Toluene diisocyanate (TDI)-induced asthma

Inflammatory and immunological responses to TDI, ovalbumin (OVA) and ovalbumin peptide OVA 323-339 in mouse models

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Scientific environment

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<td>AHR</td>
<td>airway hyper-responsiveness</td>
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<td>APCs</td>
<td>antigen-presenting cells</td>
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<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<td>granulocyte colony-stimulating factor</td>
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<td>LMW compounds</td>
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<tr>
<td>MBP</td>
<td>major basic protein</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MIP-2</td>
<td>macrophage inflammatory protein-2</td>
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<tr>
<td>MSA</td>
<td>mouse serum albumin</td>
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<td>OA</td>
<td>occupational asthma</td>
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<tr>
<td>OVA</td>
<td>ovalbumin</td>
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<tr>
<td>OVA 323-339</td>
<td>ovalbumin peptide 323-339</td>
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<tr>
<td>Penh</td>
<td>enhanced pause</td>
</tr>
<tr>
<td>TDI</td>
<td>toluene diisocyanate</td>
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<td>VEGF</td>
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Summary

Among three major diisocyanates: TDI, methylenediphenyl diisocyanate (MDI) and hexamethylene diisocyanate (HDI), TDI has been regarded as the leading cause of diisocyanate-induced asthma. The principal feature of TDI-induced asthma is the airway inflammation with influx of neutrophils, lymphocytes and eosinophils. This in turn leads to the variable airway hyper-responsiveness. TDI-induced asthma can share many similar features with allergic asthma, such as increased total and specific IgE serum levels, activated CD4+ T cells, eosinophils and mast cells, increased levels of IL-4 and IL-5 and airway remodeling.

So far, the pathogenic mechanism of TDI-induced asthma is still far from clear. It has been commonly accepted that TDI sensitization involves binding of TDI to carrier molecules. Specific IgE to diisocyanate has been regarded to be helpful in the diagnosis of occupational asthma. Particularly, increased immunoglobulin IgG \(_1\) was suggested to be associated to TDI exposure and the protein-conjugated TDI can be a useful marker for progress of TDI-asthma. In the past decades, the reports on the phenotype of immune response to TDI have been quite controversial. Maestrelli et al. reported that increased levels of Th2 cytokines were detected in the airways and bronchial mucosa of TDI asthmatics. However, some authors such as Lummus et al. observed Th1-like response characterized by an increase in the number of neutrophils and the levels of IFN-\(\gamma\) and IL-8.

In order to better address those issues, we established a TDI BALB/c mouse model by subcutaneous sensitization and intranasal challenge to investigate the inflammatory and immune responses that occurred in the development of TDI-induced asthma. In this model, the Th2 mode was involved since the TDI mice demonstrated Th2-like immune responses with significantly increased serum total IgE, IL-4 and decreased IFN-\(\gamma\). Airway remodeling was initiated in the early phase of the development of the TDI asthma. However, neutrophil-dominant pulmonary inflammation in TDI-induced mouse model of asthma suggested a different inflammatory phenotype from the eosinophil-dominant allergic responses induced by high molecular weight chemicals, such as OVA and OVA 323-339.

There are several different routes of exposure from which the professional workers finally could develop asthma. The skin might function as one of most possible initiative site for
sensitization and later on, the final target tissue, airways became hyper-sensitive and asthma occurred. In this context, the improved mouse model by repeated epicutaneous sensitization and intranasal challenge was established. Similar inflammatory and immune responses were triggered as in the mice by subcutaneous sensitization. Furthermore, followed by exposure to TDI, increased airway hypersensitivity was observed when indicated by Penh with the method of plethysmography. It seems that IgG\textsubscript{1} may not be ignored as well as IgE in the development of TDI-induced asthma, at least in mouse model of asthma.
1. **Introduction**

