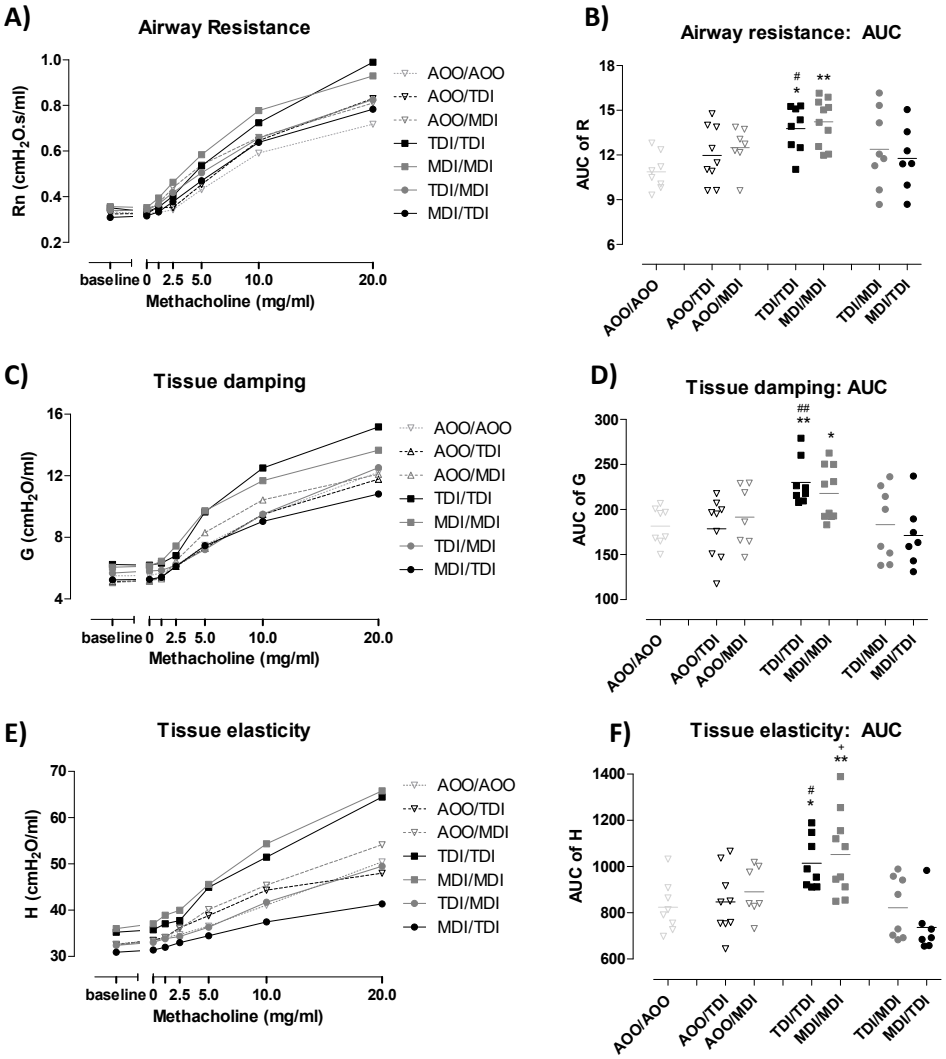

Fig. 1: Airway and tissue hyperreactivity. Airway and tissue hyperreactivity. Methacholine responsiveness was assessed 24 h after the oropharyngeal challenge. The airway (**a**, **b**) (Rn—airway resistance) and tissue reactivity (**c**–**f**) (G—tissue damping and H—tissue elasticity) to methacholine was measured using a forced oscillation technique. **a**, **c**, **e** Show mean values of Rn, G and H; **b**, **d**, **f** show individual values and the group mean of the area under the curve (AUC) of Rn, G and H against methacholineconcentrations between 0 and 20 mg/ml. Experimental groups are identified by two symbols, the *first symbol* indicates the dermal treatment on days 1 and 8, whereas the *second symbol* indicates the oropharyngeal challenge on day 15. n = 7–10 per group. *$p < 0.05$, **$p < 0.01$ compared to the AOO/AOO control group. #$p < 0.05$, ##$p < 0.01$ compared with the AOO/TDI control group. +$p < 0.05$ compared to the AOO/MDI group.

Regarding lung tissue hypersensitivity, there was a significantly increased tissue damping (G) compared to the vehicle-treated group. The TDI/TDI-treated group was also increased compared to their control group AOO/TDI. Furthermore, there was an increase in tissue elasticity (H) in both the TDI/TDI and MDI/MDI groups, compared to the full control group (AOO/AOO) and their own non-sensitized but challenged control group (AOO/TDI and AOO/MDI, respectively) (fig. 1c–f).

Broncho-alveolar lavage

Following AHR measurements, a broncho-alveolar lavage (BAL) was performed. Figure 2 shows the total number of macrophages, neutrophils and eosinophils in the BAL fluid. The total number of macrophages in the completely MDItreated mice is significantly lower compared to the vehicle- treated control group (AOO/AOO). The total number of neutrophils and eosinophils was significantly increased in completely TDI- and MDI-treated mice, compared to the vehicle-treated group (AOO/AOO) and their own non-sensitized but challenged control group (AOO/TDI and AOO/MDI, respectively). In the cross-reactivity groups, only a limited inflammation was present, which was not significant.

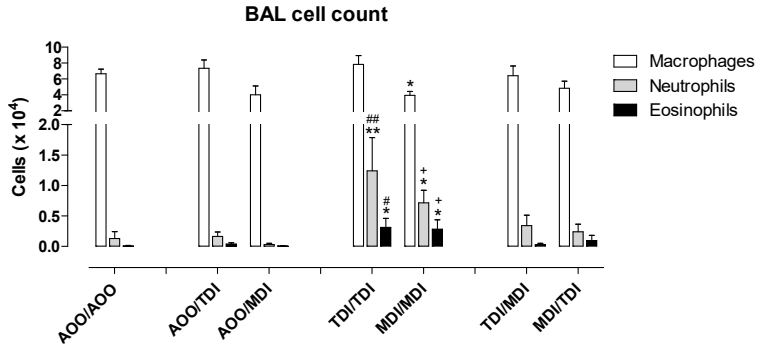

Fig. 2: Broncho-alveolar lavage (BAL) cell count. Total numbers of macrophages, neutrophils and eosinophils were assessed in BAL 24 h after the oropharyngeal challenge. Experimental groups are as in Fig. 1. *n* = 7–10 per group. *Bars* show the mean and SEM. **p* < 0.05, ***p* < 0.01 compared to the AOO/AOO control group. #*p* < 0.05, ##*p* < 0.01 compared to the AOO/TDI control group. +*p* < 0.05 compared to the AOO/MDI group.

Auricular lymphocyte analysis

The lymphocyte subpopulation of the auricular lymph nodes was assessed by FACSArray. Figure 3 shows the total number of CD3+ (T lymphocytes), CD3+CD4+ (Th lymphocytes), CD3+CD4+CD25+ (activated/regulatory Th lymphocytes), CD3+CD8+ (Tc lymphocytes) and CD19+ (B lymphocytes) cells in auricular lymph nodes. In the auricular lymph nodes of all mice sensitized with either TDI or MDI, there was a significantly increased number of both T and B lymphocytes, compared to the vehicle-treated group (AOO/AOO) and their own non-sensitized but challenged control group (AOO/TDI and AOO/MDI, respectively). The increase in T lymphocytes was due to a significant increase in all three subpopulations (Th lymphocytes, ctivated/regulatory Th lymphocytes and Tc lymphocytes).

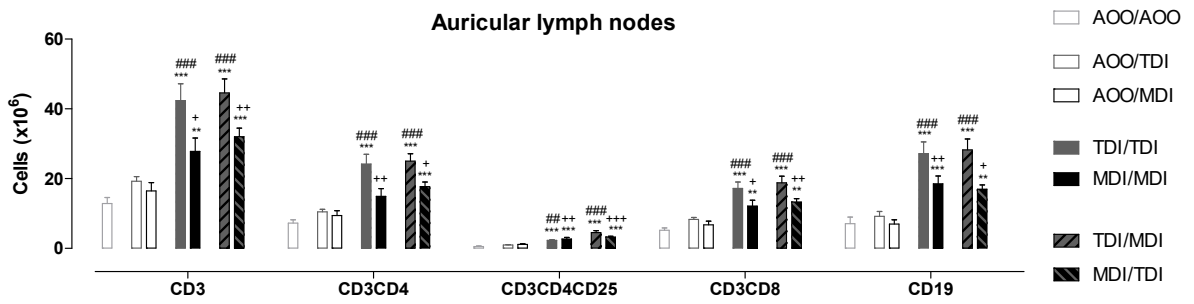

Fig. 3: Lymphocyte subpopulations in auricular lymph nodes. Auricular lymphocytes were stained with anti-CD3+ (T lymphocytes), anti- CD3+CD4+ (Th lymphocytes), anti-CD3+CD4+CD25+ (activated/ regulatory Th lymphocytes) and anti-CD3+CD8+ (Tc lymphocytes) or stained with a single anti-CD19+ (B lymphocytes). Experimental groups are as in Fig. 1. *n* = 7–10 per group. *Bars* show the mean and SEM. ***p* < 0.01, ****p* < 0.001 compared with the AOO/AOO control group. ##*p* < 0.01, ###*p* < 0.001 compared to the AOO/TDI control group. +*p* < 0.05, ++*p* < 0.01, +++*p* < 0.001 compared to the AOO/ MDI group.

Auricular lymphocytes were cultured (42 h) with ConA (2.5 µg/ml). The levels of ex vivo release of IL-4 (fig. 4a), IL-13 (fig. 4b) and IL-10 (fig. 4c) and IFN-γ (fig. 4d) in the supernatant are measured using cytometric bead array. Figure 4 shows that all mice sensitized with TDI or MDI have a significant increase in IL-4, IL-13, IL-10 and IFN- γ, compared to the vehicle-treated group (AOO/AOO) and their own non-sensitized but challenged control group (AOO/TDI and AOO/MDI, respectively).

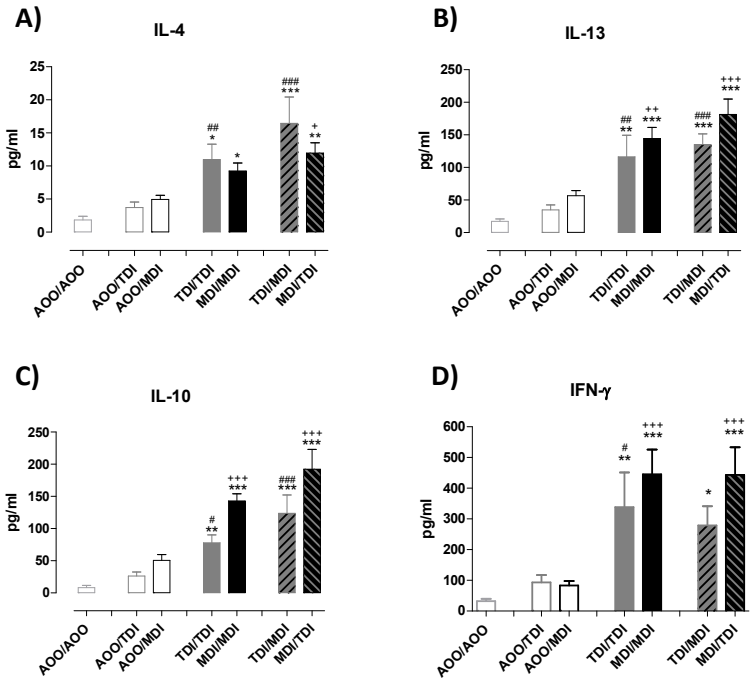

Fig. 4: *Ex vivo* cytokine production of auricular lymphocytes. Levels of IL-4 (**a**), IL-13 (**b**), IL-10 (**c**) and IFN-γ (**d**) were measured by cytometric bead array in supernatant of auricular lymphocytes. Experimental groups are as in Fig. 1. *n* = 7–10 per group. *Bars* show the mean and SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to the AOO/AOO control group. #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 compared to the AOO/TDI control group. +*p* < 0.05, ++*p* < 0.01, +++*p* < 0.001 compared to the AOO/MDI group.

Total serum IgE

Total serum IgE was measured using standard ELISA assays. In fig. 5, the concentration of total serum IgE is presented. All mice sensitized to TDI and MDI show a significant increase in IgE, compared to the vehicle treated group (AOO/AOO) and their own non-sensitized but challenged control group (AOO/TDI and AOO/MDI, respectively).

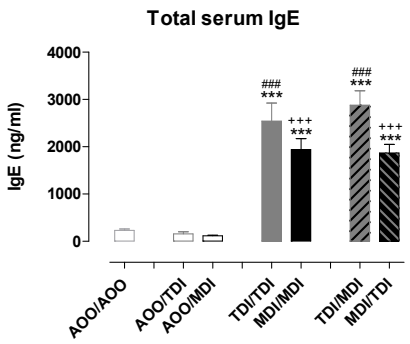

Fig. 5: Total serum immunoglobulin E. Blood was collected 24 h after the oropharyngeal challenge. Total serum IgE was measured using standard ELISA assays. Experimental groups are as in Fig. 1. *n* = 7–10 per group. *Bars* show the mean and SEM. ***$p < 0.001$ compared to the AOO/AOO control group. ###$p < 0.001$ compared to the AOO/TDI control group. +++$p < 0.001$ compared to the AOO/ MDI group.

Discussion

4,4′-Methylene diphenyl diisocyanate (MDI) and toluene- 2,4-diisocyanate (TDI) are widely used chemicals in the polyurethane industry. Both diisocyanates are often used in the same plants, sometimes even together in the same production process [23]. Since the structural and binding similarities and the contradictory result in humans concerning cross-reactivity, it is of importance to test possible cross-reactivity in our mouse model of chemical- induced asthma.

Exposure to diisocyanates is associated with adverse health effects such as sensitization and asthma [24]. Our results show that TDI is a potent asthmogen, which is in agreement with previous results obtained by our group and others [25–31]. Furthermore, we demonstrated that MDI has the same asthmogenic potency as TDI in this mouse model of chemical-induced asthma. Yet, the most important novelty of this study is the fact that in both crossed groups either sensitized with TDI and challenged with MDI or sensitized with MDI and challenged with TDI, no airway/ tissue hyperreactivity or BAL inflammatory response could be found, thus showing no cross-reactivity.

Previously, De Vooght *et al.* [32] showed that applying the same doses of TDI for sensitization and challenge, which give an asthma-like response in BALB/c mice, does not have the same result in the non-atopic C57Bl/6 mice, which remained similar to the control treatment [32]. Here, we demonstrated that by increasing the dermal sensitization concentration of TDI (0.3 % in BALB/c mice to 2 % in C57Bl/6 mice), we are able to induce an asthma-like response in the C57Bl/6 mouse strain. An advantage is the wide availability of different KO C57Bl/6 mice strains for future mechanistic studies. Furthermore, C57Bl/6 mice may reflect asthma development in non-atopic persons.

Based on the selected doses of MDI and TDI, we established dermal sensitization of the mice. This was demonstrated by the results of the different lymphocyte subpopulations, found in the auricular draining lymph nodes, which were significantly increased in mice sensitized to MDI or TDI. This was indicated by an increase in T helper ($CD3^+CD4^+$), T cytotoxic ($CD3^+CD8^+$), activated/ regulatory Th ($CD3^+CD4^+CD25^+$) and B lymphocytes ($CD19^+$). The cytokine release of ConA-stimulated lymphocytes displayed an increased mixed Th1/Th2 cytokine response, which is evidenced by an increased expression of Th2 cytokines IL-13, IL-4, IL-10 as well as Th1 cytokine IFN-γ. This is in agreement with the previous findings [26,29,33]. The sensitization of the mice is also confirmed by an increase in total IgE after skin exposure of both MDI and TDI. This was also described by other groups for both chemicals [6,9,26,30]. The role of IgE in diisocyanate-induced asthma is still under debate [34].

Twenty-four hours after the single challenge, we observed that only mice receiving an antigen-specific oropharyngeal challenge with either TDI or MDI (TDI/TDI; MDI/MDI) resulted in lung inflammation characterized by mainly neutrophils and eosinophils, which is consistent with previous results using

BALB/c mice [33]. The predominant neutrophilic inflammation is also observed in patients with TDI-induced asthma and other TDI-induced animal models of asthma [26,30,31,35–37]. For the first time, we confirm that also MDI induces an asthma-like response in this mouse model, indicated by an increase in eosinophils and mainly neutrophils in BAL fluid. Previously, other animal models showed increased levels of mainly neutrophils or eosinophils in BAL fluid after multiple exposures of MDI [6,9]. This is in accordance with human studies, where MDI has been causally related to induce asthma, with symptoms such as variable airflow limitation, eosinophilic bronchitis and respiratory irritant effects after exposures on the work floor [10,38,39].

Besides the lung inflammation, airway hyperreactivity (Rn) to increasing concentrations of methacholine (AHR) was measured 24 h later after the single challenge. Both TDI/TDI and MDI/MDI groups showed AHR compared to the complete control group. Furthermore, we also showed a significant increase in the TDI/TDI and MDI/MDI groups of tissue damping (G) and tissue elasticity (H), which is indicative for lung tissue hypersensitivity. This can be explained by the presence of epithelial necrosis and epithelial shedding after an oropharyngeal challenge as shown by De Vooght *et al*.[33].

Unlike the TDI/TDI and MDI/MDI group, the cross-reactivity groups (TDI/MDI and MDI/TDI) presented no asthma-like response. Despite successfully sensitized, no statistical changes in airway hyperreactivity and tissue hypersensitivity compared to the vehicle control group could be found. Furthermore, only a nonsignificant lung inflammation was present in the BAL. Yet, some mice in the cross-reactivity groups do show positive responses. This might be due to experimental variation, but it could also be due to actual cross-reactivity as shown for other chemicals [16]. Human studies on cross-reactivity currently remain indecisive. Several research groups indicated that respiratory reactions can be elicited by other diisocyanates to which the workers were sensitized [8,21]. Using inhalation provocation tests, O'Brien *et al.* demonstrated that four persons only exposed to TDI exhibited a bronchoconstrictive response to MDI inhalation [8]. As already indicated, this was also seen in our results, and some mice in the cross-reactivity groups (TDI/ MDI and MDI/TDI) showed AHR and neutrophils in BAL 24 h after the challenge. The crossed bronchial responses may be linked with crossed immunoreactivity. Immunological studies showed mutual cross-reactivity among isocyanate- bound human serum albumin (HSA) to which the workers were not sensitized [18–22,40]. Also Ruwona *et al*. showed that murine monoclonal antibodies toward TDIHSA cross-react with MDI-HSA and HDI-HAS [41]. Furthermore, Lemons *et al.* produced a murine IgM mAb with broad specificity toward the most commonly used isocyanates TDI-HSA, MDI-HSA and HDI-HSA [42].

On the other hand, there is evidence in exposed workers that there is no crossed respiratory or immunological reaction to different types of diisocyanates, which is in line ith the main findings of this study [7,17,43]. Mapp *et al.* demonstrated that in two patients sensitized to MDI, no drop of forced

expiratory volume in 1s (FEV1) was elicited after an inhalation challenge to TDI [7]. The absence of respiratory cross-reactivity was also confirmed by immunological evidence. IgG antibodies to MDI, of subjects with asthma induced by MDI, appear to be specificor MDI without cross-reactivity with TDI [17]. Furthermore, Wisnewski and Liu developed six different murine monoclonal antibodies which all bind to MDI-conjugated proteins, but not to TDI or HDI protein conjugates [12].

The absence of cross-reactivity can be explained by the different reactivity of the functional group (diisocyanates) of MDI and TDI. Moreover, TDI has a higher affinity for lysine residues (e.g., on albumin), and therefore, MDI binds to less lysine residues compared to TDI [2,24]. Also, the different stereo isomers of MDI and TDI may lead to a different binding to albumin, which makes it further difficult to generate the same epitope, although not exclusive. Theoretically, MDI and TDI have the ability to cross-react due to the communalities in albumin-binding sites.

In this study, we established three major conclusions. First of all, we successfully optimized our mouse model of chemical-induced asthma in the C57Bl/6 mouse strain. Secondly, we conclude that MDI responses are similar to those of TDI in this model. Thirdly, the most important finding of this study is the absence of cross-reactivity between MDI and TDI.

Acknowledgments: This work was supported by a grant of the Interuniversity Attraction Poles Program-Belgian State-Belgian Science Policy [IUAP P7/30].

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

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Chapter 4

Dermal exposure determines the outcome of repeated airway exposure in a long-term chemical-induced asthma-like mouse model

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Abstract

Introduction: Exposure to diisocyanates is an important cause of occupational asthma (OA) in the industrialized world. Since OA occurs after long-term exposure to diisocyanates, we developed a chronic mouse model of chemical-induced asthma where TDI was administered at two different exposure sites.

Aim: Study the presence of DC, T- and B- cells and effect of exposure route after long-term airway isocyanate exposure, with or without prior skin exposure.

Method: On days 1 and 8, BALB/c mice were dermally treated (20µl/ear) with 0.5% TDI or the vehicle AOO (3:2). Starting from day 15, they received under light anesthesia five times in a week, for five successive weeks, intranasal instillations with 0.1% TDI or AOO (3:2). One day after the last instillation airway hyperreactivity (AHR) to methacholine was assessed, followed by an evaluation of pulmonary inflammation, structural lung changes and immune-related parameters such as lymphocyte subpopulations ($CD4^+$, $CD8^+$, $CD25^+$ and $CD19^+$) and *in vitro* cytokine production profile (IL-4, IL-13, IL-10 and IFN-γ) in auricular and cervical lymph nodes. Blood was sampled to determine total serum IgE, IgG_1, IgG_{2a} and IL-13. Dendritic cells (DC), T- and B- cells were assessed in the lungs and ALN.

Results: Our data indicate that mice repeatedly exposed via the nasal mucosa to TDI results in systemic sensitization, a mixed Th1/Th2 type immune response, without the presence of AHR. However, when mice are first sensitized with TDI via the skin, repeated nasal mucosa exposure to TDI leads to a pronounced Th2 response and AHR.

Conclusion: Dermal exposure to TDI determines the respiratory outcome after repeatedly respiratory exposure to TDI.

Keywords: Toluene diisocyanates, BALB/c mice, dendritic cells, paucigranulocytic asthma, IgE, airway hyperreactivity, Th2/Th1 response

Introduction

Exposure to diisocyanates is an important cause of occupational asthma (OA). Diisocyanates are low molecular weight (LMW) compounds, used in spray foam insulation, plastic packaging and paints. They may act as haptens and bind with self-proteins to form functional antigens recognized by the immune system [1,2]. To date, a growing body of animal and workplace observational studies suggest that skin exposure to isocyanates elicits immune sensitization. After sensitization, very low airway exposures, can elicit asthma [3–6]. As a consequence, asthma still continues to occur even after reducing the airborne exposure below the occupational exposure limit (according to the American Conference of Governmental Industrial Hygienists 2015 the TLV is 0.001ppm) [4].

Previously, we showed in a mouse model that dermal TDI sensitization can lead to asthma-like features after one TDI specific airway challenge, characterized by airway hyperreactivity (AHR), airway inflammation and Th1/Th2 immune response, which are mediated by the TRPA1 receptor, IL-13, B-lymphocytes and neutrophils [7–13]. However, despite the importance of unraveling the mechanistic pathways, the clinical relevance of such an acute asthma model is limited.

Sub-chronic and chronic pulmonary exposure to diisocyanates has been studied before in rat and mouse to mimic human asthma [14–17]. While T- and B- lymphocytes and the secreted cytokines have been investigated in diisocyanate asthma, little is known about the involvement of dendritic cells (DC) in this type of asthma [18]. DC are the most important antigen presenting cells (APC) responsible for initiating an immune response. They are located near the epithelium, where they can sense and capture inhaled antigens present in the environment. After collecting, DC process the allergens into small peptides and migrate to the T cell zone of the local draining lymph nodes. There they present the processed antigens via the major histocompatibility complexes (MHC class I and II) to naive T cells, inducing polarized T cells [19,20]. In total, three distinct DC subsets are defined in the lung each with their own surface markers and functional specialization: conventional DC (cDC), which can be further divided in $CD11b^+$ and $CD103^+$ cDC, plasmacytoid DC (pDC) and monocyte derived DC (moDC) [21]. Each of those DC subset are geared to induce a particular helper T cell respons. These responses are context dependent: environmental signals (house dust mite, cigarette smoke, diesel exhaust particles etc…), dose, routes of exposures and cell-released molecular patterns influence the function of DC which emphasize the plasticity of DC [21–24].

We tested the importance of exposure route (dermal/respiratory) and the presence of DC, B- and T-cells in a chronic mouse model consisting of long-term nasal mucosa exposure, with or without a brief prior skin exposure.

Material and methods

Reagents

Toluene-2,4-diisocyanate (TDI) (98%, CAS584-84-9), acetyl-β-methylcholine (methacholine) and acetone were obtained from Sigma-Aldrich (Bornem, Belgium). Pentobarbital (Nembutal®) was obtained from the internal animalium at the KU Leuven. The vehicle acetone olive oil (AOO) used to dissolve TDI consists of a mixture of 2 volumes acetone and 3 volumes of olive oil (highly refined, low acidity, CAS8001-25-0) obtained from Sigma Aldrich. Concentration of TDI is given as percentage (v/v) in AOO.

Mice

Male BALB/c mice (6-8 weeks old) were obtained from the internal stock of the animalium (KU Leuven, Belgium). All mice were housed in a conventional animal house with 12h dark/light cycles, in filter top cages. They received lightly acidified water and pelleted food ad libitum. All experimental procedures were approved by the local Ethical Committee for animal experiments (P063/2015).

Experimental protocol

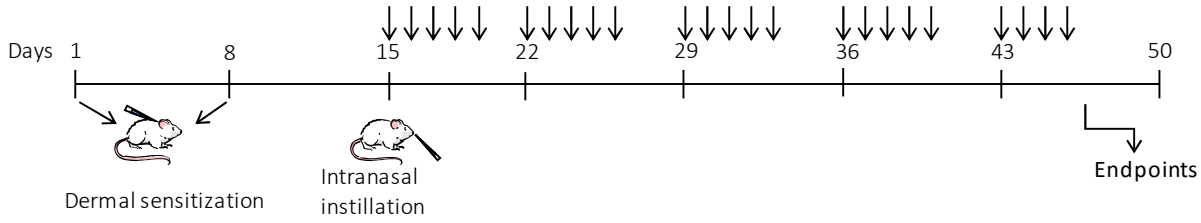

Illustration 1: chronic exposure protocol.

On days one and eight mice received dermal applications of 20 µl of 0.5% TDI or the vehicle AOO on the dorsum of both ears. From day 15, mice received, under light isoflurane anesthesia, intranasal instillations (20 µl) of 0.1% TDI or the vehicle AOO, 5 times a week for 5 consecutive weeks. All experimental groups are indicated with two symbols: the first symbol indicates the dermal treatment on days one and eight, whereas the second symbol indicates the intranasal instillations. The AOO/AOO group never received TDI, the AOO/TDI group only received intranasal TDI and the TDI/TDI group received TDI dermally and intranasally. Each group consisted of 6 to 17 mice.

Analysis in different sets of experiments

In this study, we have three different sets of experiments. In the first set, we evaluated the AHR, histological changes in the lung, IgE and IgG in blood serum, IgE and inflammation in BAL and T- and B-cells with *in vitro* cytokine secretion of the auricular and cervical lymph nodes (n=6-7/group). To increase the number of the auricular and cervical lymph nodes, we added a second set where we evaluated the T- and B- cells with *in vitro* cytokine secretion of the auricular and cervical lymph nodes (n=4-6/group).

To analyze the DC, we added a third set. In the latter set we analyzed IgE in blood serum (to confirm sensitization), inflammation in BAL and DC, T- and B- cells in lung cells (n=8/group). In a subgroup of this set we also evaluated the T- and B- cells in the auricular and cervical lymph nodes (n=4/group).

Airway and lung tissue hyperreactivity measurements

Twenty-four hours after the last instillation, airway and lung tissue reactivity to methacholine was measured using a forced oscillation technique (FlexiVent 7.1, SCIREQ, Montreal, Canada). Mice were anesthetized with pentobarbital (70 mg/kg body weight, Nembutal®). Applying the 'quick prime 3'(QP3) perturbation, airway resistance (Rn), and tissue elasticity (H) to increasing concentrations of methacholine (0, 1.25, 2.5, 5, 10, 20 mg/ml) was measured. After each concentration, the QP3 perturbation was performed 5 times spread over 2 minutes. If the COD (Coefficient of Determination) of a QP3 perturbation was lower than 0.90, the measurement was excluded and not used to calculate the average. For each mouse Rn and H were plotted against methacholine concentration and the area under the curve (AUC) was calculated to perform statistical analysis.

Lung, lymph node and serum analysis

Serum sampling

After measuring airway hyperreactivity to methacholine, mice were sacrificed. Blood was sampled from the retro-orbital plexus, centrifuged (14000 g, 4 °C, 10 min) and serum samples were stored at -80°C until analysis.

Broncho-alveolar lavage (BAL)

The lungs were lavaged, *in situ*, three times with 0.7 ml sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cell count) and the BAL fluid was centrifuged (1000 g, 10 min). For differential cell counts, 250 µl of the resuspended cells (100.000 cells/ml) were spun (300 g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

Single cell suspension

The pulmonary circulation was rinsed with saline/EDTA to remove the intravascular pool of cells. A cell suspension of the lungs was obtained using digestion medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol [Gibco; Invitrogen, Paisley, United Kingdom], 100 U/mL penicillin–100 mg/mL streptomycin [Invitrogen], 1 mg/mL collagenase type 2 [Worthington Biochemical, Lakewood, NY], and 0.02 mg/mL DNase I [grade II from bovine pancreas; Boehringer Ingelheim, Ingelheim, Germany]) for 45 minutes at 37°C and 5% CO2. Red blood cells were lysed by

using ammonium chloride buffer. Cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/ml) in PBS.

The left and right retro-auricular as well as the two cervical lymph nodes from the same mice were pooled and kept on ice in RPMI-1640 (1X) + GlutaMAX™-I (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 µm) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml tissue culture medium (RPMI-1640 (1X) + GlutaMAX™-I). After centrifugation (1000 g, 10 min), cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/ml) in complete tissue culture medium (RPMI-1640 (1X) + GlutaMAX™-I supplemented with 10% heat-inactivated fetal bovine serum, 10 mg/ml streptomycin/penicillin).

Flow cytometry

T- and B- Cells

Five-hundred thousand cells from the auricular/cervical lymph nodes and lung were stained with anti-$CD3^+$ (APC), anti-$CD4^+$ (APC-Cy7), anti-$CD8^+$ (PerCP-Cy5.5) and anti-$CD25^+$ (PE), or received a single staining with anti-$CD19^+$ (PE) labeled antibodies, according to standard procedures (BD Biosciences, Erembodegem, Belgium). Percentages of labeled cells were determined by performing flow cytometry (FACSArray, BD Biosciences, Erembodegem, Belgium) on at least 10^5 cells.

Dendritic cells

To minimize non-specific bindings, two million cells of lung and auricular lymph nodes were preincubated with anti-CD16/CD32 (Clone 2.4G2) (from BD Biosciences, San Jose, Calif). After live/dead staining using the Zombie Aqua™ Fixable Viability Kit, cells were labeled with combinations of anti-mouse fluorochrome-conjugated mAbs against CD45 (clone 30-F11), CD11c (N418), MHCII (M5/114.15.2), CD11b (M1/70), CD103 (2E7), CD64 (X54-5/7.1) or Siglec-H (551) (all from Biolegend, San Diego, Calif). Data acquisition was performed on a LSR Fortessa flow cytometer running DIVA software (BD Biosciences, San Jose, Calif). FlowJo software (TreeStar, Inc, Ashland, Ore) was used for data analysis. We composed our DC panel based on those of sharen provoost *et al.* and Maud Plantinga *et al.* [21,24,25].

Cytometric bead array

Cells of the auricular and cervical lymph nodes were seeded into 48-well culture plates at a density of 10^6 cells/ml and incubated in complete RPMI-1640 (1X) + GlutaMAX™-I medium for 42h with 2.5 µg/ml of concanavalin A (ConA) (Sigma–Aldrich, Bornem, Belgium). Cell suspension were centrifuged (1000 g, 10 min) and supernatant was stored at −80 °C. Concentrations of interleukin (IL)-4, IL-10, IL-13 and interferon gamma (IFN-γ) were measured via the LSR Fortessa (BD Biosciences, Erembodegem, Belgium). Detection limits were 0.2 pg/ml, 0.3 pg/ml, 9.6 pg/ml, 2.4 pg/ml and 0.5 pg/ml, respectively.

Enzyme-linked Immunosorbent Assay (ELISA)

The OptEIA Mouse IgE set from pharmingen (BD Biosciences) was used to measure total IgE in serum (diluted 1/70 for AOO/AOO and 1/1000 for the AOO/TDI and TDI/TDI treated group) and BAL (undiluted). Measurements were performed according to the manufacturer's instructions. For the measurement of total serum IgG_1 (diluted 1/8000 or $1/10^6$) and IgG_{2a} (diluted1/4000), plates were coated using purified rat anti-mouse IgG_1 (Cat: 553445, BD PharmingenTM) and purified rat anti-mouse IgG_{2a} (Cat: 553446, BD PharmingenTM). A standard was created using purified mouse IgG_1 (Cat: 557273, BD PharmingenTM) and purified mouse IgG_{2a} (Cat: 553454, BD PharmingenTM). Further measurements were performed according to the manufacturer's (BD Pharmingen) instructions with the use of biotinylated anti-mouse avidin horseradish peroxidase conjugate (HRP rat anti-mouse IgG_1, Cat:559626, biotin rat anti-mouse IgG_{2a}, Cat: 553388, and streptavidin HRP, Cat: 554066, BD PharmingenTM).

Histology

Lungs were instilled with 4% formaldehyde until full inflation of all lobes. An experienced pathologist evaluated lung injury on slides stained with hematoxylin and eosin (H&E), in a blinded manner.

Data analyses

The data are presented as means with standard deviation (SD), or as individual mice and group mean. Normality of distribution was assessed by the Kolmogorov-Smirnov test, followed by a one-way parametric ANOVA combined with a bonferroni multiple comparison post hoc test. Dose–response curves (AHR) were analysed using two-way parametric ANOVA, followed by a Bonferroni multiple comparison post hoc test (Graph Pad Prism 5.01. Graphpad Software Inc, San Diego, USA). A level of $p<0.05$ (two tailed) was considered significant.

Results

Markers of sensitization

Dendritic cells in auricular lymph nodes

Figures 1B and C show a significant increase of DC, CD11b$^+$ cDC in both groups receiving TDI (AOO/TDI and TDI/TDI) compared to the control group (AOO/AOO). Moreover, there is an increasing trend of moDC and a decreasing trend of CD103$^+$ cDC and pDC in both TDI-treated groups (fig. 1C). Total amount of leukocytes were not significant increased (fig. 1A).

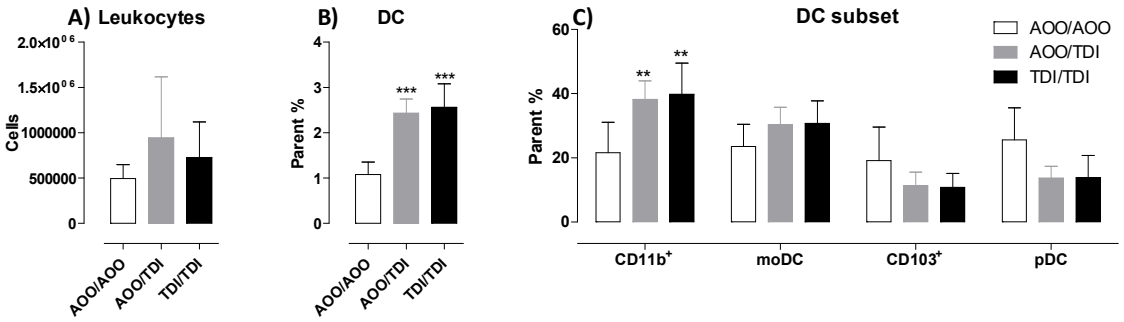

Fig. 1: Leukocytes and dendritic cells ALN. Cells were analyzed using a flow cytometer. Leukocytes were assessed as CD45$^+$ cells (**a**). DC were detected as low auto fluorescent MHCII$^+$CD11c$^+$ DC (**b**). Fig **c** demonstrates the parent % of CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^-$CD103$^+$ cDC (CD103$^+$ cDC), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^+$CD103$^-$ conventional DC (CD11b$^+$ cDC), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^+$ CD64$^+$ monocyte derived DC (moDC), and CD45$^+$ low auto fluorescent MHCII$^+$ CD11c$^+$ SiglecH$^+$ plasmacytoid DC (pDC). Experimental groups are identified by two symbols; the first symbol indicates the dermal treatment on days 1 and 8, whereas the second symbol indicates the intranasal instillations. n=8 per group. Table shows the mean with SD. **p<0.01 and ***p<0.001 compared with the AOO/AOO control group.

T- and B- cells in auricular and cervical lymph nodes

Figure 2A shows that mice treated with TDI (AOO/TDI and TDI/TDI) have significantly more T-(CD3$^+$) and B-(CD19$^+$) cells in the auricular lymph nodes than the AOO/AOO control group. Moreover, all T cell subpopulations (CD4$^+$ T helper, CD4$^+$CD25$^+$ activated/regulatory T cells and CD8$^+$ cytotoxic T cells) were significantly increased in both TDI-treated groups. In the cervical lymph nodes (fig. 2B), in general lower numbers of T- and B- cells were found in the TDI-treated mice. CD19$^+$ B cells were increased in both TDI treated groups, while CD8$^+$ cytotoxic T cells were only increased in the AOO/TDI treated group (fig. 2B).

Ex vivo cytokine secretion

Ex vivo stimulation of auricular lymphocytes with concanavalin A resulted in significantly increased concentrations of IL-4, IL-13, IL-10 and IFN-γ in the TDI/TDI treated group (fig. 3A-D). While in the AOO/TDI treated group, a significant increase in IL-13 and IFN-γ was measured (fig. 3A-D). The *ex vivo* cytokine production of the cervical lymphocytes was substantially lower than in the auricular lymph nodes (fig 3E-H). In the TDI/TDI treated group, increased concentrations of IL-4, IL-13 and IL-10 were

found, while in the AOO/TDI treated group, only IL-13 was significantly increased (fig. 3E-G). The IFN-γ levels were not significantly changed (fig. 3H).