Occupational asthma is the most commonly reported occupational lung disease in many industrialized countries [1-2]. Occupational exposure has been shown to induce both respiratory and contact hypersensitivity reactions [3-5]. Diisocyanates, highly reactive low molecular weight compounds (LMW), are widely used in the polyurethane industry. Diisocyanates have been widely used for manufacture of polyurethane foams, coatings, elastomers, adhesives and many other products [6]. With the increasing demand, the diisocyanate production has been in steady rise for decades [1, 3]. Diisocyanates have been reported as one of the leading causes of occupational asthma, among which TDI, MDI and HDI are the most common triggers. Estimates on the prevalence of isocyanate-induced asthma among exposed workers varies from 0-15% [3, 7]. The principal feature of TDI-induced asthma is the airway inflammation with influx of lymphocytes, eosinophils and neutrophils [8]. This in turn leads to the variable airway hyper-responsiveness (AHR). Furthermore, many features with allergic asthma, such as elevated total and specific IgE serum levels, airway inflammation characterized by activated CD4+ T cells, eosinophils and mast cells, increased levels of IL-4 and IL-5 and airway remodeling can also be observed in most of TDI-induced asthma [9].

Murine models have been widely used to study atopic asthma. Mouse OVA models have successfully mimicked many features of atopic asthma: eosinophilic airway respiratory inflammation, Th2 cell responses, and airway hyper-responsiveness, and have greatly contributed to our understanding of the immune mechanisms in atopic asthma [10]. So far only limited animal models of diisocyanate asthma can be regarded to be adequately successful for studies of TDI-induced asthma. Studies, most commonly in guinea pigs, have demonstrated the acute inhalational toxicity and immunogenicity of diisocyanates, as well as features characteristic of human asthma, including airway inflammation, AHR, and mucus hyper-secretion following sensitization and respiratory tract exposure [1, 11-12]. Interestingly, it has been reported an upper respiratory tract inflammation with predominantly neutrophilic infiltration in BALF [13].
1.1 Definition of hypersensitivity and allergy

The term hypersensitivity is used to describe the objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons [14-15].

Allergy is a hypersensitivity reaction initiated by specific immunologic mechanisms and can be antibody or cell-mediated. In the patient with symptoms from mucosal membranes in the airways and gastrointestinal tract, where the antibody belongs to the IgE isotype, these patients may be said to have IgE-mediated allergy. In a more chronic stage of IgE-initiated inflammation, the inflammatory reaction causing symptoms is dominated by allergen-specific lymphocytes. Because of the hypersensitivity, the allergic symptoms can also be induced or aggravated by non-immunological factors such as infections, irritant, exercise and so forth [15]. In the conglomerate of non-IgE-mediated allergy, the inflammation can be mediated by allergen-specific lymphocytes, as in contact dermatitis, or by antibodies of the IgG isotype, as in anaphylaxis caused by immune complexes containing dextran and the classical serum sickness [15].

Fig.1. Allergy and hypersensitivity
1.2 Asthma

Asthma is a disease characterized by episodic airflow obstruction that at least partially is reversible, lung inflammation and particularly airway’s hyper-responsiveness (AHR). Both the structure and function of the airways are altered in asthma. Airways’ remodeling in asthma induces not only structural changes but also fundamental changes in the relationships between and among various airway constituents [16]. Asthma resulting from immunological reactions should be called allergic asthma. Most cases are initiated by IgE antibodies, and if there is a wish to highlight that fact, a proper term is IgE-mediated allergic asthma. The importance of other immunologic mechanisms in initiating the inflammation associated with allergic asthma needs further investigation. About 80% of childhood asthma and > 50% of adult asthma has been reported to be allergic. The mechanisms initiating non-allergic asthma are not well defined, although similar inflammatory changes occur in both forms of asthma [15].

Fig.2. Allergy and asthma.