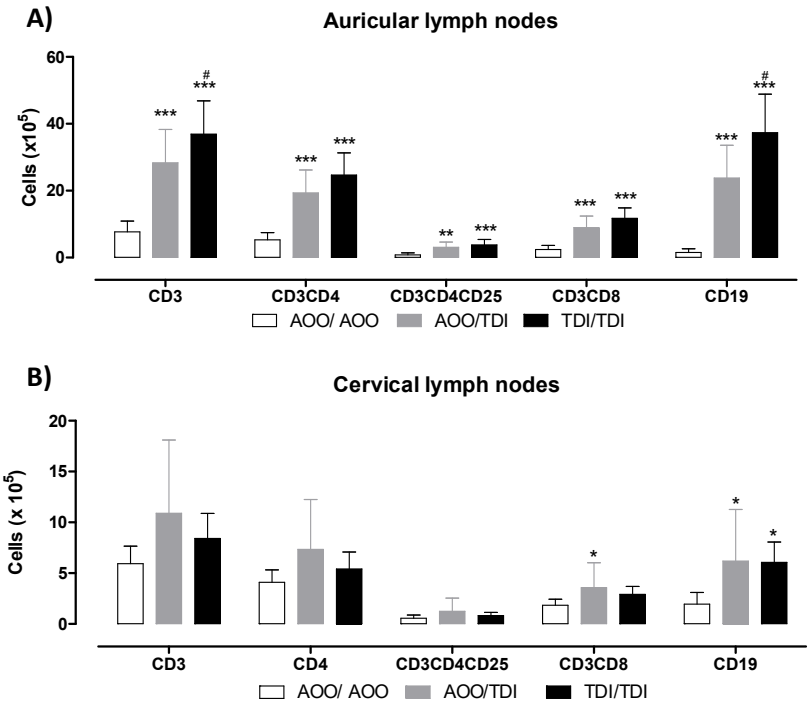

Fig. 2: Lymphocyte subpopulations in auricular and cervical lymph nodes. Lymphocyte subpopulations in ALN (**a**) and CLN (**b**) were measured using flow cytometry. Lymphocytes were stained with anti-CD3$^+$ (T lymphocytes), anti-CD3$^+$CD4$^+$ (Th-lymphocytes), anti-CD3$^+$CD4$^+$CD25$^+$ (activated/regulatory Th-lymphocytes) and anti-CD3$^+$CD8$^+$ (Tc-lymphocytes) or stained with a single anti-CD19$^+$ (B-lymphocytes). Experimental groups are as described in fig. 1. n= 14 per group. Bars show the mean with SD. *p<0.05, **p<0.01 and ***p<0.001 compared with the AOO/AOO control group. $^{\#}$p<0.05 compared to the AOO/TDI treated group.

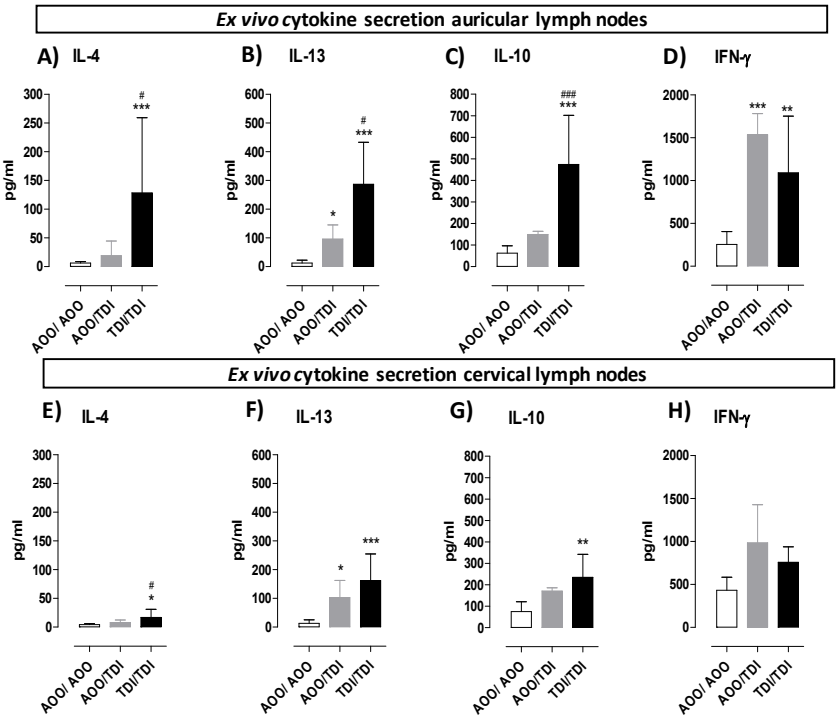

Fig. 3: *Ex vivo* cytokine production by auricular and cervical lymphocytes. Levels of IL-4 (**a, e**), IL-13 (**b, f**), IL-10 (**c, g**) and IFN-γ (**d, h**) were measured by Cytometric Bead Array in supernatant of auricular and cervical lymphocytes. Experimental groups are as described in fig. 1. n= 10-11 per group for the ALN, and 6-10 for the CLN. Bars show the mean and SD. *p<0.05, **p<0.01 and ***p<0.001 compared with the AOO/AOO control group. $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001 compared to AOO/TDI.

Immunoglobulines and serum IL-13

Figure 4A shows that mice which received weekly intranasal instillations with TDI (AOO/TDI group), resulted in a significant increase of total serum IgE compared to the control (AOO/AOO) group. However, prior TDI skin sensitization, followed by TDI-intranasal instillations (TDI/TDI group) caused a much higher increase of IgE in serum, compared to the AOO-treated control group (fig. 4A). Besides the serum, IgE was also significantly increased in the BAL fluid of the TDI/TDI group (fig. 4D). IgG_1 was significantly increased and IgG_{2a} was significantly decreased in the serum of the TDI/TDI group compared to the control (AOO/AOO) group (fig. 4B and C). IL-13 in serum showed a step-wise significant increase in both TDI-treated groups (AOO/TDI and TDI/TDI), compared to the AOO control group. Nevertheless, the IL-13 levels in the TDI/TDI group was much higher compared to AOO/TDI group (fig. 4E).

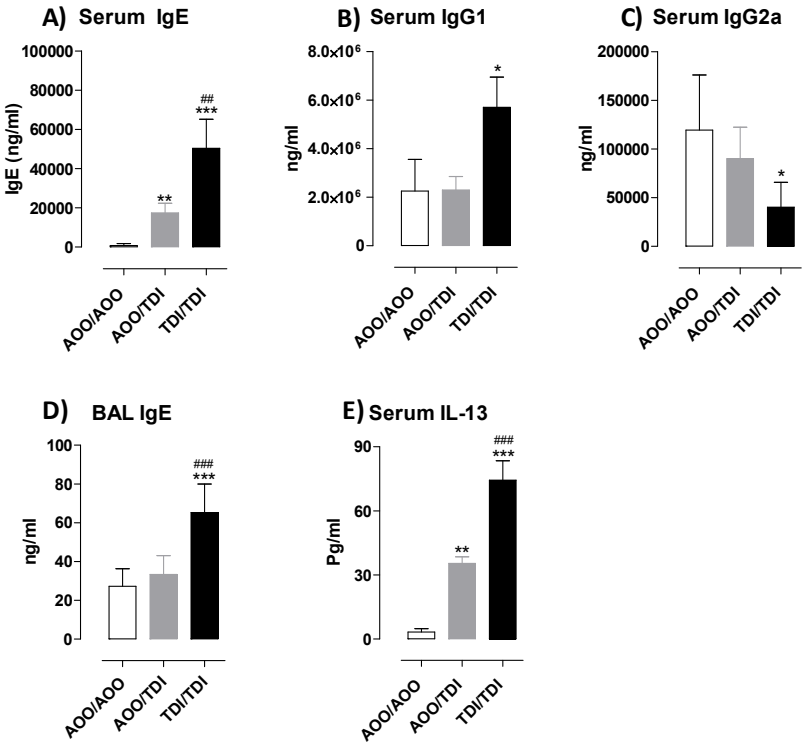

Fig. 4: Total levels of immunoglobulin (Ig) E, IgG_1 and IgG_{2a} and IL-13. Serum IgE (**a**), BAL IgE (**d**), serum IgG1 (**b**), serum IgG2a (**c**), and serum IL-13 (**e**) were measured using ELISA. Blood and BAL were collected 24h after the last intranasal challenge. Experimental groups are as described in fig. 1. n= 6-7 per group for serum IgG2a, IgG1, IL-13 and BAL IgE, and n=14-15 for serum IgE. Bars show the mean and SD. *p<0.05, **p<0.01 and ***p<0.001 compared with the AOO/AOO control group. $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to AOO/TDI.

Airway response

Airway hyperreactivity

Figures 5A and C show the dose-response increase of methacholine-induced airway resistance and tissue elasticity, while figures 5B and D show the area under the curve of the individual mice and the group average. Airway hyperreactivity (AHR) in the TDI/TDI treated group were significant increased compared to both other groups (AOO/TDI and AOO/AOO).

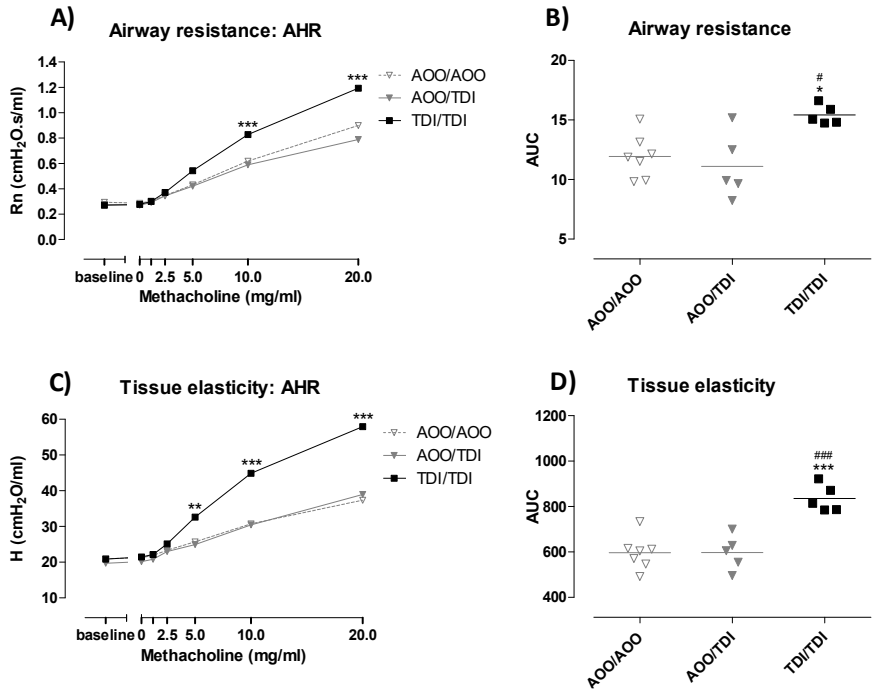

Fig. 5: Airway and tissue hyperreactivity. Methacholine responsiveness was assessed 24h after the last intranasal challenge. The airway reactivity to methacholine was measured using a forced oscillation technique. Fig **a** and **c** show the mean values of Rn and H, respectively. Fig **b** and **d** show individual values and the group mean of the area under the curve (AUC) of Rn and H against methacholine concentrations between 0 and 20 mg/ml, respectively. Experimental groups are as described in fig. 1. n= 5-7 per group. *p<0.05, **p<0.01, ***p<0.001 compared with the AOO/AOO control group. #p<0.05 and ###p<0.001 compared to AOO/TDI treated group.

Broncho-alveolar lavage and lung histology

The AHR in the TDI/TDI treated group was not accompanied by an increase of inflammatory cells (neutrophils, eosinophils or lymphocytes) in the BAL fluid, where only macrophages were present (data not shown). Yet, histological analysis revealed that long-term intranasal exposure to TDI resulted in limited increased inflammation round the blood vessels , epithelial shedding and alveolar widening, although not significant (fig. 6A-C).

T- and B- cells in the lung

Table 1 shows a significant increase in $CD3^+D8^+$ cytotoxic T cells in the TDI/TDI treated group, compared to the AOO/AOO group. Yet, no statistical differences were found in $CD3^+$ T helper cells of the TDI-treated groups (AOO/TDI and TDI/TDI), compared to the AOO/AOO control group.

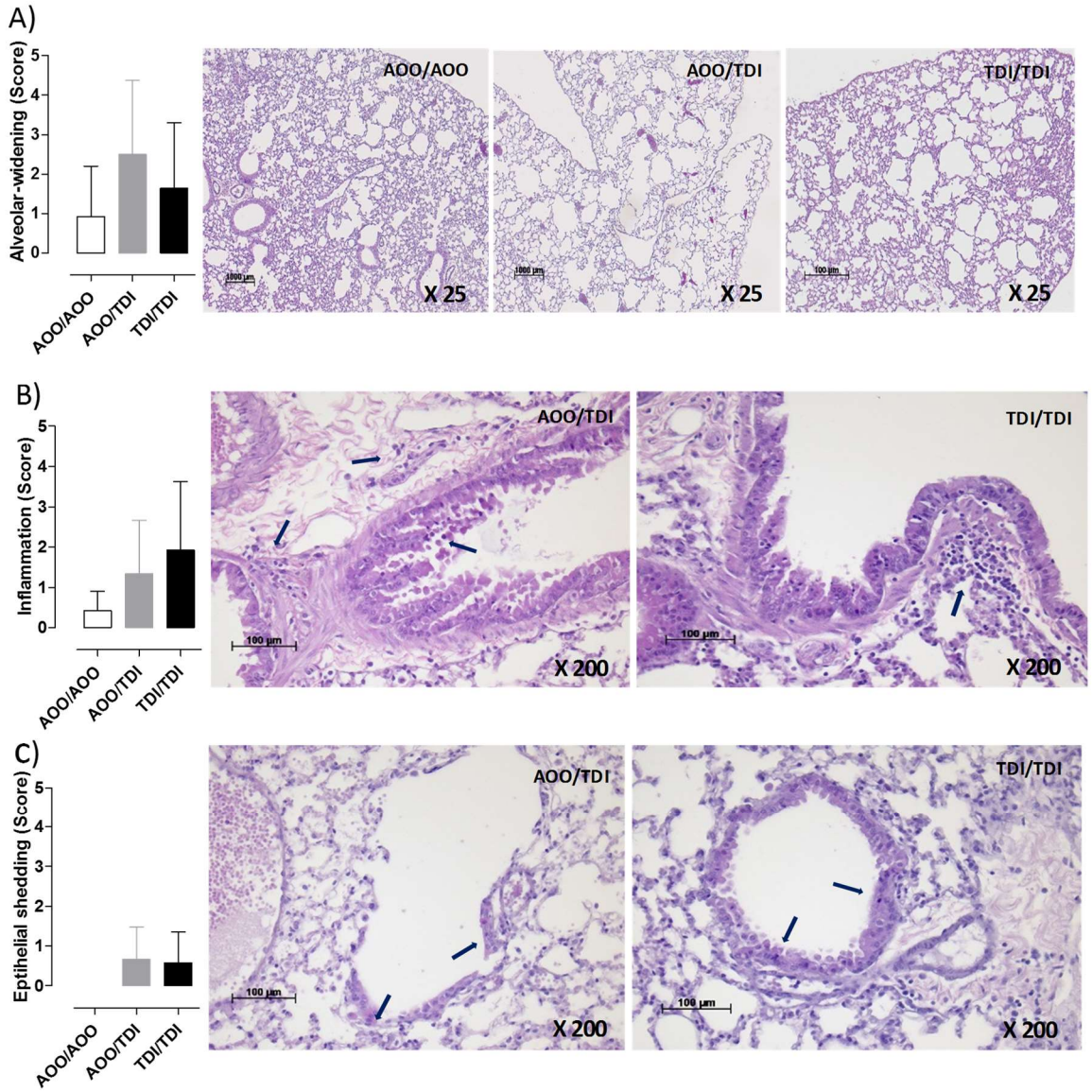

Fig. 6: Lung histology. H&E staining was performed on lung slices 24 after the last intranasal instillation. Alveolar widening (fig. 6**a**, 25x amplification), inflammation (fig. 6**b**, 200x amplification) and epithelial shedding (fig. 6**c**, 200x amplication) were validated. Experimental groups are as described in fig.1. n= 6-7 per group. Bars show the mean with SD.

Table 1: Lymphocyte subpopulations in the lung.

T cells lung (%)	AOO/AOO	AOO/TDI	TDI/TDI
$CD3^+$	9,09 ± 1,97	10,38 ± 1,76	10,77 ± 2,21
$CD3CD4^+$	5,9 ± 1,38	6,76 ± 1,30	6,83 ± 0,98
$CD3CD4CD25^+$	0,82 ± 0,37	1,12 ± 0,53	0,96 ± 0,22
$CD3CD8^+$	2,63 ± 0,66	3,12 ± 0,62	**3,50 ± 0,96***
$CD19^+$	12,16 ± 3,59	9,69 ± 2,38	10,64 ± 3,42

Lung cells were stained with anti-$CD3^+$ (T lymphocytes), anti-$CD3^+CD4^+$ (Th lymphocytes), anti-$CD3^+CD4^+CD25^+$ (activated/regulatory Th lymphocytes) and anti-$CD3^+CD8^+$ (Tc lymphocytes) or stained with a single anti-$CD19^+$ (B lymphocytes). Experimental groups are as described in fig. 1. n= 12-14 per group. Bars show the mean with SD. *p<0.05 compared with the AOO/AOO control group.

Dendritic cells in the lung

Figure 7D shows that only the moDC subpopulation in the lung from the TDI/TDI treated group is significantly increased compared to the AOO-treated control group. There were no significant changes in total number of leukocytes, dendritic cells, nor in the DC subpopulations: 103^{+}cDC, $CD11b^{+}$cDC, pDC and macrophages (fig. A-D).