1.3 Occupational asthma

Occupational asthma (OA) is a type of asthma due to causes and conditions attributable to a particular work environment rather than to stimuli encountered outside the workplace [17]. As the most commonly reported occupational lung disease in many of industrialized countries [1-2], OA approximately accounts for 9% to 25% of cases of new-onset adult asthma [17-21]. There is a general agreement that OA can be distinguished as two types: immunologic OA (ie. allergic) and non-immunologic OA (ie. non-allergic). Immunologic OA appears after a latency period of exposure necessary for acquiring immunologic sensitization to the causal agent, though so far an immunologic mechanism for some agents has not yet been proven to induce asthma after a latency period. Non-immunologic OA generally occurs after acute exposure to high dose of irritants, is far less frequent and is characterized by lack of latency period [17]. This type of OA now has been termed as “irritant-induced asthma” [22], which includes reactive airway dysfunction syndrome after massive exposure to irritants, work-exacerbated asthma as a condition in which asthma is pre-existing or concurrent but exacerbated by occupational exposure [17]. The current thesis’ focus is on immunologic asthma. Occupational exposure has been shown to induce both respiratory and contact hypersensitivity reactions [3-5]. Like non-occupational asthma, OA is probably the result of multiple genetic, environmental and behavioral influences.

Many aspects of the pathophysiology of OA are controversial. Issues that frequently stimulate debate are the characteristics of immune responses induced by chemical respiratory allergens and the nature of the immunologic effector mechanisms [17].

1.4 Chemicals and asthma

More than 350 agents have been reported to cause OA. Although there are fewer low molecular weight (LMW) chemicals than high-molecular-weight (HMW) agents in the list of occupational respiratory sensitizers, LMW still presents an important subset of etiologic agents, including approximately 100 separate chemical entities. Recent data indicate that LMW chemicals account for more new cases of OA caused by sensitization than HMW agents [17, 23]. Since there are about 30,000 registered chemicals, it can be estimated that 1
in 300 is asthmagenic. It is therefore reasonable to believe that asthmagenic chemicals have some important features in common. Quantitative structure-activity relations have been used to correlate chemical function with chemical structure, on the assumption that chemical structure is a determinant of chemical reactive properties and biological activity. So far the molecular mechanism for LMW respiratory sensitizers is still uncertain, therefore quantitative structure-activity relationships seems to be a relatively appropriate assumption [24-25].

1.5 High molecular weight (HMW) compounds

HMW compounds (MW > 1000 daltons) are groups of occupational agents like proteins, polysaccharides, and peptides that induce allergic response by producing specific IgE antibodies and sometimes specific IgG antibodies. In most instances, positive immediate skin test reactions can be elicited with extracts of the offending agents and specific IgE antibodies to these antigens can be detected. Clinically, the patients are usually atopic and have a history of allergic rhinitis and eczema. They usually describe asthma symptoms occurring within a few minutes of exposure. Inhalation challenge tests with HMW occupational allergens induce an isolated immediate asthmatic reaction or biphasic reaction (immediate and late), seldom an isolated late asthmatic reaction. There are no differences in the pathogenetic mechanisms of asthma due to common occupational allergens and asthma due to common inhalant allergens such as house dust mite or pollen allergens [26-27].

1.6 Low molecular weight (LMW) compounds

Many LMW compounds (MW< 1000 daltons) have been shown to cause occupational asthma. In some instances, LMW agents are believed to act as haptens and form immunogenic complexes with carrier proteins. Specific antibodies to LMW chemicals are more difficult to detect though they are still likely to be involved in the pathogenesis of the disease. Especially, in the case of isocyanates, specific IgE antibodies are found in only a small proportion of affected subjects [26, 28-29].
Diisocyanates, highly reactive LMW compounds, are widely used in industry for the production of, for example, polyurethane foam, varnish, paint, elastomers, isolation materials, adhesives and many other products [6, 9, 30]. Diisocyanates contain two highly reactive groups (– N = C = O), which can react with – OH, – SH, and – NH₂ groups of endogenous proteins [31-32]. It is known that diisocyantes are potential sensitizers and are able to bind to albumin, tubulin, keratin and glutathione in the skin or the mucosal surface [30, 33-38]. When the isocyanate groups (– N = C = O) of diisocyanate come into contact with an aqueous environment, amine groups (– NH₂) are formed spontaneously, with release of CO₂ [39].