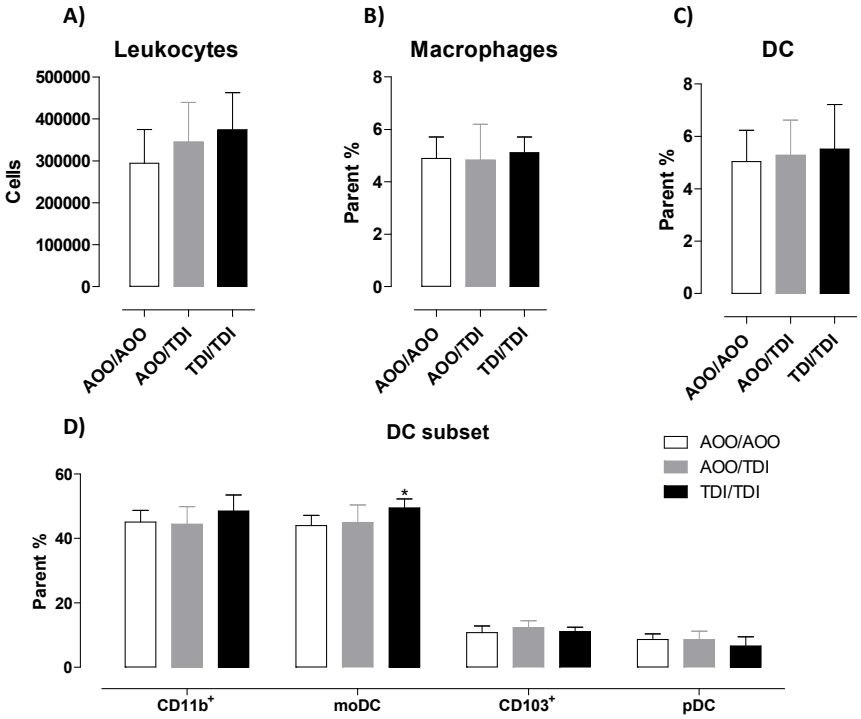

Fig. 7: Leukocytes, macrophages and DC subpopulations in the lung. Cells were analyzed using flow cytometry as $CD45^{+}$ leukocytes (**a**), $CD45^{+}$ low auto fluorescent $MHCII^{+}CD11c^{+}$ DC (DC) (**c**), $CD45^{+}$ low auto fluorescent $MHCII^{+}CD11c^{+}CD11b^{-}$ $CD103^{+}$ cDC ($CD103^{+}$ cDC), $CD45^{+}$ low auto fluorescent $MHCII^{+}CD11c^{+}CD11b^{+}$ $CD103^{-}$ conventional DC ($CD11b^{+}$ cDC), $CD45^{+}$ low auto fluorescent $MHCII^{+}CD11c^{+}CD11b^{+}$ $CD64^{+}$ monocyte derived DC (moDC), and $CD45^{+}$ low auto fluorescent $MHCII^{+}$ $CD11c^{+}$ $SiglecH^{+}$ plasmacytoid DC (pDC) (**d**). Macrophages were analyzed as auto fluorescent cells (**b**). Experimental groups are as described in fig. 1. n=8 per group. Figure shows the mean with SD. *p<0.05 compared with the AOO/AOO control group.

Discussion

To date, many efforts have been done to elucidate the underlying mechanisms of chemical-induced asthma. Yet, most of the work is performed in acute mouse models of chemical-induced asthma, with limited numbers of airway exposures [8,12,13,17,26–28]. However, it takes weeks to years before initial onset of work related asthma symptoms occurs. As such, we aimed to investigate immunological parameters after long-term isocyanate exposure. Therefore, in contrast to our well-known acute mouse model consisting of dermal sensitization followed by one single airway exposure, we developed a chronic exposure model consisting of long-term airway exposures for five successive weeks, with or without prior brief skin exposure. Using this long-term model we were able to study both the effect of exposure sites (skin/respiratory) and the presence of immunological parameters such as dendritic cells, leukocytes, cytokines, T cells and immunoglobulins after long-term airway exposure. We showed in this study that TDI skin exposure in combination with repeated TDI airway exposure results in a pronounced Th2 hypersensitivity and AHR. Mice only exposed via the nasal mucosa to TDI, without prior dermal contact, showed a more Th1/Th2 balanced systemic response without the induction of airway hyperreactivity.

As expected, the TDI/TDI treated group showed evidence of pronounced systemic sensitization. Interestingly, the AOO/TDI group showed also partly similar signs of systemic sensitization. It has already been described that TDI is able to migrate from the skin to the T cell rich area of the cortex in the dermal lymph nodes, where it is shown to be co localized with APC [29]. This suggests that after TDI uptake, the APC migrate to the local lymph nodes and present TDI to the local T cells. Here we show that DC, T- and B- cells were significantly increased in both TDI treated groups. This was illustrated by a similar significant increase of cytotoxic T cells, regulatory T cells, helper T cells, B cells, $CD11c^+$ and $CD11b^+$ cDC, and an increasing trend of moDC in the ALN of both TDI-treated groups. As $CD103^+$ and pDC both can induce T regulatory cells that damp allergic immune responses, the decrease of both cells confirms the sensitization to TDI [20]. As we focused on the panel composition of DC in the lung, we are aware that we might have missed some dermal DC subpopulations in the TDI/TDI group [30].

The sensitization is also proven by a significant increase of serum IgE in both groups. Yet, in the TDI-dermally sensitized mice, the IgE serum concentration was three times higher than the AOO/TDI treated group. This suggests that contact of a sensitizer, with two different exposure sites, one skin and one airway mucosa, results in a much stronger immune response compared to only TDI airway exposure. Previously, Ban *et al.* and Matheson *et al.* showed similar results, where inhalation exposure resulted in a significant increase of total serum IgE [14,16].

Another important immunological difference between AOO/TDI and TDI/TDI treated groups is illustrated by the cytokines. The AOO/TDI treated group showed a predominant Th1 response; indicated

by a very high increase of *ex vivo* IFN-γ secretion and increase of IL-13 concentration both in serum and *ex vivo* secretion. In contrast, the TDI/TDI treated group had a similar Th1 response, but a much more pronounced Th2 phenotype response, demonstrated by a very high *ex vivo* Th2 cytokine secretion (IL-4, IL-10 and IL-13), along with high IL-13 serum levels. The pronounced Th2 response in the fully TDI-treated group is also demonstrated by an increase of serum IgG_1, along with IgE and decrease of IG_{2a} in the serum. Again, this indicates that skin sensitization in combination with airway exposure leads to a pronounced Th2 response. Previously, Ban *et al* 2006 showed similar results when topical sensitization was followed by tracheal instillation, leading to a Th2 type lung inflammatory response, whereas sensitization via the airways was not effective [16].

Yet, although both TDI treated groups (AOO/TDI and TDI/TDI) showed pronounced sensitization, only the TDI/TDI treated group showed AHR. Surprisingly, the AHR was not accompanied by a neutrophilic, nor an eosinophilic airway inflammation in BAL, and only very limited histological alteration in the blood vessels was found. An explanation could be that the delivered volume/dose of TDI into the lungs was too limited. To determine the volume of the intranasal instillation, we based ourselves on findings of Southam *et al.*, who demonstrated that the instillation volume and anesthesia affect the distribution of substances in the lungs [31]. Therefore, we performed the instillation under isoflurane anesthesia and increased the instillation volume to 40 µl. At this volume, 50-60% of the substance is distributed to the lungs [31]. Yet, these distribution ranges are only demonstrated for substances dissolved in aqueous solutions. The use of the viscous vehicle acetone/olive oil will also limit the substance distribution and volume that reaches the lower airways [32].

These results suggest that the Th2 immune response, induced by the combined skin and (upper) airway exposure, is necessary to induce AHR. Probably, as we showed previously in an acute mouse model of chemical-induced asthma, a complex interplay between neurogenic mechanisms (TRPA1 and NK1 receptor), innate immune mechanisms (mast cells) and adaptive immune mechanisms are involved in the induction of airway hyperreacitivity [26]. Notably, the IgE levels of the TDI/TDI treated group were three times higher compared to the AOO/TDI treated group, even five weeks after the initial dermal sensitization phase. Moreover, the levels of total serum IgE are 20 times higher than in our acute mouse model of chemical-induced asthma [8,26]. The literature already showed a significant correlation between total serum IgE and the presence of asthma and AHR. As such, high levels of IgE can induce ongoing mast cell activation, which is crucial in our chemical-induced asthma mouse model [26,33–35]. We measured the presence of IgE directly in the BAL fluid, which was significantly increased in TDI/TDI treated group. Furthermore, a key-role is attributed to IL-13 for inducing AHR. We also measured very high levels of the Th2 marker IL-13 in serum in the TDI/TDI treated group, confirming previously published research

[36]. The importance of initial topical exposure to chemicals on the airway response has been established for a while [6,8,11,16,26,37–40].

An important novelty in our research is that we investigated whether dendritic cells, the most 'professional' APCs in the lung and responsible for initiating an immune response and the induction of an asthmatic response, still play a role in the effector phase 46 days after the initial TDI contact [21,24,25]. As this is still unstudied in isocyanate-induced asthma, we analyzed the different DC and T cell subsets in the lung [22,41]. Although there was an increasing trend of some subpopulations in both AOO/TDI and TDI/TDI groups, there was no significant increase of DC in the lung after long-term exposures to TDI. This could be because DC, after allergen capture, migrate to the T cell area of the draining mediastinal lymph nodes (MLN) within twelve hours via a CCR7 dependent manner [41]. Although there was no significant increase of DC, there was a shift between the DC subpopulations, illustrated by a significant increase of moDC. As they do not express CCR7, moDC do not migrate well to the draining nodes leading to an accumulation into the lung [42]. moDC have the capacity to initiate and maintain a Th2 immune response which can explain the Th2 type sensitization in this chronic mouse model [21]. The absence of DC in the lung after a long-term exposure protocol is in contrast with the findings of Provoost *et al*., Lambrecht *et al*. and Johnson *et al.* [21,24,25,43]. The differences in response can be due to the different allergen exposure (house dust mite (Lambrecht *et al*. and Johnson *et al*.) and diesel exhaust particles (Provoost *et al.*)) and different airway exposure. Provoost *et al.* and Lambrecht *et al*. both used an intratracheal aspiration technique, which leads to a higher distribution to the lungs (80%) compared to the intranasal instillation method (50–60%) [21,24,31,44]. Although Johnson *et al.* and lambrecht *et al*. used HDM as a potent allergen, both treatment protocols were different. Johnson *et al.* used a long-term treatment protocol in which mice were intranasally exposed 5 days/week for seven consecutive weeks with 25 µg HDM, while Lambrecht *et al.* used a short intratracheal instillation protocol in which mice were instilled once with 100 µg HDM. Although our treatment protocol is partly similar to Johnson *et al.*, we were not able to detect an increase of DC in the lungs of mice treated with TDI. Therefore it is possible that the response in the lung is further influenced by the used vehicle:aqueous vehicle (saline or PBS), versus our much more viscous vehicle (Olive oil) which furthermore influence the distribution of the agent to the lungs[32].

Our findings indicate that repeated airway exposure to TDI causes immunological alterations indicative of sensitization, but are not accompanied by physiological changes indicative of asthma (i.e. AHR); however, the same type of exposure does lead to AHR if there has been previous dermal contact. These results implicate that long-term airway exposure to low TDI concentrations only results in asthma after previous accidental skin exposure, suggesting that dermal protection is important to protect TDI-induced OA.

Acknowledgments : This project was supported by a grant of the Interuniversity Attraction Poles Program-Belgian State-Belgian Science Policy- project P7/30, and by a grant of the Flemish Research Foundation (research grant 1504912N).

Conflict of interest: The authors declare no conflict of interest.

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Chapter 5

Importance of dendritic cells in chemical-induced lung responses

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Importance of dendritic cells in chemical-induced lung responses

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Abstract

Introduction: Toluene diisocyanate (TDI) is a known lung irritant when exposure to high concentrations occurs, while exposure to low concentrations can lead to immune-mediated occupational asthma (OA). However, both lung responses are difficult to differentiate in the clinic.

Aim: To determine differences between the immune-mediated and irritant TDI-induced lung response, we mimicked both phenotypes in a mouse model in which we assessed several lung parameters.

Methods: On days one and eight, BALB/c mice were dermally treated (20µl/ear) with 0.5% TDI or the vehicle AOO (3:2). Starting from day 15, they received on days 15, 17, 19, 22 and 24 an oropharyngeal challenge with 0.01% TDI (1:4). One day after the last challenge, airway hyperreactivity (AHR) to methacholine was assessed, followed by an evaluation of pulmonary inflammation and immune-related parameters including lymphocyte subpopulations and their *ex vivo* cytokine production profile, blood immunoglobulins and dendritic cells (DC).

Results: DC are recruited to inflamed lungs after multiple TDI airway exposure. However, prior dermal exposure determines the type of airway inflammation. Without prior skin exposure, the airway inflammation is dominated by mainly neutrophils, while prior skin exposure and consequently systemic immune sensitization, results in a shift from neutrophilic to eosinophilic airway inflammation accompanied with AHR.

Conclusion: These results show that multiple airway exposures to low TDI concentrations are able to initiate an adaptive immune response indicated by an increased amount of DC in the lung. Additionally, Prior dermal exposure influences the inflammatory and physiological lung response.

Keywords: Toluene diisocyanates, BALB/c mice, dendritic cells, Severe TDI-induced asthma

Introduction

Asthma is a chronic inflammatory disorder of the airways with bronchial hyper-responsiveness to a variety of non-specific stimuli, persistent lung inflammation and variable airflow obstruction [1]. In most of the cases, asthma begin in childhood, in association with IgE-dependent sensitization to common environmental allergens. Yet, asthma can also emerge later in life. This late onset asthma in older adults tends to be more severe than asthma that develops in younger age [2].

Adult onset asthma is in 5 to 25% the result of exposure on the workplace, also defined 'occupational asthma' (OA) [3]. OA can be classified in several sub phenotypes. High molecular weight (HMW) (such as flour, enzymes, latex, house dust mite,…) and some low molecular weight (LMW) agents (platinum salts, acid anhydrides,…) develop immune-mediated sensitization through the development of specific IgE, as similar seen in allergic asthma. On the other hand, some LMW compounds (diisocyanates and western red cedar) develop an immunologically mediated response in which IgE is not consistently seen [4,5]. Moreover, the role of IgE in this phenotype of OA, is still under debate and the immune-mediated mechanisms are less well understood [6]. The airway inflammation in both immunological phenotypes is postulated to be similar and characterized by mainly eosinophils [4]. OA can also develop via a less well-known non-immunological mechanism caused by exposure to high concentrations of irritant chemicals, called irritant-induced asthma. This phenotype of OA is recognized by a predominant neutrophilic airway inflammation [4,5].

Toluene diisocyanates (TDI), a low molecular weight compound, are an important cause of chemical-induced OA. High acute exposure to diisocyanates is known to cause irritant-induced asthma. Besides the acute toxicity/effects, diisocyanates are potent immune sensitizers resulting in immune-mediated asthma. Both asthma phenotypes are difficult to differentiate clinically [7]. As diisocyanates are LMW compounds, they can act as haptens and bind to endogenous proteins before they are recognizable by the immune system. For a long time it was believed that airways were the key route of exposure in the development of TDI-induced asthma, however, to date, animal and human studies support the fact that skin exposure is an important route for sensitization. Once sensitized, low airborne concentrations can trigger an asthmatic response [8,9].

Diisocyanate-induced asthma is often phenotyped by a severe neutrophilic airway inflammation and difficult to treat [10,11]. Continuous exposure on the work floor results in worsening of symptoms such as airway obstruction and non-specific bronchial hyperresponsiveness. Therefore, the general therapeutic recommendation is to remove the workers from exposure to the causal agent [12]. Yet, long-term follow up studies have shown that in 50% of TDI asthmatic patients taking anti-asthmatic medication, symptoms still are present even after cessation of exposure [13–17]. Moreover, airway remodeling is shown to be decreased but airway inflammation and AHR persist to exist [16,17]. In most of the studies, the

persistence of asthma is associated with the duration of exposure to TDI and the symptoms at work before diagnosis [15]. The causal reason for the persistent asthmatic symptoms in some subjects remains unclear.

Dendritic cells (DC) are important antigen presenting cells in the lung, bridging the innate and adaptive immunity. They have been shown to be crucial during both sensitization and effector phase of allergic asthma [18,19]. However, the presence of DC in the lung tissue of diisocyanate-induced asthma has never been studied in an inflammatory phenotype.

In this study, we investigated the differences in systemic (IgE, IgG and *ex vivo* cytokines) and lung response (airway hyperreactivity, DC, inflammation and histological changes) after multiple oropharyngeal instillations, with and without prior sensitization, which mimic immune-mediated asthma-like response and irritant-induced inflammation, respectively.

Material and methods

Reagents

Toluene-2,4-diisocyanate (TDI) (98%, CAS584-84-9), acetyl-β-methylcholine (methacholine) olive oil and acetone were obtained from Sigma-Aldrich (Bornem, Belgium). Pentobarbital (Nembutal®) was obtained from the internal animalium at the University of Leuven. The vehicle acetone olive oil (AOO) is used to dissolve TDI. For the sensitization phase, a mixture of 2 volumes acetone and 3 volumes of olive oil was used. For the challenge phase, a mixture of 1 volume actone and 4 volumes of olive oil was used. Concentration of TDI is given as percentage (v/v) in AOO.

Mice

Male BALB/c mice (6-8 weeks old) were obtained from the internal stock of the animalium (University of Leuven, Belgium). All mice were housed under a conventional animal house with 12h dark/light cycles. They were housed in filter top cages and received lightly acidified water and pelleted food ad libitum. All experimental procedures performed in mice were approved by the local Ethical Committee for animal experiments (P063/2015).

Experimental protocol

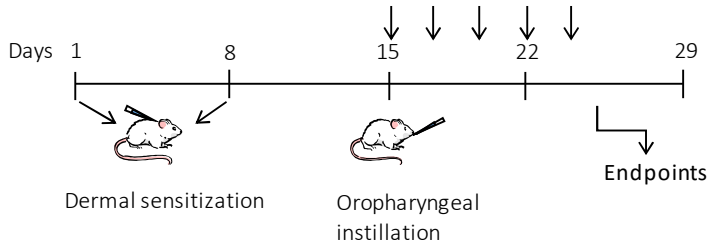


Illustration 1: sub-chronic exposure protocol

On days one and eight, mice received dermal applications of 20 µl 0.5% TDI or the vehicle AOO (2:3) on the dorsum of both ears. On days 15, 17, 19, 22 and 24 mice received an oropharyngeal instillation with 20 µl 0.01% TDI or the vehicle AOO (1/4). All experimental groups are indicated with two symbols: the first symbol indicates the dermal treatment on days one and eight, whereas the second symbol indicates the oropharyngeal instillations.

In this study, we have two different sets of experiments. In the first set, we evaluated the AHR, lung histology and IgE, IgG, and IL-13 in blood serum. Furthermore we measured the cytokine secretion of T- and B- cells in the auricular lymph nodes (n=8-9/group). To study the DC in this mouse model, we added a second set were we performed a BAL to assess the lung inflammation, and studied the DC, T- and B-cells in the ALN and lung tissue. Furthermore, to confirm sensitization, we analyzed IgE in this set (n=8/group). So depending on the endpoint, each group consisted of 6 to 17 mice.

Airway and lung tissue hyperreactivity measurements

Twenty-four hours after the last challenge, airway and lung tissue reactivity to methacholine was measured using a forced oscillation technique (flexiVent 6.3, SCIREQ, Montreal, Canada). Mice were anesthetized with pentobarbital (70 mg/kg body weight, Nembutal®). Using the 'quick prime 3' (QP3) perturbation, airway resistance (Rn) and tissue elasticity (H) to increasing concentrations of methacholine (0, 1.25, 2.5, 5, 10, 20 mg/ml) was measured. After each concentration, the QP3 perturbation was performed 5 times spread over 2 minutes. If the COD (coefficient of determination) of a QP3 perturbation was lower than 0.90, the measurement was excluded and not used to calculate the average. For each mouse Rn and H were plotted against the methacholine concentration and the area under the curve (AUC) was calculated to perform statistical analysis.

Lung, lymph node and serum analysis

Serum sampling

After measuring airway hyperreactivity, mice were sacrificed. Blood was sampled from the retro-orbital plexus, centrifuged (14000 g, 4 °C, 10 min) and serum samples were stored at -80°C until analysis.