With the increasing demand, the diisocyanate production has been in steady rise for decades [1, 3]. Diisocyanate has been reported as one of the leading causes of occupational asthma, among which TDI, MDI and HDI are the most common triggers. Estimates on the prevalence of isocyanate-induced asthma among exposed workers varies from 0-15% [3, 7]. The principal feature of TDI-induced asthma is the airway inflammation with influx of lymphocytes, eosinophils and neutrophils [8]. This in turn leads to the variable AHR. Furthermore, TDI-induced asthma may share many features with allergic asthma, such as elevated total and specific IgE serum levels, airway inflammation characterized by activated CD4+ T cells, eosinophils and mast cells, increased levels of IL-4 and IL-5 and airway remodeling [9].

OA due to diisocyanate exposure has following properties in many common aspects: the 5% to 10% prevalence among workers in the industry, the predominant occurrence of late asthmatic reaction (isolated or late component of the biphasic reaction) on an inhalation challenge lest, the persistence of asthma despite removal from exposure in a large proportion of sensitized subjects, and the lack of predilection for atopic subjects. Despite the low prevalence of specific IgE antibodies to the hapten conjugates in affected subjects, the clinical features of isocyanate- and plicatic acid-induced asthma are regarded as those of allergic diseases. Immune response mounted by these guinea pigs to diisocyanate protein conjugates was heterogeneous and involved multiple specificities for hapten, carrier protein, and new antigenic determinants. Because TDI is a highly reactive material, it is difficult to know what type of conjugate it might form when it is taken up by the body system. The inability to detect specific IgE and IgG antibodies may be due to our inability to produce an
appropriate antigen. The immunologic mechanism responsible for the asthmatic reaction has yet to be clearly elucidated [26].

In contrast, mouse model-associated approaches are based on the finding that chemicals have the potential to cause respiratory allergy. Isocyanate skin exposure in various animal models induces systemic Th2-like sensitization that leads to asthma-like responses of the lung on subsequent specific inhalation challenges [6, 17, 40-41]. Interestingly, skin exposures may result in a greater lung inflammatory response after inhalation challenge in mice [42]. Data supporting the hypothesis that the skin may be a relevant site for systemic sensitization to certain occupational allergens in human beings are largely indirect. OA induced by MDI has been described in human beings, who appear to develop IgE-mediated asthma after dermal exposure to MDI [43].

Although several efforts to use animal models have been made, the pathogenesis of TDI-induced asthma remains largely unclear. In the first publication, we described a TDI mouse model with subcutaneous injection followed by intranasal challenge, which demonstrated a typical IgE-mediated Th2 immune response [44]. However, some authors observed a Th1-like immune response characterized by an increase in the number of neutrophils and the increased levels of IFN-γ and IL-8 [45]. They considered that IgE antibody to diisocyanate might be associated with high dose exposure and less severe outcome [23]. In order to further investigate immune response and the inflammatory phenotype which occurred in the development of TDI-induced asthma, we established a TDI asthma mouse model by epicutaneous sensitization and intranasal challenge, which is adjuvant free and was constructed on the base of type I allergy mouse model [46-49].

1.7 Some concepts in allergy and immunology

1.7.1 Hapten

The concept of haptens emerged from the work of Karl Landsteiner[50] who also pioneered the use of synthetic haptens to study immunochemical phenomena.
A hapten is a small molecule that can elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one that does not elicit an immune response by itself. In general, only large molecules, infectious agents, or insoluble foreign matter can elicit an immune response in the body. Once the body has generated antibodies to a hapten-carrier adduct, the small-molecule hapten may also be able to bind to the antibody, but it will usually not initiate an immune response, only the hapten-carrier adduct can do this. Sometimes the small-molecule hapten can even block immune responses to the hapten-carrier adduct by preventing the adduct from binding to the antibody, a process called hapten inhibition.

1.7.2 Protein antigens and immune responses

Proteins are continually synthesized in the cells. These include normal proteins (self) and microbial invaders (non-self). A histocompatibility complex (MHC) molecule inside the cell takes a fragment of those proteins and displays it on the cell surface. (The protein fragment is sometimes compared to a sausage in a hot dog, and the MHC protein to the bun.) When the MHC-protein complex is displayed on the surface of the cell, it can be presented to a nearby immune cell, usually a T cell or natural killer (NK) cell. If the immune cell recognizes the protein as non-self, it can kill the infected cell, and other infected cells displaying the same protein [51].

1.7.3 MHC and antigen-presenting cells

There are two general classes of major MHC molecules: Class I and II. Class I MHC molecules are found on almost all cells and present proteins to cytotoxic T cells. Class II MHC molecules are found on certain immune cells themselves, chiefly macrophages and B cells, also known as antigen-presenting cells (APCs). These APCs ingest microbes, destroy them, and digest them into fragments. The Class II MHC molecules on the APCs present the fragments to helper T cells, which stimulate an immune reaction from other cells [51].
1.7.4 T cells or T lymphocytes

T lymphocytes belong to a group of white blood cells known as lymphocytes, and play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, such as B cells and natural killer cells (NK cells) by the presence of a special receptor on their cell surface called T cell receptors (TCR). The abbreviation T, in T cell, stands for thymus, since this is the principal organ responsible for the T cell's maturation. A large number of different subsets of T cells have been discovered, each with a distinct function [52].

1.7.5 T helper cell

T helper cells (Th cells) are a sub-group of lymphocytes that play an important role in establishing and maximizing the capabilities of the immune system. Th cells are involved in activating and directing other immune cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages, among other functions. It is this diversity in function and their role in influencing other cells that gives T helper cells their name. These cells are unusual in that they have no cytotoxic or phagocytic activity; they cannot kill infected host cells (also known as somatic cells) or pathogens, and without other immune cells they would usually be considered useless against an infection. These cells are also known as CD4+ T cells because they express the CD4 protein on their surface. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of Antigen Presenting Cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response. These cells can differentiate into one of several subtypes, including Th1, Th2, Th3, Th17, regulatory T (Treg) or follicular helper T (TFH) cells, which secrete different cytokines to facilitate a different type of immune response. The mechanism by which T cells are directed into a particular subtype is poorly understood, though signaling patterns from the APC are thought to play an important role [52].
1.7.6 Mast cells

Mast cells are the main effector cells of especially the early phase of the allergic reaction and in immediate asthmatic response. Through antigen cross-linking to high affinity IgE receptors on the cell surface, mast cells discharge several groups of mediators, such as histamine. It has also been reported that mast cell-derived lipid mediators including leukotrienes and prostaglandins play an important role in the late asthmatic response and AHR [53-54]. Furthermore, mast cells can secrete inflammatory cytokines such as IL-4, IL-5, IL-6, IL-13 and TNF-α, which have a potential role in cellular infiltrates and airway inflammation [55].

Moreover, some studies in vitro demonstrated that mast cells produce various mediators such as transforming growth factor (TGF)-β1 [56] and matrix metalloproteinases (MMPs) [57], which may contribute to the development of airway remodeling [58].
Fig.3. Activation of T cells.

The T lymphocyte activation pathway is triggered when a T cell encounters its cognate antigen, coupled to a MHC molecule, on the surface of an infected cell or a phagocyte. T cells contribute to immune defenses in two major ways: some direct and regulate immune responses; others directly attack infected or cancerous cells.