Broncho-alveolar lavage (BAL)

The lungs were lavaged, *in situ*, three times with 0.7 ml sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cell count) and the BAL fluid was centrifuged (1000 g, 10 min). For differential cell counts, 250 µl of the resuspended cells (100,000 cells/ml) were spun (300 g, 6 min) (Cytospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried and stained (Diff-Quik® method, Medical Diagnostics, Düdingen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

Single cell suspension

Lung cells

The pulmonary circulation was rinsed with saline/EDTA to remove the intravascular pool of cells. Cell suspension of the lungs were obtained using digestion medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol [Gibco; Invitrogen, Paisley, United Kingdom], 100 U/mL penicillin–100 mg/mL streptomycin [Invitrogen], 1 mg/mL collagenase type 2 [Worthington Biochemical, Lakewood, NY], and 0.02 mg/mL DNase I [grade II from bovine pancreas; Boehringer Ingelheim, Ingelheim, Germany]) for 45 minutes at 37°C and 5% CO2. Red blood cells were lysed by using ammonium chloride buffer. Cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/ml) in PBS.

Lymph node cells

Retro-auricular lymph nodes were pooled and kept on ice in RPMI-1640 (1X) + GlutaMAX^{TM}-I (Invitrogen, Merelbeke, Belgium). Cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 µm) (BD Bioscience, Erembodegem, Belgium) and rinsing with 10 ml tissue culture medium (RPMI-1640 (1X) + GlutaMAX^{TM}-I). After centrifugation (1000 g, 10 min), cells were counted using a Bürker hemocytometer and resuspended (10^7 cells/ml) in complete tissue culture medium (RPMI-1640 (1X) + GlutaMAX^{TM}-I supplemented with 10% heat-inactivated fetal bovine serum, 10 mg/ml streptomycin/penicillin).

Flow cytometry

Lymphocyte subpopulations

Five-hundred thousand cells from the auricular lymph nodes and lung were stained with anti-CD3$^+$ (APC), anti-CD4$^+$ (APC-Cy7), anti-CD8$^+$ (PerCP-Cy5.5) and anti-CD25$^+$ (PE), or received a single staining with anti-CD19$^+$ (PE) labeled antibodies, according to standard procedures (BD Biosciences, Erembodegem, Belgium). Percentages of labeled cells were determined by performing flow cytometry (FACSArray, BD Biosciences, Erembodegem, Belgium) on at least 10^5 cells.

Dendritic cell subpopulations

To minimize nonspecific bindings, two million cells of lung and auricular lymph nodes, were preincubated with anti-CD16/CD32 (Clone 2.4G2) (from BD Biosciences, San Jose, Calif). After live/dead staining using the Zombie Aqua™ Fixable Viability Kit, cells were labeled with combinations of anti-mouse fluorochrome-conjugated mAbs against CD45 (30-F11), CD11c (N418), MHCII (M5/114.15.2), CD11b (M1/70), CD103 (2E7), CD64 (X54-5/7.1) or Siglec-H (551) (all from Biolegend, San Diego, Calif). Data acquisition was performed on a LSR Fortessa flow cytometer running DIVA software (BD Biosciences, San Jose, Calif). FlowJo software (TreeStar, Inc, Ashland, Ore) was used for data analysis.

Cytometric bead array

Cells of the auricular lymph nodes were seeded into 48-well culture plates at a density of 10^6 cells/ml and incubated in complete RPMI-1640 (1X) + GlutaMAX^{TM}-I medium for 42h with 2.5 µg/ml of concanavalin A (ConA) (Sigma–Aldrich, Bornem, Belgium). Cell suspension were centrifuged (1000 g, 10 min) and supernatant was stored at −80 °C. Concentrations of interleukin (IL)-4, IL-10, IL-13 and interferon gamma (IFN-γ) were measured via the LSR Fortessa (BD Biosciences, Erembodegem, Belgium). Detection limits were 0.2 pg/ml, 0.3 pg/ml, 9.6 pg/ml, 2.4 pg/ml and 0.5 pg/ml, respectively.

Enzyme Linked Immuno Sorbent Assay (ELISA)

The OptEIA Mouse IgE set from pharmingen (BD Biosciences) was used to measure total serum (diluted 1/70) IgE. Measurements were performed according to the manufacturer's instructions. For the measurement of total serum IgG_1 (diluted 1/8000 or 1/20000) and IgG2a (diluted1/1000), plates were coated using purified rat anti-mouse IgG1 (Cat: 553445, BD PharmingenTM) and purified rat anti-mouse IgG2a (Cat: 553446, BD PharmingenTM). A standard was created using purified mouse IgG_1 (Cat: 557273, BD PharmingenTM) and purified mouse IgG2a (Cat: 553454, BD PharmingenTM). Further measurements were performed according to the manufacturer's (BD Pharmingen) instructions with the use of biotinylated anti-mouse avidin horseradish peroxidase conjugate (HRP rat anti-mouse IgG1, Cat:559626, biotin rat anti-mouse IgG2a, Cat: 553388, and Streptavidin HRP, Cat: 554066, BD PharmingenTM).

Histology

Lungs were instilled with 4% formaldehyde until full inflation of all lobes. An experienced pathologist evaluated lung injury on slides stained with hematoxylin and eosin (H&E), in a blinded manner.

Data analyses

The data are presented as means with standard error of the mean (SD), or as individual mice and group mean. Normality of distribution was assessed by the Kolmogorov-Smirnov test, followed by a one-way parametric ANOVA combined with a bonferroni multiple comparison post hoc test (Graph Pad Prism 5.01. Graphpad Software Inc, San Diego, USA). A level of $p<0.05$ (two tailed) was considered to be significant.

Results

Airway response

Airway hyperreactivity

Figures 1A and C show the dose-response increase of methacholine-induced airway resistance and tissue elasticity. Figures 1B and D show the area under the cure of each individual mice. Mice, sensitized and challenged with TDI (TDI/TDI treated group) showed a significant increase of airway hyperreactivity (AHR) and tissue elasticity compared to both AOO/AOO and AOO/TDI treated groups.

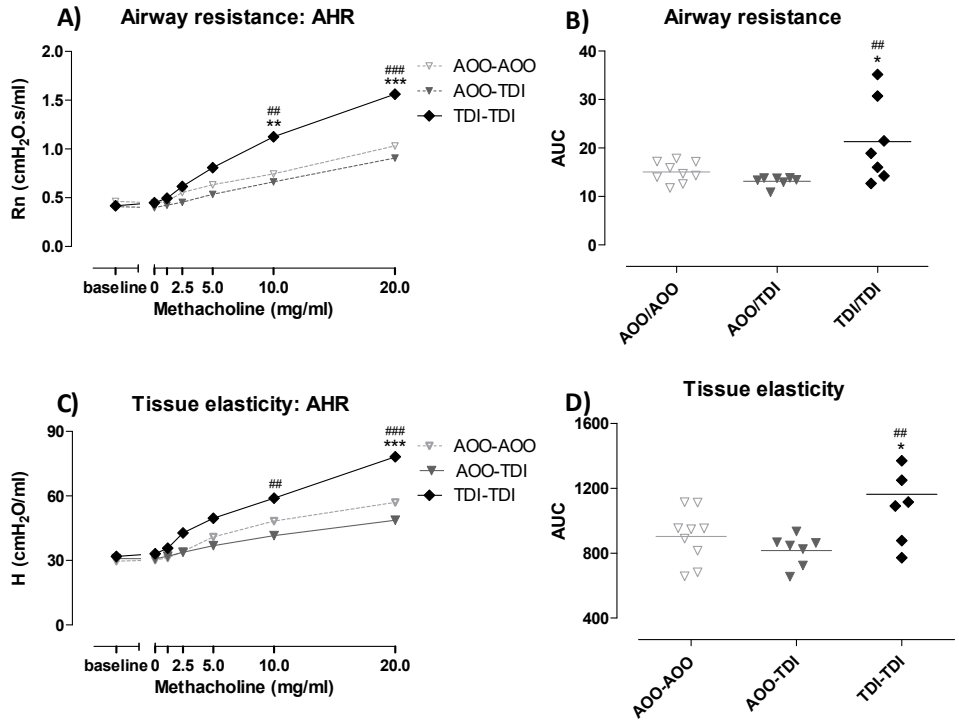


Fig. 1: Airway and tissue hyperreactivity. Methacholine responsiveness was assessed 24h after the last oropharyngeal challenge. The airway reactivity to methacholine was measured using a forced oscillation technique. Fig **a** and **c** show the mean values of Rn and H, respectively. Fig **b** and **d** show individual values and the group mean of the area under the curve (AUC) of Rn and H against methacholine concentrations between 0 and 20 mg/ml. Experimental groups are identified by two symbols; the first symbol indicates the dermal treatment on days 1 and 8, whereas the second symbol indicates the oropharyngeal instillations. n=8-9 per group. **p<0.01, ***p<0.001 compared with the AOO/AOO control group. ##p<0.01 and ###p<0.001 compared with the AOO/TDI treated group.

Broncho-alveolar lavage and lung histology

Figure 2 shows the differential cell count of the BAL. Mice not sensitized and only exposed via the airways to TDI (AOO/TDI treated group) showed a significant increase of neutrophils compared to the AOO/AOO and TDI/TDI treated groups. Mice both sensitized and challenged with TDI (TDI/TDI) showed a highly significant influx of eosinophils in the BAL compared to both AOO/AOO and AOO/TDI treated groups. Macrophages were significant decreased in the AOO/TDI treated group compared to the AOO/AOO control group.

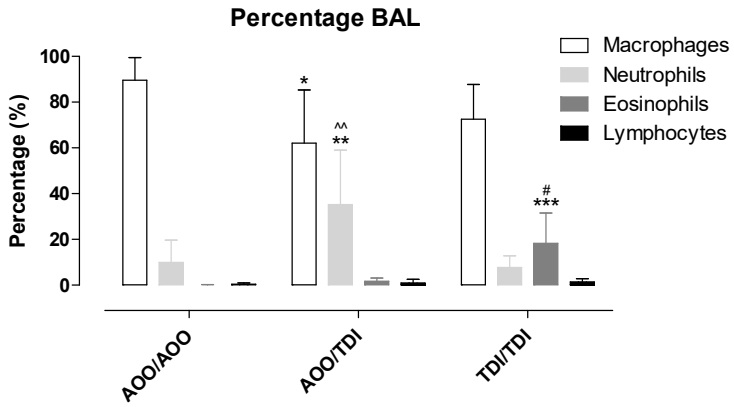


Fig. 2: Broncho-alveolar lavage (BAL) cell count. Percentages of macrophages, neutrophils, eosinophils and lymphocytes were assessed in BAL 24h after the last oropharyngeal instillation. Experimental groups are as in figure 1. n=8 per group. Bars show the mean and SD. *p<0.05, **p<0.01, ***p<0.01 compared to the AOO/AOO control group. #p<0.05 compared with the AOO/TDI treated group. ^^p<0.01 compared with the TDI/TDI treated group.

These inflammatory results are confirmed by histological analysis, shown in figure 3 by an increase of alveolar widening (fig. 3A), inflammation (fig. 3B) and epithelial shedding (fig. 3C), in both TDI treated groups, nevertheless, the response was only significant in the fully treated TDI group (TDI/TDI) compared to both AOO/AOO and AOO/TDI treated groups (fig. 3A-C).

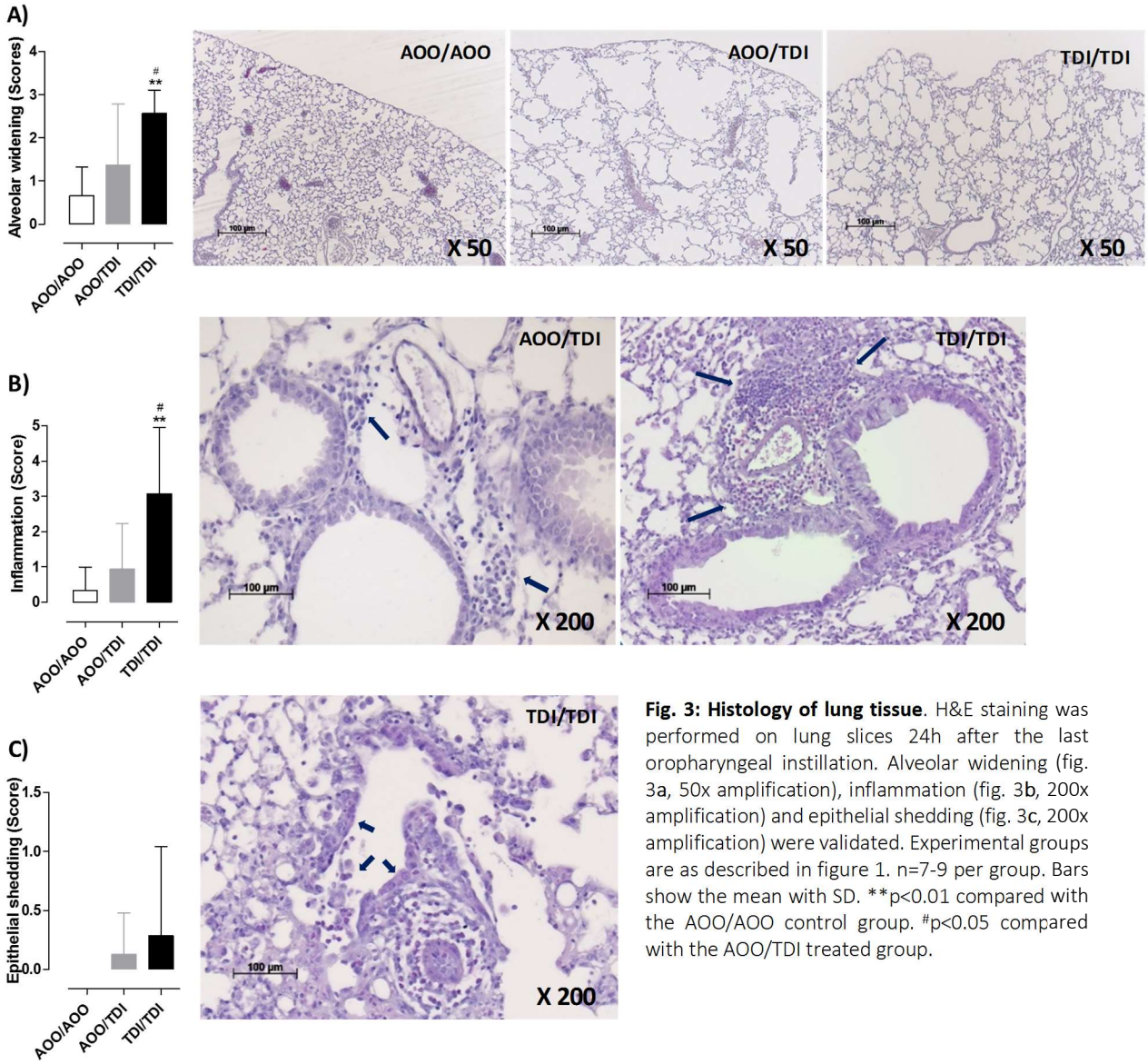


Fig. 3: Histology of lung tissue. H&E staining was performed on lung slices 24h after the last oropharyngeal instillation. Alveolar widening (fig. 3a, 50x amplification), inflammation (fig. 3b, 200x amplification) and epithelial shedding (fig. 3c, 200x amplification) were validated. Experimental groups are as described in figure 1. n=7-9 per group. Bars show the mean with SD. **p<0.01 compared with the AOO/AOO control group. #p<0.05 compared with the AOO/TDI treated group.

Markers of sensitization

Lymphocyte subpopulations in auricular lymph nodes

Figure 4 shows that TDI-sensitized mice (TDI/TDI) have a significant increase of all T cell subpopulations ($CD4^+$ helper T cells, $CD4^+CD25^+$ activated/regulatory T cells and $CD8^+$ cytotoxic T cells) compared to the AOO/AOO and AOO/TDI treated groups.

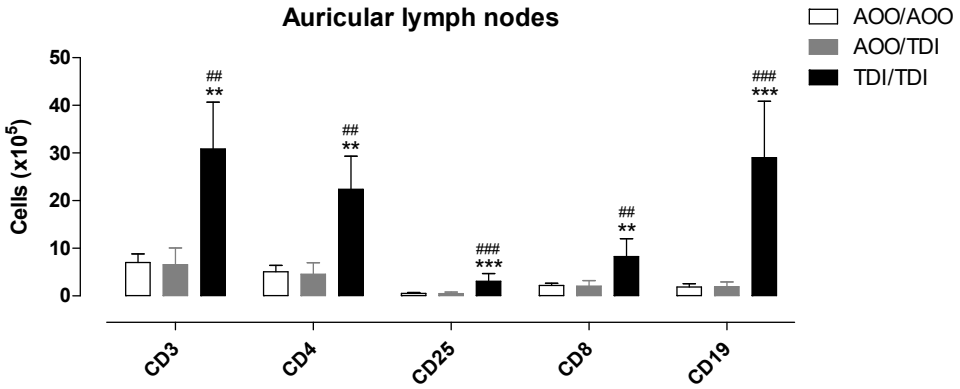


Fig. 4: Lymphocyte subpopulations in auricular lymph nodes. Lymphocyte subpopulations in auricular lymph nodes were measured using flow cytometry. Auricular lymphocytes were stained with anti-$CD3^+$ (T lymphocytes), anti-$CD3^+CD4^+$ (Th-lymphocytes), anti-$CD3^+CD4^+CD25^+$ (activated/regulatory Th-lymphocytes) and anti-$CD3^+CD8^+$ (Tc-lymphocytes) or stained with a single anti-$CD19^+$ (B-lymphocytes). Experimental groups are as described in figure 1. n=8 per group. Bars show the mean with SD. **p<0.01, ***p<0.001 compared with the AOO/AOO control group. ##p<0.01, ###p<0.001 compared with the AOO/TDI treated group.

Ex vivo cytokine secretion of the auricular lymph nodes

Lymphocytes of the auricular lymph nodes were *ex vivo* stimulated with concanavalin A. Figure 5 shows a significant higher secretion of IL-4 (fig. 5A), IL-13 (fig. 5B), IL-10 (fig. 5C) and IFN-γ (fig. 5D) from the auricular lymphocytes of TDI/TDI treated mice compared to both AOO/AOO and AOO/TDI treated groups.

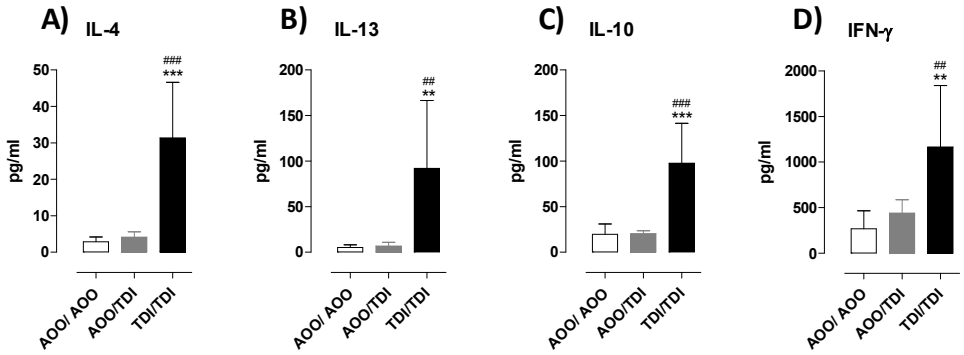


Fig. 5: *Ex vivo* cytokine production of auricular lymphocytes. Levels of IL-4 (**a**), IL-13 (**b**), IL-10 (**c**) and IFN-γ (**d**) were measured by Cytometric Bead Array in supernatant of auricular lymphocytes. Experimental groups are as described in figure 1. n=7-8 per group. Bars show the mean and SD. **p<0.01, ***p<0.001 compared with the AOO/AOO control group. ##p<0.01, ###p<0.001 compared with the AOO/TDI treated group.

Dendritic cells in auricular lymph nodes

As DC are important during the sensitization phase, we determined the number of DC in the auricular lymph nodes. Figures 6A to E show the total amount of leukocytes and different subpopulations of DC

in the ALN. There is no significant increase of total amount of leukocytes in the ALN in the TDI/TDI group compared to the control group AOO/AOO and AOO/TDI treated group (fig. 6A). Nevertheless, the dendritic cells (DC) (fig. 6B) and CD11b$^+$ conventional dendritic cells (cDC) (fig. 6C) are significant increased in the TDI/TDI treated group compared to the control group (AOO/AOO) and AOO/TDI treated groups. CD103$^+$cDC and pDC (fig. 6D and E) were only significant increased in the TDI/TDI group compared to the AOO/AOO treated group.

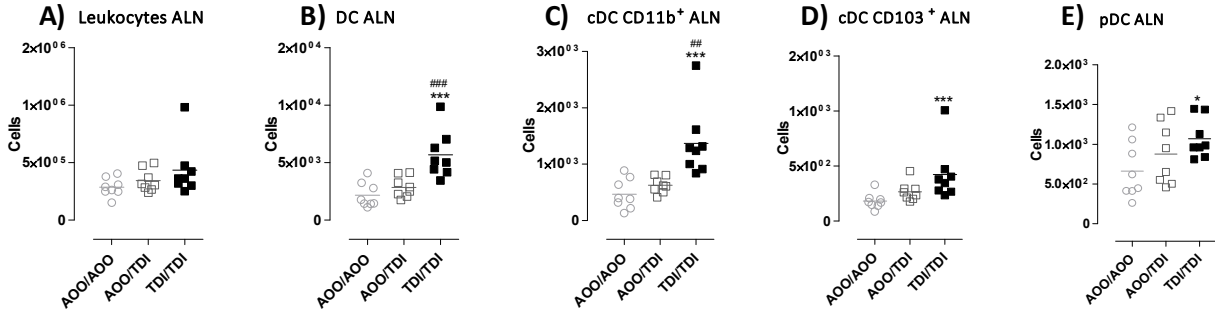


Fig 6.: leukocytes and antigen presenting cells in auricular lymph nodes. Leukocytes and antigen presenting cells in auricular lymph nodes were measured using flow cytometry. Cells were analyzed as CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$ DC (DC) (**b**), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^-$CD103$^+$ cDC (CD103$^+$ cDC) (**d**), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^+$CD103$^-$ conventional DC (CD11b$^+$ cDC) (**c**) and CD45$^+$ low auto fluorescent MHCII$^+$ CD11c$^+$SiglecH$^+$ plasmacytoid DC (pDC) (**e**). Experimental groups are as described in figure 1. n=8 per group. Figures show the individual values and group mean. *p<0.05, ***p<0.001 compared with the AOO/AOO control group. ##p<0.01, ###p<0.001 compared with the AOO/TDI treated group.

Dendritic cells in the lung

Figure 7 shows the total amount of leukocytes (fig. 7A), macrophages (fig. 7B), DC (fig 7C) and DC subpopulations (fig. 7D-G) in the lung. There is a significant increase of leukocytes in both groups treated with TDI (AOO/TDI and TDI/TDI) compared to the AOO/AOO control group (fig. 7A). The DC (fig. 7C), CD11b$^+$ cDC (fig. 7D), CD103$^+$cDC (fig. 7E) and moDC (fig. 7F) are also significantly increased in both TDI treated groups, compared to the AOO/AOO control group. Furthermore, pDC were increased in both TDI treated group, but was only significant in the AOO/TDI treated group (fig. 7G). Macrophages did not show significant differences (fig. 7B).

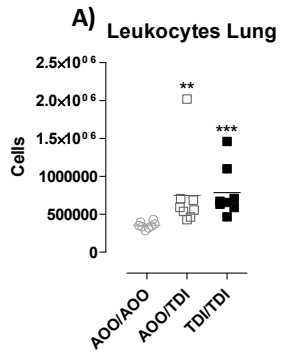
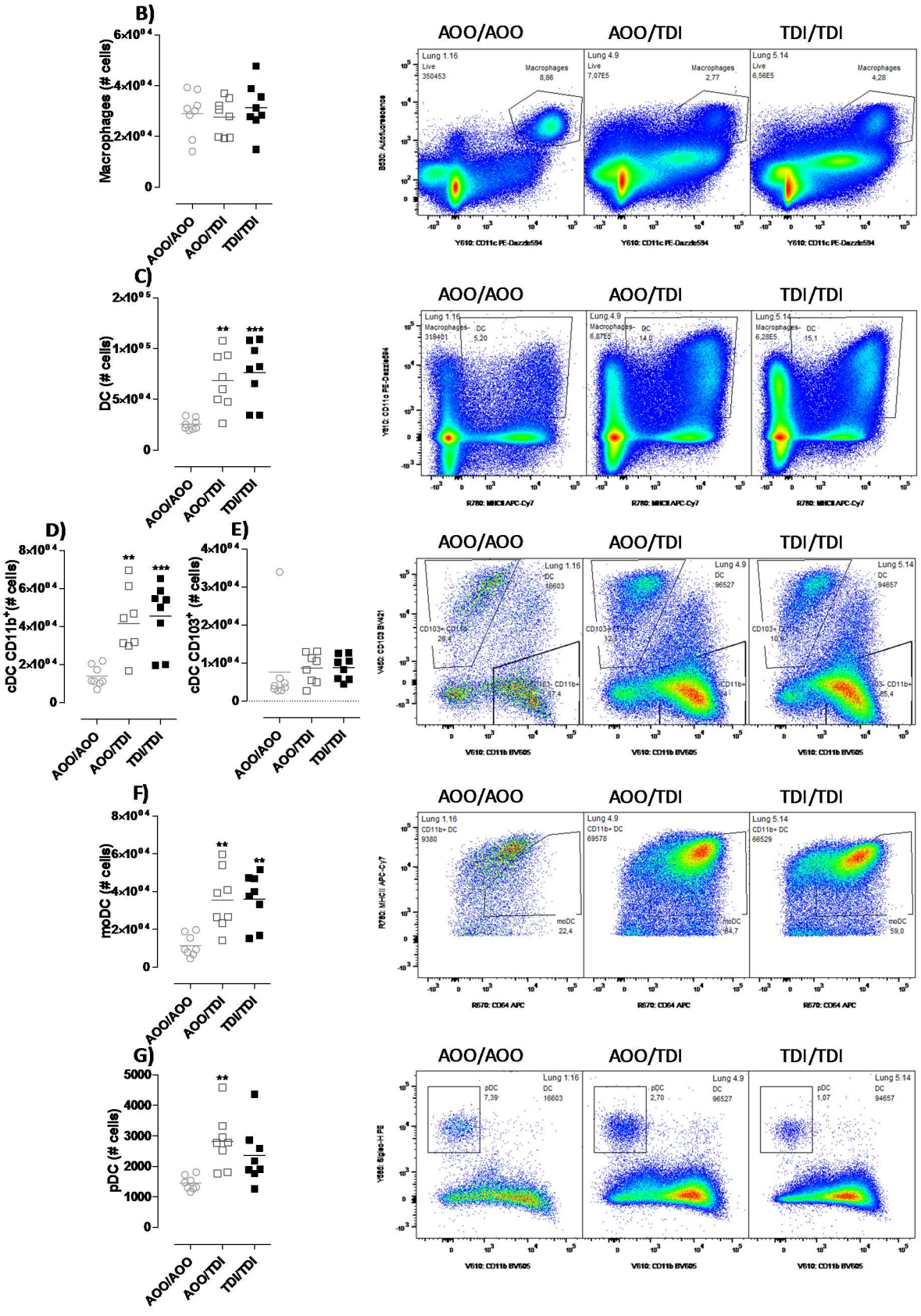


Fig. 7: Leukocytes and antigen presenting cells in the lung. Leukocytes and antigen presenting cells in the lung were measured using flow cytometry. Cells were analyzed as CD45$^+$ leukocytes (**a**), auto fluorescent macrophages (**b**) CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$ DC (DC) (**c**), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^-$CD103$^+$ cDC (CD103$^+$ cDC) (**e**), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^+$CD103$^-$ conventional DC (CD11b$^+$ cDC) (**d**), CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$CD11b$^+$CD64$^+$ monocyte derived DC (moDC) (**f**) and CD45$^+$ low auto fluorescent MHCII$^+$CD11c$^+$SiglecH$^+$ plasmacytoid DC (pDC) (**g**). Experimental groups are as described in figure 1. n=8 per group. Figures show the individual values and group mean. Facs plots were shown using one representative mice (closed to the group mean) for each group. **p<0.01, ***p<0.01 compared with the AOO/AOO control group.


B)
Macrophages (# cells)
AOO/AOO
AOO/TDI
TDI/TDI
C)
DC (# cells)
D)
cDC CD11b+ (# cells)
E)
cDC CD103+ (# cells)
F)
moDC (# cells)
G)
pDC (# cells)
Lung 1.16
Lung 4.9
Lung 5.14
Macrophages
DC
CD11b+ DC
pDC
moDC

Serum analysis

The serum analysis shows a significant increase in total serum IgE (fig. 8A) and IgG1 (fig. 8B) in TDI sensitized and challenged mice (TDI/TDI), compared to the AOO/AOO and AOO/TDI treated groups. There was no difference in total serum IgG2a and serum IL-13 between the different groups (data not shown).

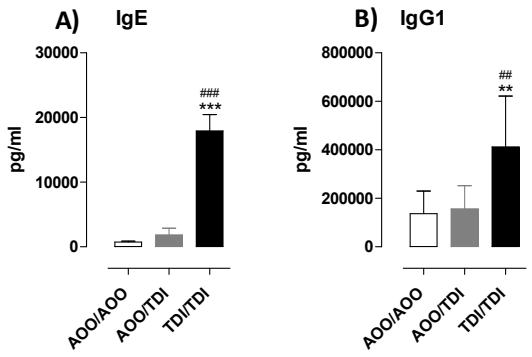


Fig. 8: Total serum immunoglobulins. Total serum IgE (**a**) and IgG1 (**b**) were measured using ELISA. Blood was collected 24h after the last oropharyngeal challenge. Experimental groups are as described in figure 1. n=15-17 for IgE and n=7-9 for IgG1 per group. Bars show the mean and SD. **p<0.01 and ***p<0.001 compared with the AOO/AOO control group. ##p<0.01, ###p<0.001 compared with the AOO/TDI treated group.

Discussion

In this study, we show that inflammatory and physiological lung responses after multiple TDI airway exposures substantially differ between dermally sensitized and non-sensitized mice. In dermally sensitized mice, a more 'classic' Th2 mediated eosinophilic asthmatic response, with airway hyperreactivity appears, mimicking immune-mediated TDI-induced asthma, while in non-sensitized mice, a pronounced neutrophilic inflammation developed, without the presence of airway hyperreactivity, which is more reprehensive for irritant-induced inflammation. However, in both exposure protocols, DC are recruited to the lungs, indicating the early initiation of an adaptive immune response via lung exposure.

Previously, we already described that dermal sensitization with TDI results in significantly proliferated auricular lymph nodes, where both T- and B- cells are stimulated. Here, we also show a significant increase of several DC subpopulations in the auricular lymph nodes. Dendritic cells are the most 'professional' APCs for initiating an immune response. As the sensitization occurred on the dorsum of both ears, and studies have shown that diisocyanates are transported via DC from the skin to the local lymph nodes, we validated the presence of different DC subpopulations in the auricular lymph nodes [20]. Depending on the exposure context, each DC subset is geared to induce particular T cell responses [21]. Both $CD11b^+$ and $CD103^+$ cDC have shown to be able to induce a Th2 and Th1 immunization, respectively [20]. Their significant increase can therefore be directly linked to the increased number of $CD4^+$ T cells in the ALN. $CD103^+$DC are also described to cross present antigens to $CD8^+$ T cells. Therefore, the significant increase of this type of DC can explain the significant increased numbers of $CD8^+$ T cells in the ALN [20]. Furthermore, the significant increase of $CD103^+$ and pDC, can be responsible for the significant increase of $CD25^+$ Tregs, which are known to diminish immune responses, and induce immune tolerance [22,23]. Both humoral and *ex vivo* cell mediated cytokine secretion of the auricular lymph nodes demonstrated that there was a mixed Th2 (IgE, IgG1, IL-4, IL-10 and IL-13)/Th1 (IFN-γ) cytokine profile. The production of Th2 cytokines was probably initiated by moDC and $CD11b^+$DC, as they are known Th2 immunity inducers. On the other hand, $CD103^+$ and $CD11b^+$cDC can be responsible for the Th1 response as they have been shown to induce a Th1 response *ex vivo* [22].

Next to their role during the sensitization phase, DC also have a crucial role during the effector phase of asthma. In an OVA model, it is shown that depletion of $CD11^+$DC during OVA airway exposures results in an abrogation of all the characteristic feature of asthma, however, these features were restored after adoptive transfer of $CD11c^+$ cells [18,24]. The role of DC in Th2 allergic mediated asthma phentoypes is crucial, however less is known about DC in a mixed Th1/Th2 asthmatic phenotype. Therefore, we assessed the DC recruitment in the airways of TDI treated mice, and studied the influence of prior dermal sensitization.

DC are recruited to the lungs of both TDI treated groups. Moreover, there is no difference in the DC subpopulations between both exposure groups. Also other studies confirmed recruitment of DC in the lung after diesel exhaust particles and HDM airway exposures [20,25–27].

Our data indicate that dermal sensitization influences the inflammatory phenotype in the lungs. Both TDI-treated groups demonstrated a clear airway inflammation, however, depending on initial dermal TDI-sensitization the phenotype of inflammation was different. Mice only oropharyngeal instilled with TDI (AOO/TDI) have a predominant neutrophilic inflammation, rather reflecting an irritant, non-allergic, immune response, while mice both dermal sensitized and oropharyngeal instilled with TDI (TDI/TDI) illustrated a predominant eosinophilic inflammation reflecting a Th2 mediated allergic immune response. The increase of inflammatory cells are in line with the increase of moDC in both groups, which are known to rapidly appear in inflammatory sites [20].

Surprisingly, despite a severe neutrophilic inflammation, AHR was only present in the fully treated TDI/TDI group. Besides differences in functional measurements and type of inflammation, there were also some significant structural changes in the lung such as an increased alveoli-widening, inflammation and epithelial shedding. These structural changes can be caused by the release of intracellular cytotoxic granules by the significant increased BAL eosinophils and the presence of the *ex vivo* IL-13 secretion [28].

The absence of AHR and diminished histological changes in the AOO/TDI group can be explained by the absence of prior dermal sensitization and the high number of pDC in the lungs [22]. We and other groups already emphasized the importance of previous skin exposure in inducing a pronounced airway response (26–28). The high number of pDC in the lung can be an explanation for the dampened lung response as they are able to induce immune tolerance via the induction of Tregs.

As a conclusion, multiple airway exposure to TDI, with or without prior dermal sensitization, results in recruitment of DC, which are indicative of the early events of an adaptive immunity, accompanied with airway inflammation. Without prior skin exposure, this inflammation is dominated by mainly neutrophils, while prior skin exposure and consequently systemic immune sensitization results in a shift from a neutrophilic to an eosinophilic airway inflammation accompanied with AHR. These findings can have a direct implication for workers exposed on the work floor to diisocyanates. Depending on the skin protective clothing and gloves use during diisocyanate exposure, a different lung response develops when later exposure of diisocyanates to the lung occurs. Moreover, it is also important to notice, that low airborne concentration, without previous skin exposure, is able to initiate immune sensitization via the lungs which is illustrated by the increased DC in the lung.

Acknowledgments : This project was supported by a grant of the Interuniversity Attraction Poles Program-Belgian State-Belgian Science Policy- project P7/30, and by a grant of the Flemish Research Foundation (research grant 1504912N).

Conflict of interest: The authors declare no conflict of interest.

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Chapter 6

General discussion

1. Introduction

Occupational asthma (OA) is a type of asthma caused by exposure to agents on the workplace. It has become one of the most common forms of occupational lung diseases in many industrialized countries and is responsible for 9-15% of adult asthma cases [1].

Diisocyanates are important causes of chemical-induced OA. They are used in the polyurethane industry for the manufacture of flexible and rigid foams, fibers, paints and varnishes. Methylene diphenyl diisocyanate (MDI) and toluene diisocyanate (TDI), together account for more than 90% of the global diisocyanate market. Using their reactive isocyanate moiety (-N=C=O), they act as haptens reacting with alcohols, phenols, amines and thiols on endogenous proteins, forming functional antigens [2]. 5 to 30% of the exposed workers are at risk to develop isocyanate-induced asthma [3]. The general therapeutic recommendation is to remove the sensitized workers from exposure to the causal agent. Nevertheless, even after many years of cessation of exposure, more than 50% of TDI asthmatic patients taking anti-asthmatic medication remain symptomatic [4,5].

At present, the immunological mechanisms of the different phenotypes of chemical-induced OA are not fully elucidated. To study the underlying mechanisms, we developed several mouse models, mimicking different phenotypes of OA. Additionally, we validated MDI for its asthmatic potency and studied the cross-reactivity with TDI.

2. Summary of the findings

2.1 Evaluation of MDI and their cross-reactivity with TDI

In a first study, we validated the asthmogenic potency of MDI and studied its cross-reactivity with TDI (chapter 3). Although MDI has a lower vapor pressure than TDI, and therefore is assumed safer in use, both human and animal studies show that exposure affects the respiratory system [6–11]. Both diisocyanates are often used in the same plants and sometimes even together in the same production process. Moreover, they have common binding preference to lysine residues on human serum albumin (HSA), indicating that cross-reactivity might occur between both chemicals [2]. In the past, several research groups focused on the immunological and respiratory cross-reactivity between MDI and TDI, yet the results remain contradictory and the number of patients included in the studies were limited [7,12–17].

Regarding the respiratory cases of MDI, their common binding places on HSA and contradictory results of cross-reactivity between MDI and TDI, we validated MDI for their asthmogenic potency and studied the asthmatic cross-reactivity with TDI in our acute mouse model of chemical-induced asthma. This

model consists of a dermal sensitization phase, induced via dermal applications on days one and eight, followed by a single oropharyngeal instillation on day 15 (fig. 6.1).

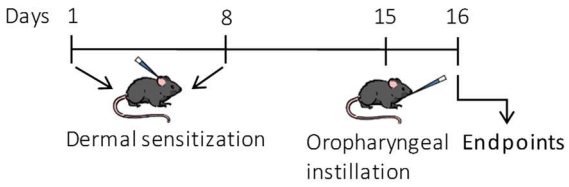

Fig. 6.1: Illustration of the acute exposure protocol.