2. AIMS OF THE STUDY

The principal aims of this work were:

1) To establish and evaluate asthma mouse models to study the phenotype of responses to TDI as compared to the responses to the protein allergens OVA and OVA peptide 323-339.

2) To define the *in vivo* and *in vitro* antigenicity or allergenicity of the protein allergen OVA and the synthesized OVA peptide 323-339.

3) To study whether different sensitization routes result into different responses with respect to antibody production, cytokine secretion and inflammatory cell infiltration.

4) To investigate the role of IgE in the inflammatory and bronchial responses to TDI.
3. MATERIALS AND METHODS

3.1 Allergens and chemicals

In order to correctly interpret the different responses monitored in this study, it is imperative to use high quality chemicals and well defined allergens for the specific exposure and sensitization.

TDI (2,4 to 2,6-isomers; ratio, 80:20; lot S36694 311) was obtained from Merck-Schuchardt, Hohenbrunn, Germany. Ovalbumin (electrophoretic purity 99%, lot 81F-8230) was purchased from Sigma Chemical Company, St Louis, MO, USA. Imject-alum (lot E164 107) was obtained from Pierce Biotechnology Inc., Rockford, IL, USA. Ovalbumin peptide, amino acid residues No. 323–339, was synthesized by semi-automatic peptide synthesizer (LKB Biochrom, Ltd, Cambridge, England) as described before by Johnsen and Elsayed [59] at the Allergy Research Group, Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway.

3.2 Experimental animals

BALB/c mice have been widely used for asthma and other allergy-related research, since they are high IgE responders and easily to acquire at lower cost. In addition, increasing amount of literatures cites the use of mouse models in studies of OA. Female BALB/c mice (approximately 20 g and 8 weeks old) were kept in a conventional animal house with 12-h dark/light cycles. They received tap water and pelleted pathogen-free food ad libitum. All experiment procedures were approved by the local Ethical Committee for Animal Research.
3.3 Sensitization and challenge protocols

Animals were allowed to acclimatize at least for 1 week in order to avoid unnecessary influence on the results originated from the stress. In this thesis three different protocols were used.

3.3.1 Subcutaneous sensitization and intranasal challenge protocol for TDI

Firstly, female BALB/c mice were sensitized by subcutaneous injection (SC) with 20 μl of neat TDI once, followed by 20 μl of TDI in olive oil (1:10) once a week for 2 weeks. Thereafter, the mice were challenged intranasally (IN) with 1% TDI dissolved in ethyl acetate:olive oil (1:4) for 3 consecutive days (see Publication I).

![Diagram of TDI mouse model](image)

Fig.4. The protocol of TDI mouse model used in Publication I. SC = subcutaneous injection, IN = intranasal challenge.

3.3.2 Intraperitoneal sensitization and intranasal challenge protocol for OVA and OVA 323-339

Female BALB/c mice were intraperitoneally injected with 25 μg OVA or OVA 323-339 absorbed on 300 μg alum once a week for 3 weeks, followed by intranasal challenge with 20 μl of 1% OVA and 1% OVA 323-339, respectively. Alum and saline control groups were sensitized and then challenged in a similar manner as the OVA 323-339 group. The protocol is illustrated as in following figure. The peptide (Pep) group is presented as an example to explain the protocols. IP: intraperitoneal injection, IN: intranasal challenge (see Publication I and II).
3.3.3 Epicutaneous sensitization and intranasal challenge for TDI and OVA

After a two-week acclimatization period, female BALB/c mice were epicutaneously sensitized according to Vaali et al. [47] with minor modifications. Briefly, allergens or vehicles were added to lightly wounded skin once a week for total of 3 weeks. The concentrations of allergens in volume of 100 μl were used as follows: 1% TDI in acetone: olive oil (3:2, AOO), AOO and 0.1% OVA in saline. Mice were challenged intranasally (IN) with 25 μl of the same allergen or vehicle every other day for a total of 3 times. Using TDI as an example, the protocol is illustrated as follows (see Publication III):