Our results demonstrated that mice sensitized and challenged with MDI resulted in a similar asthma-like phenotype as previously shown with TDI, suggesting that both diisocyanates are potent asthmogens [18–20]. However, MDI seems to be a somewhat weaker sensitizer compared to TDI since we needed to increase the sensitization and challenge concentration of MDI to induce similar results [21]. This acute mouse model is phenotyped by a predominant neutrophilic airway inflammation, airway hyperreactivity (AHR) and a mixed T helper (Th)1/Th2 response. Concerning cross-reactivity, we have found no or limited indications of cross-reactivity between TDI and MDI in this acute model.

2.2 Developing phenotypes of chemical-induced asthma

In the second part of this thesis, we aimed to develop different phenotypes of chemical-induced asthma. A gap in knowledge is the lack of a mouse model of chemical-induced asthma that shows robust lung inflammation and lung remodeling. Additionally, as dendritic cells (DC) are known to be important in both sensitization and elicitation phase of Th2-mediated ovalbumin (OVA)-induced asthma, we wanted to investigate the recruitment of DC in the lungs of a mixed Th1/Th2 phenotype of chemical-induced asthma [22].

2.2.1 Chronic nasal mucosa exposure model

In a first model, we aimed to develop a pronounced asthmatic response characterized by a pronounced AHR, airway inflammation and airway remodeling using TDI as model agent. Mice were dermally sensitized to TDI or vehicle treated on days one and eight followed by long-term nasal mucosa exposure, five days/week for five consecutive weeks (fig. 6.2), which is based on a mouse model of HDM-induced asthma that shows the anticipated outcome [23]. To differentiate the influence of skin versus long-term airway exposure, we included three different groups in this study: mice only weekly exposed to TDI in the airways (AOO/TDI) and mice exposed to both skin and airways (TDI/TDI). Both groups were compared to the complete control group, receiving AOO both on the skin and in the airways (AOO/AOO) (chapter 4).

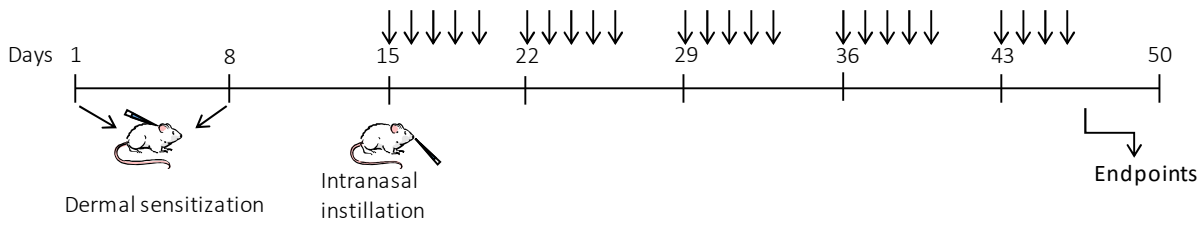

Fig. 6.2: Illustration of the chronic exposure protocol.

Yet unexpectedly, airway inflammation and remodeling were absent in all groups. Only pronounced AHR was present in the TDI/TDI treated group, in fact mimicking the clinically relevant paucigranulocytic phenotype of chemical-induced asthma more than the anticipated Th2 inflammation-mediated asthma. Our data show that mice repeatedly exposed via the nasal mucosa to TDI (AOO/TDI group) results in systemic sensitization, indicated by a proliferation of the local lymph nodes (LN) (consisting of DC, T- and B- cells), increase of total serum IgE and *ex vivo* mixed Th1 (IFN-γ) /Th2 (IL-13) response of the LN cells, however, AHR is absent. Additional preceding TDI skin exposure (TDI/TDI group) results in a higher systemic sensitization, and a predominant Th2 response (high increase of *ex vivo* IL-13, IL-4, IL-10 and serum IL-13, IgE, IgG_1 and decreased serum levels IgG_{2a}) with the presence of AHR. DC were not migrated to the lungs in any of the groups.

2.2.2 Sub-chronic oropharyngeal exposure model

The absence of inflammation and airway remodeling using the intranasal challenge made us switch to the oropharyngeal challenge method as previously described by De Vooght *et al.* [18]. By challenging deeper into the lung, we aimed to induce a pronounced inflammatory asthmatic response. Mice were dermally sensitized on days one and eight followed by five oropharyngeal challenges on days 15, 17, 19, 22 and 24 (fig. 6.3). Similar as for the first chronic study, three exposure groups were included: one receiving five oropharyngeal instillations with TDI (AOO/TDI), and one additionally exposed to TDI via the skin (TDI/TDI). Both groups were compared to the complete control group, receiving AOO both on the skin and in the airways (AOO/AOO) (chapter 5).

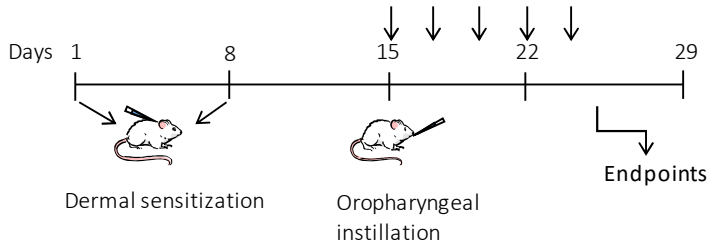

Fig. 6.3: Illustration of the sub-chronic exposure protocol.

As a result both groups receiving multiple airway exposures to TDI, with (TDI/TDI) and without (AOO/TDI) prior dermal sensitization, showed an increasing recruitment of DC in the lung, which is indicative for the initiation of an adaptive immunity, accompanied with airway inflammation. Without prior skin

exposure, the inflammation in the lung is mainly dominated by neutrophils, while prior skin exposure and consequently systemic immune sensitization results in a shift from a neutrophilic to an eosinophilic airway inflammation accompanied with AHR.

3. Relevance and limitations

3.1 Cross-reactivity MDI and TDI

MDI and TDI are both used in the polyurethane production and together account for more than 90% of the global diisocyanate market. As TDI and MDI are both used in the same plants and sometimes even together in the same production process, it is possible that employees can become dermally sensitized to MDI or TDI, and then exposed to low airborne concentrations of the other diisocyanate, which is an additional risk factor for the development of isocyanate-induced asthma. To date it is already well-described in animal and human studies that both diisocyanates are potent asthmogens [7–10,24,25].

3.1.1 MDI less potent than TDI

Originally, our lab used BALB/c mice to validate chemicals and study the pathophysiology of chemical-induced asthma in an acute mouse model (fig. 6.1). However, due to the lack of the wide availability of genetic modified BALB/c mouse strains and therefore limitations in mechanistic studies, the C57Bl/6 mouse strain was introduced. Yet, De Vooght *et al*. reported that the TDI concentrations used in the BALB/c mouse, namely 0.3% for the dermal sensitization and 0.01% for the oropharyngeal challenge, do not result in an asthma-like response in the C57Bl/6 mouse strain [26]. Therefore, we needed to adjust the TDI treatment concentrations in the C57Bl/6 mice to 2% for the dermal sensitization. The oropharyngeal challenge concentration remained the same (0.01%). To study the cross-reactivity between TDI and MDI, we additionally optimized the treatment concentrations for MDI in the acute C57Bl/6 mouse model of chemical-induced asthma. To induce similar asthma-like responses as TDI, we needed to increase the dermal sensitization and challenge concentration to 3% and 0.04% MDI, respectively. As a conclusion, both MDI and TDI are potent asthmogens, however TDI seems somewhat more potent than MDI.

3.1.2 Cross-reactivity: yes or no?

Cross-reactivity is a phenomenon that occurs in daily practice via cross-reactive IgE's of homologue proteins such as mushrooms and airborne molds, pollen (bee and weed), different types of legumes (lupine and peanut) and between homologue chemicals such as methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI) [27–30]. As MDI and TDI have structural similarities and common binding places

on HSA we tested whether a crossed-asthmatic reaction exists between both diisocyanates. To our surprise, we could not show cross-reactivity between TDI and MDI in our acute mouse model.

3.1.3 Concerns about cross-reactivity

One could argue that there are signs of possible cross-reactivity in some mice. In both TDI/MDI and MDI/TDI treated groups, some mice show signs of AHR and lung inflammation, which might be attributable to cross-reactivity. Yet since the AOO/TDI and AOO/MDI control groups also show substantial variations in the lung parameters, we remain conservative in our conclusion.

The lack of cross-reactivity can be explained by the fact that, due to their higher –N=C=O moiety reactivity towards endogenous proteins, TDI has a higher accessibility (34 lysine residues) on HSA than MDI (only 19 lysine residues) [2]. Due to these many binding places on HSA and consequently large amount of possibilities for epitope formation, it is unlikely that TDI and MDI form the same epitopes. In addition, the different stereo isomers of MDI and TDI may lead to a different binding to albumin, which makes it further difficult to generate the same epitope. However, as all lysine binding places on HSA of MDI are the same as TDI, we cannot fully exclude similar epitope formation. It is possible that by increasing the number of airway exposures, as we did in chapter 5 (fig. 6.3), the chances of similar epitope formations increase and cross-reactivity may be present. Additional to the binding possibilities to HSA and different stereo isomers, epitope formation can be influenced by the predominant proteins present in different tissues. In studies investigating immunological cross-reactivity between diisocyanates, MDI and TDI were only used in conjugation with the same endogenous proteins [13,31,32]. This is different to our study where we used 'pure' MDI or TDI to expose both skin and lung. Each tissue contains different predominant proteins potential, resulting in several different diisocyanate-protein conjugates in both tissues. Due to the potential high number of permutations, it is possible that, in the limited number of animals taken up in the cross-reactivity experiments, the epitopes formed during skin exposure differed simply by chance from the epitope formed during lung exposure. To overcome the drawback of the presence of different predominant proteins between tissues, we could have sensitize and challenge the mice with preformed TDI/MDI conjugates, using the same protein and reaction conditions.

3.2 Phenotypes of chemical-induced asthma

Since asthma is driven by genetic and environmental factors, it can result in different clinical outcomes, called phenotypes. Due to the complexity and heterogeneity of asthma, it is unlikely that one single mouse model can mimic all the features of asthma. By adjusting the treatment protocol, dosing and choice of mouse strains different phenotypes of asthma can be developed [33,34].

Several attempts to induce a severe chemical-induced inflammatory asthma phenotype have been described. Matheson *et al.* developed a chronic TDI mouse model in which C57BL/6 mice were exposed via the airways 5 times a week, for six consecutive weeks followed 2 weeks later by a single airway challenge [35]. Lee *et al*. developed a model in which rats were intranasal treated with TDI for five consecutive days, followed by three inhalation exposures [36]. Scheerens *et al*. sensitized BALB/c mice dermally via weekly exposures to TDI for six consecutive weeks followed by one intranasal challenge on week 7 [37]. Herrick *et al*. developed a hexamethylene diisocyanates (HDI) model in which BALB/c mice were dermally sensitized on days one and eight followed by four intranasal challenges within two weeks. Wisnewski *et al*. used the same treatment protocol to study the effect of MDI [25]. Recently, Pauluhn *et al.* developed a TDI model in which Brown Norway and Wistar rats where dermally sensitized on days one and eight, followed by repeated (maximum four times) inhalation challenges varying from 10-60 min.

Although most groups showed lung inflammation, much less signs of airway remodeling including epithelial shedding, smooth muscle hyperplasia and collagen deposition were found compared to OVA and HDM mouse models of asthma [23,38,39]. Therefore, we aimed to induce a severe phenotype of chemical-induced asthma with infiltration of inflammatory cells in the lung, AHR and airway remodeling.

We developed a mouse model based on a chronic house dust mite model in which BALB/c were intranasally exposed to HDM for five days/week for seven consecutive weeks resulting in AHR, eosinophilic airway inflammation, DC recruitment in the lung and airway remodeling consisting out of goblet cell hyperplasia, collagen deposition and accumulation of contractile tissue [23]. Since we know that dermal exposure is an important route of sensitization in TDI-induced asthma, we adapted the first two weeks of the HDM protocol to a dermal sensitization (fig. 6.2) [24].

3.2.1 Challenge method determines phenotypical outcome

In contrary to our hypothesis we found absolutely no signs of airway inflammation and airway remodeling after five weeks intranasal exposures to TDI. However, adding prior dermal sensitization resulted in AHR. One could argue, by using such long-term exposure mouse model, the absence of airway inflammation and airway remodeling could be due to the induction of tolerance. However, pilot studies demonstrated that even after two or three weeks of intranasal exposure, with or without dermal sensitization, airway inflammation is still absent (data not shown).

The absence of airway inflammation and remodeling in this treatment regime is probably due to the combinational use of the intranasal instillation method and the viscous vehicle acetone olive oil in which TDI is dissolved. Southam *et al*. demonstrated that, using an intranasal challenge technique, the lung distribution of the compound is heavily influenced by the instilled volume and level of anesthesia. At least 30 to 35 µl of the test substance is necessary to reach the lower airways. Additionally, studies

demonstrated that aqueous vehicles (saline or PBS) flow more easily to the lower airways, while the viscous vehicle olive oil only seems to affects the nasal cavity [40,41]. Yet, as diisocyanates are not stable in water and hydrolyze rapidly, it was required to use the viscous non-aqueous vehicle acetone olive oil.

Considering these findings and compensate the drawback of the use of a viscous vehicle, we increased the intranasal instillation from 20 µl (volume in the acute mouse model) to 40 µl and sedated the animals during instillation [11,40,41]. Yet, inflammatory lung response and airway remodeling remained absent, which is in contrast with the findings of Johnson *et al.*. Although similar treatment protocols are used, the different responses can be explained by the use of different allergens (house dust mite versus diisocyanates) and vehicles (saline versus olive oil) [23].

As the intranasal instillation using viscous vehicles mainly targets the upper airways, we decided to use another mouse model to target the lower airways by bypassing the nose, using an oropharyngeal instillation technique described by De Vooght *et al*. [18]. By bypassing the nose it has been shown to have a higher distribution to the lungs (80%) compared to the intranasal instillation method (50–60%) [40–44]. Originally, for the intranasal instillation a concentration of 0.1% TDI was used, however, this concentrations caused too much mucosal irritation when given via oropharyngeal instillation. Therefore, the concentration was decreased to 0.01% TDI, which gave no signs of irritation. To perform this instillation, mice were anesthetized using isoflurane and held vertically. Next, TDI solution was pipetted at the end of the tongue. By grasping the tongue, and meanwhile closing the nose, the mice were prevented from swallowing. Although the oropharyngeal instillation technique is not invasive, the procedure is more intensive compared to the intranasal instillation. Oropharyngeal instillation targets more the lower airways and requires anesthesia. Consequently, it is not possible to execute the procedure on a daily basis. Therefore we decided to instill the mice every other day (3 times a week), for only two weeks long (fig. 6.3). Although the total dose was much less compared to the chronic nasal instillation protocol, the oropharyngeal instillation technique was suitable to induce a severe lung inflammation. Using a similar challenge technique, Provoost *et al.* and Plantinga *et al*. also founded inflammatory lung responses after exposure to diesel exhaust particles and HDM, respectively [42,43].

3.2.2 Skin exposure determines phenotypical outcome

During the last years, it has become clear that skin exposure is an important route of sensitization. Once sensitized via the skin, low airborne concentrations can elicit an asthmatic response [24]. Moreover, both multiple exposure models (intranasal and oropharyngeal exposure) show that dermal exposure influence the phenotypical outcome in the lungs.

Chronic nasal mucosa exposure model

In this chronic model, we increased the sensitization concentration from 0.3% TDI (used in our acute BALB/c mouse model) to 0.5% to prolong the sensitization effects during the following intranasal challenges. Although nasal mucosa exposure to TDI results in systemic sensitization, shown by proliferated auricular lymph nodes, cytokine production profile and increased serum IgE titers, lung response remained absent. However, by dermally sensitizing mice with TDI prior to the intranasal exposures, AHR was present leading to a paucigranulocytic phenotype of asthma. The presence of AHR can be attributed to the systemically increase of Th2 mediators IgE (serum and BAL) and IL-13 (serum and *ex vivo* secretion). It is suggested that high levels IgE can induce ongoing mast cell activation, which consequently can result in a release of mediators such as IL-13. In parallel, mast cell can be activated by neurogenic mechanisms. Devos *et al*. demonstrated that, in a paucigranulocytic phenotype of asthma, TDI directly activates TRPA1 expressed on airway sensory nerves. This binding will lead to a release of neuropeptides such as substance P (SP). SP will consequently bind on its NK1 receptor expressed on the surface of mast cells, leading additionally to mast cell activation and a release of mediators such as IL-13, a cytokine shown by our and other groups to be directly linked with AHR [38].

One could argue that we have induced the same phenotype as already studied by Devos *et al.*, however using this chronic exposure model we found some surprising interesting results. Depending on the site of exposure a different immune response developed. Nasal mucosa exposure resulted in a predominant Th1 response; indicated by a very high increase of *ex vivo* IFN-γ secretion and a low Th2 response indicated by the increase of IL-13 concentration both in serum and *ex vivo* secretion. Moreover, nasal mucosa exposure resulted in a significant increase of the auricular lymph nodes indicating that besides the ears the nose also drains these lymph nodes. However, adding dermal exposure, Th1 response is accompanied with a predominant Th2 response, demonstrated by a very high *ex vivo* Th2 cytokine secretion (IL-4, IL-10 and IL-13) and increase of serum IgE, IgG_1 (a Th2 marker), IL-13 and decrease of $IgG2_a$ (a Th1 marker). This indicates that skin sensitization in combination with airway exposure leads to a pronounced Th2 response, which is also suggested by Ban *et al*., while nasal mucosa exposure results in a predominant Th1 response [45].

The relevance of this study is, although airway inflammation and airway remodeling remained absent, that long-term low airborne TDI exposure leads to immune sensitization but do not results in an asthma-like response. However, when the skin is exposed to TDI and sensitization occurs, TDI airway exposures finally lead to AHR. This emphasizes the importance of dermal protection when working with TDI on the work floor in order to prevent TDI-induced OA.

Sub-chronic oropharyngeal exposure model

The results of the chronic intranasal exposure model indicate that skin exposure influences the presence of AHR and type of immune response. Using our oropharyngeal instillation model, we additionally are able to investigate the influence of prior dermal exposure on the type of airway inflammation.

Depending on the presence of dermal exposure, a different inflammatory phenotype developed. Mice only instilled with TDI in the airways developed a neutrophilic inflammation, which is indicative of an irritant inflammatory response, while by adding prior dermal exposure the same airway instillations results in a mainly eosinophilic airway inflammation accompanied with AHR, indicative for immune-mediated inflammatory phenotype of asthma.

3.2.3 Dendritic cells in chemical-induced asthma

Dendritic cells (DC) are one of the most important antigen presenting cells (APC) responsible for initiating an immune response. They are located near the epithelium, where they can sense and capture inhaled antigens present in the environment. After phagocytosis, DC process the allergens into small peptides and migrate to the T cell zone of the local draining lymph nodes. There they present the processed antigens via the major histocompatibility complexes (MHC class I and II) to naive T cells, inducing polarized T cells [46,47].

Distinct DC subsets are defined in the lung each with their own surface markers and functional specialization: conventional dendritic cells (cDC), which can be further divided in $CD11b^+$ and $CD103^+$ cDC, plasmacytoid DC (pDC) and monocyte derived DC (moDC) [42]. Each of these DC subsets are equipped to induce particular T helper cell responses. These responses are context dependent: environmental signals (house dust mite, cigarette smoke, diesel exhaust particles etc…), dose, route of exposure and cell-released molecular patterns all influences the function of DC which emphasize the plasticity of DC [42,43,48,49]. DC are shown to be crucial during the sensitization and elicitation phase of Th2 mediated OVA induced asthma [22,50]. Since the DC subtypes were not yet studied in chemical-induced asthma, we investigated the recruitment of DC in our chronic and sub-chronic mouse models of chemical-induced asthma.

Long-term intranasal exposures, with or without previous skin sensitization, resulted in recruitment of DC in the local-auricular lymph nodes, which was accompanied by an increase of T- and B- cells. However, DC were not present in the lungs of the paucigranulocytic phenotype of asthma. As already explained in the previous paragraph, this can be due to intranasal challenge method.

In the multi-oropharyngeal exposure protocol, DC were only present in the ALN after dermal sensitization. However, multi-oropharyngeal airway exposures resulted in airway inflammation accompanied with the recruitment of DC ($CD11b^+$, moDC and pDC) in the lung. The recruitment of the

DC subpopulations is slightly different depending on the exposed compound (irritant versus allergen). Three days after one high exposure to the allergen HDM (not specified which der P extract is used), mainly moDC and lower amount (not significant) of $CD11b^+$ were present in the lungs [42]. While after three exposures to the irritant diesel exhaust particle, $CD11b^+$, $CD11b^-$cDC and moDC were increased in the lungs [43]. In both models, pDC were not increased, which is a main difference with our results. These results confirm that the role of DC are context dependent (type of agent, exposure protocol, administered concentrations…).

Although AHR and the type of inflammation depend on prior sensitization this is not the case for the DC recruitment to the lung. DC are recruited to the lungs of both irritant induced inflammation and immune mediated inflammatory phenotypes of asthma. The DC recruitment in the irritant-induced inflammatory phenotype indicates the potential initiation of an adaptive immune response in the lung.

Taking the results of the DC in the chronic mouse model into account, DC only recruit to lung after inflammatory stimuli [23,43,51].

3.2.4 Future perspectives

Chronic nasal mucosa exposure model

We need to take into account that by increasing the number of intranasal airway exposures the phenotypical outcome of both groups (AOO/TDI and TDI/TDI) can be changed. Therefore, an interesting next step would be to investigate if longer airway exposures (> five weeks) could lead to AHR without prior skin exposure. To answer this question, we need to increase the amount of intranasal challenges until the same levels IgE are reached as in the present TDI/TDI treated group. Another important aspect, which is not addressed in this thesis, is determining at which time point the dermal contact is crucial in the induction of TDI-induced asthma. Therefore, we can first perform a chronic airway exposure and subsequently expose them via the skin. Alternatively, we can first expose mice via the airways for a certain period, followed by skin exposure and subsequently expose them again via the airways. Moreover, it would be interesting to study the effect of simultaneous skin and airway exposure.

Sub-chronic oropharyngeal exposure model

Although we were able to induce an inflammatory phenotype of asthma, airway remodeling was still absent. To study the underlying mechanisms of this asthma feature, we could increase the amount of multiple oropharyngeal instillations.

The next step of this study would be the investigation of the contribution of DC in the induction of inflammation and AHR during the effector phase of chemical-induced asthma. This can be achieved by conditional deplete DC selectively during the airway exposures by adding diphtheria toxin (DT) in

transgenic (Tg) mice in which the CD11c promotor element drives the expression of the monkey DT receptor [51].

After it has been shown that DC are crucial in the development of chemical-induced asthma, it would be of interest to study which chemokine ligand-chemokine receptor interaction is involved in the recruitment of DC to the lung during TDI exposure. For pulmonary tissue, there is no unique chemokine receptor that mediates DC recruitment and depending on the inflammatory stimulus, various chemokine ligand-chemokine receptor interactions appear to be implicated [52]. In response to specific stimuli, lung epithelial cells produce chemokines that direct DC towards the mucosa upon binding to their chemokine receptors present on DC. For example, upon cigarette smoke it has been shown that CCR5 and CCR6, receptors for CCL5 and CCL20, respectively, seems to be crucial in DC lung recruitment. Provoost *et al.* demonstrated that, after exposure to diesel exhaust particles, moDC recruit to the lung in a CCR2-and to a less extent CCR6 dependent manner [52]. As chemokine receptors are responsible for the recruitment of the DC to the lung, they are suggested to be a potent target for drugs development [51]. Therefore, it would be interesting to study the specific chemokine receptors involved in the recruitment of DC during TDI exposure. This could be achieved by using specific chemokine receptor knockout mice. Depending on the CCR involved in the DC recruitment after specific stimuli, different CCR blockers could be developed leading to personalized medicine.

3.2.5 General limitations of both exposure models

A first limitation of this study is the pulmonary dosing. We are aware that the inhalation exposure would mimic better the real life human exposure compared to the intranasal and oropharyngeal instillation techniques. However, inhalation exposure requires the use of specified equipment, it is technically demanding and time-consuming. Furthermore, it requires high amounts of chemicals, which could increase the risk of sensitization of the handler to the chemical.

Another limitation of the chronic intranasal study is the fact that, although it is known in the literature that viscous solutions during an intranasal instillation mainly affects the nasal cavity, we did not studied the presence of inflammatory and dendritic cells in the nasal mucosa.

The marker composition of the lung dendritic cell sub populations were based on the panel composition of both Provoost *et al.* and Plantinga *et al*. [42,43,52]. However, in the discrimination between $CD11b^+$ cDC and the moDC we did not used the marker CD64 in combination with MAR 1 (high-affinity IgE α chain receptor (FcεRIα)). MAR 1^+ staining is used to compensate for the possible reduced CD64 staining intensity during migration of DC to the local lymph nodes. Therefore it is possible that we did not detected all moDC in the auricular lymph nodes [42].

The fourth limitation concerned the different markers of the dendritic cell subpopulations between the skin and lung. In this study, we focused on the panel composition of DC in the lung; yet, the same DC panel was used for the analysis of DC in the auricular lymph nodes that are probably migrated from the skin. Therefore, we are aware that we might have missed some dermal DC subpopulations in the auricular lymph nodes of TDI treated groups [42,43,48,56].

4. Conclusion

In this study, we found three main conclusions:

- Both TDI and MDI are potent asthmogens, however, MDI is somewhat less potent as TDI. There is no cross-reactivity between both chemicals in an acute mouse model.
- Skin exposure is necessary to induce AHR and determines the systemic immune response (Th1 versus Th2) and type of lung inflammation (eosinophilic versus neutrophilic).
- DC only recruit to the lung after inflammatory stimuli.

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Summary

Asthma is a heterogeneous airway disease, characterized by chronic airway inflammation, variable airway remodeling, reversible airway obstruction and non-specific airway hyperreactivity (AHR). The respiratory symptoms include wheezing, shortness of breath, chest tightness and cough. In adults, 9-15% of all asthma cases are attributable to exposure to agents on the workplace, called occupational asthma (OA). OA is therefore one of the most common forms of occupational lung disease in many industrialized countries.

Based on the presence or absence of an asymptomatic latency period, OA can be divided into two phenotypes. First, immunological OA appears after a latency period of exposure (months to years) necessary for acquiring immune sensitization to the causal agent. This phenotype of OA encompasses OA induced by an immunoglobulin (Ig)E mediated mechanism (most high- and some low- molecular weight agents), and OA in which the IgE mechanism has not been consistently demonstrated (low molecular weight agents such as western red cedar, acrylates and isocyanates). Second, non-immunological OA, in which asthma occurs after an acute exposure to high concentrations of an irritant, without a latency period, termed irritant-induced asthma.

Diisocyanates are reactive chemicals, used in the polyurethane industry for the manufacture of flexible and rigid foams, fibers, paints and varnishes. They are one of the most important causes of OA. Low airborne levels of diisocyanates, even below the established permissible exposure level, can trigger asthmatic reactions in individuals sensitized to diisocyanates. An important difference between protein-induced allergic asthma and chemical-induced asthma, is that the chemicals first need to bind to endogenous proteins before they become recognizable by the immune system resulting in a different, not yet fully delineated mechanism of action.

In this doctoral thesis, we used an established acute mouse model of chemical-induced asthma (chapter 3). This model consists of a dermal sensitization phase followed by a single airway exposure. This acute exposure model results in AHR and airway inflammation that is mainly dominated by neutrophils in conjunction with the presence of eosinophils. This acute mouse model has been suitable to validate chemicals for their asthmatic potency and to study several key players in chemical-induced asthma. However, due to the short-term exposure time, there are some limitations in this model such as the absence of a robust lung inflammation and airway remodeling. Therefore, we developed a chronic (chapter 4) and a sub-chronic (chapter 5) mouse model of chemical-induced asthma.

1. Study of the cross-reactivity between MDI and TDI (chapter 3)

In the first part of this thesis, we optimized the acute mouse model using 2,4-toluene diisocyanate in C57Bl/6 mice, and validated the sensitization and asthmogenic potency of 4,4-methylene diphenyl diisocyanate (MDI). Both TDI and MDI were potent sensitizers and asthmogens in the C57Bl/6 mice. However MDI was, compared to TDI, a somewhat weaker dermal sensitizer and asthmogen. Additionally, we investigated whether there is cross-reactivity between TDI and MDI. We studied the cross-reactivity between TDI and MDI by dermally sensitizing mice with TDI followed by an airway challenge with MDI and vice versa. In this acute model, with a single airway challenge, we did not observed cross-reactivity between TDI and MDI.

2. Development of a chronic and sub-chronic mouse model and study the presence of dendritic cells

2.1 Development of a chronic intranasal mouse model (chapter 4)

In the second part of this thesis, we attempted to obtain a severe chemical-induced asthmatic phenotype with pronounced lung inflammation and airway remodeling. Chronic intranasal exposure (daily intranasal instillation exposure – 5 days a week - for five consecutive weeks) resulted in a predominant T helper (Th1) systemic immune response, without AHR nor recruitment of inflammatory and dendritic cells (DC). By adding prior dermal sensitization, intranasal exposures resulted in a predominant systemic Th2 immune response accompanied with AHR. Still, the AHR was not accompanied with airway inflammation nor recruitment of DC in the lung, mimicking the clinically relevant paucigranulocytic phenotype of asthma. As a conclusion, dermal contact with TDI prior to long-term respiratory exposures determines the outcome of systemic immune sensitization and the presence of AHR.

2.2 Development of a sub-chronic oropharyngeal mouse model (chapter 5)

Since we were unable to find airway inflammation in the chronic intranasal mouse model, the last part of this thesis focused on the development of a mouse model using an oropharyngeal instillation technique to expose the lower airways to TDI. Multiple oropharyngeal TDI exposures resulted in airway inflammation accompanied with DC recruitment to the lung, which is indicative of the initiation of an adaptive immune response in the lung. However, the type of inflammation is shaped by the route of exposure. Without skin exposure the inflammation is dominated by mainly neutrophils, which is indicative for irritant-induced inflammation, while prior skin sensitization with TDI resulted in an eosinophilic airway inflammation accompanied with AHR, which is indicative for an immune-mediated inflammatory phenotype of asthma. As a conclusion, DC are recruited to the lungs in the presence of inflammation, and pre-dermal contact with TDI determines the type of lung inflammation (eosinophilic versus neutrophilic) and the presence of AHR.

Samenvatting

Astma is een heterogene luchtwegaandoening, gekenmerkt door chronisch luchtwegontsteking, luchtwegremodelering, reversibele luchtwegobstructie en niet-specifieke bronchiale hyperreactiviteit (BHR). De klinische symptomen zijn piepende ademhaling, kortademigheid, beklemmend gevoel aan de borststreek en hoesten. In de volwassen populatie wordt 9-10% van de astmagevallen toegeschreven aan beroepsmatige blootstellingen, ook wel beroepsastma (BA) genoemd. BA wordt eveneens aanzien als de meest voorkomende werk-gerelateerde longziekte.

BA kan, door middel van de aan of afwezigheid van een asymptomatische latentieperiode, onderverdeeld worden in twee fenotypes. Het eerste fenotype, immunologisch gemedieerd BA genoemd, ontstaat na een latentieperiode van maanden tot jaren. Tijdens deze periode worden personen gesensibiliseerd aan het causaal agens. Dit fenotype van astma kan zowel geïnduceerd worden door immunoglobuline (Ig)E gemediëerde mechanismen (door hoog- en enkele laag molecuulgewicht agens) als door niet-IgE gemediëerde mechanismen (door laag molecuulgewicht agens zoals western red cedar, acrylaten en isocyanaten). Het tweede fenotype, niet-immunologisch gemedieerd BA genoemd, ontstaat na een korte hoge blootstelling aan een irriterende stof. Dit fenotype is niet gekenmerkt door een latentieperiode en wordt irritant geïnduceerd astma genoemd.

Diisocyanaten zijn reactieve chemicaliën die gebruikt worden in de polyurethaan industrie voor de productie van polyurethaanschuim, verven, vernissen, en vormen een belangrijke oorzaak van BA. Eens een persoon gesensibiliseerd is, kunnen zeer lage diisocyanaat concentraties in de lucht, zelfs onder de wettelijk vastgelegde grenswaarden, een astma-aanval uitlokken. In tegenstelling tot hoog molecuulgewichten, worden chemicaliën met een laag moleculairgewicht pas herkenbaar door het immuunsysteem na binding met endogene proteïnen. Het immunologisch mechanisme waarmee chemicaliën met een laag moleculairgewicht astma veroorzaken is nog niet volledig gekend.

In deze doctoraatsthesis hebben we een bestaand acuut muismodel van chemisch-geïnduceerd astma gebruikt (hoofdstuk 3). Dit model bestaat uit een dermale sensibilisatie fase gevolgd door één enkele luchtwegblootstelling die resulteert in immuun sensibilisatie, BHR en luchtweginflammatie. Deze laatste bestaat voornamelijk uit neutrofielen en beperkt ook uit eosinofielen. Aan de hand van dit acuut model zijn al verschillende chemicaliën gevalideerd voor hun astmatische potentie. Hiernaast is het acuut model ook gebruikt om het onderliggend mechanismen van chemisch-geinduceerd BA te onderzoeken. Het nadeel van dit acuut model is dat robuuste longontsteking en luchtwegremodelering afwezig zijn, wat ook belangrijke kenmerken van astma zijn. Om ook deze kenmerken van astma te kunnen onderzoeken hebben we een chronisch (hoofdstuk 4) en een sub-chronisch (hoofdstuk 5) muismodel van chemisch-geïnduceerd astma ontwikkeld.

1. Studie van de kruisreactiviteit tussen MDI en TDI (hoofdstuk 3)

In het eerste gedeelte van deze thesis hebben we het acuut model geoptimaliseerd in de C57Bl/6 muizenstam gebruikmakend van de model agens 2,4-tolueendiisocyanaat (TDI). Vervolgens hebben we de sensibiliserende en astmatische potentie van 4,4'-methyleendifenyldiisocyanaat (MDI) gevalideerd. De resultaten toonden aan dat zowel TDI als MDI potente astmogenen zijn, maar MDI is een zwakkere dermale sensibilisator en astmogeen dan TDI. Tevens hebben we de kruisreactiviteit tussen TDI en MDI onderzocht. Hiervoor zijn de muizen dermaal gesensibiliseerd met TDI gevolgd door een luchtwegblootstelling aan MDI en omgekeerd. In dit acuut model, met één enkele luchtwegblootstelling, vonden we geen kruisreactiviteit tussen TDI en MDI.

2. Ontwikkeling van een chronisch en sub-chronisch muismodel en het bestuderen van de aanwezigheid van dendritische cellen

2.1 Ontwikkeling van een chronisch intranasaal muismodel (hoofdstuk 4)

In het tweede gedeelte van deze thesis hebben we geprobeerd om een fenotype van ernstig chemisch-geïnduceerd astma te ontwikkelen die gekarakteriseerd is door een uitgesproken luchtweginflammatie en luchtwegremodelering. Dagelijkse intranasale blootstellingen aan TDI (5 dagen per week voor 5 opeenvolgende weken) resulteerden in een sterke systemische T helper (Th)1 immuunrespons zonder BHR, zonder longinflammatie en afwezigheid van dendritische cellen (DC) in de long. Indien de muizen eerst dermaal werden gesensibiliseerd met TDI, resulteert deze dagelijkse intranasale blootstelling in een sterke systemische Th2 immuunrespons en BHR, maar nog altijd zonder longinflammatie en DC in de long. Dit muismodel bootst het klinisch relevant 'paucigranulocytair' fenotype van chemisch-geïnduceerd astma na. Gebruikmakend van dit model voor TDI-gemediëerde pulmonaire reacties kunnen we concluderen dat voorgaand dermaal contact met TDI de immunologische respons beïnvloed en de aanwezigheid van BHR bepaalt na een chronische luchtwegblootstelling aan lage concentraties TDI.

2.2 Ontwikkeling van een sub-chronisch orofaryngeaal muismodel (hoofdstuk 5)

Aangezien dat luchtweginflammatie niet aanwezig was in het chronisch intranasaal model, werd in het laatste gedeelte van deze thesis gefocust op het ontwikkelen van een muismodel waarin we een andere methode voor luchtwegblootstelling gebruiken, namelijk de orofaryngeale instillatie techniek. Aan de hand van deze instillatie kunnen we de lagere luchtwegen blootstellen. Meerdere orofaryngeale bloostellingen aan TDI resulteerden in een migratie van inflammatoire cellen en DC naar de long, deze laatste wijst op de initiatie van een adaptief immuunsysteem. Indien de muizen eerst dermaal werden gesensibiliseerd met TDI resulteerden de orofaryngeale TDI instillaties in luchtwegontsteking, die voornamelijk gekenmerkt was door eosinofielen en BHR. Deze longrespons wijst op een immuun-

gemedieerd inflammatoir fenotype van astma. In afwezigheid van deze dermale sensibilisatie resulteerden de orofaryngeale instillaties in een luchtwegontsteking die voornamelijk gekenmerkt werd door de aanwezigheid van neutrofielen, zonder BHR. Deze luchtweg respons wijst op irritant-geïnduceerde inflammatie. Uit deze resultaten kunnen we concluderen dat DC naar de long migreren in aanwezigheid van inflammatoire cellen. Eveneens kunnen we concluderen dat voorgaande dermale sensibilisatie het type van luchtwegontsteking (eosinofielen of neutrofielen) en de aanwezigheid van BHR bepaalt.

SHORT CURRICULUM VITAE

Lore Pollaris was born on October 2th 1989 in Genk, Belgium. After graduating from secondary school at Onze-Lieve-Vrouwlyceum genk in 2007, she started her training in Biomedical Sciences at the faculty of Medicine at the Katholieke Universiteit Leuven, Belgium, where she graduated cum laude in 2013. Under supervision of Prof. Jeroen Vanoirbeek and Prof. Peter Hoet, she started her PhD concerning a mouse model of chemical-induced asthma in September 2013, in the Laboratory of Occupational and Environmental Toxicology, Center for Environment and Health at the Katholieke Universiteit Leuven.

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