Fig.5. The protocols of OVA peptide or OVA mouse model used in Publication I and II

Fig.6. The protocol of improved TDI mouse model used in Publication III. EC = epicutaneous sensitization, Plets = plethysmographic recordings.
3.4 Sampling of serum and bronchoalveolar lavage fluid (BALF)

On the last day of our protocol, the mice were given intraperitoneal injections of Pentothal (sodium thiopental) and killed by right ventricular exsanguinations. The blood was collected and then centrifuged at 1000 g for 10 min. The serum samples were stored at -20 °C until use. After right ventricular exsanguinations, the trachea was cannulated with a 20G catheter and the lungs were lavaged three times with aliquots of 0.5 ml of sterile saline per mouse. The collected BALF was centrifuged at 400 g, and the supernatant was stored at -20 °C for cytokine and antibody analysis.

3.5 Quantification of total IgE

To evaluate whether the mice were sensitized, total IgE in the sera and BAL fluid was quantified with a sandwich ELISA protocol provided by mouse IgE ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA). The measurements were performed according to manufacturer’s manual. The detection limit of the kit was 3.9–250 ng/ml (see Publication I and II).

3.6 Detection of specific antibodies to allergens in serum.

In order to characterize the sensitization of the mice, specific immunoglobulin IgE, IgG1 and IgG2A to TDI, OVA and OVA 323-339 were assayed by in-housed ELISA as previously described [47]. As for the detection of IgE to OVA 323-339, biotinylated OVA 323-339-BSA (EZ-Link NHHS-PEO4-Biotinylation Kit; Pierce, Biotechnology, Rockford, IL, USA) [47, 60] was applied. The results were expressed as optical density (OD) (see Publication II and III).
3.7 Quantification of IL-4 and IFN-γ proteins

The most important markers for Th1 and Th2 balance were evaluated by IL-4 and IFN-γ protein assays. These were quantified from both serum and BAL fluid by a sandwich ELISA protocol (R&D Systems, Minneapolis, MN, USA). Buffers and diluents used between steps in this assay were provided by the same company. The kit detection limits for IL-4 given by the manufacturer was 7.8-250 pg/ml and for IFN-γ 9.4-600 pg/ml (see Publication I and II).

3.8 Multiplex sandwich immunoassay of cytokine proteins

For more detailed characterization of the Th1 and Th2 balance, multiplex analyses of 8-plex MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore Corporate, Billerica, MA, USA) were performed strictly according to the manufacturer’s instruction using a Luminex xMAP system (Luminex Corporation, 12212 Technology Blvd Austin, TX, USA). Serum samples were diluted 1:1 for the measurement. Standard calibration curves ranged from 3.2–10,000 pg/ml (see Publication III).

3.9 BALF cytology assays

For studies of target tissue pathophysiology, BALF was centrifuged at 1500 rpm for 15 min, and the cell pellet was collected and re-suspended with 30 μl of PBS. Total cell count was done with a Burkers hemocytometer and then the rest of cells were equally smeared to a slide by Shandon Cytospin and Papanicolaou staining was conducted. Differential cell numbers were counted under light microscope (see Publication III).

3.10 Spleen cell culture.

In order to evaluate whether OVA or OVA 323-339 can stimulate T cell proliferation, mice splenocytes were isolated and cultivated in the presence or absence of the immunogens. The spleens were minced into small pieces, splenocytes were dissected free and then
collected from interface after Ficoll-paque centrifugation. The viability of the collected cells was ≥ 95% by use of the trypan blue exclusion method. Separated splenocytes were cultured on 24-well cell culture clusters with saline or allergens in RPMI-1640 at 37 °C 5% CO₂, final volume of 2 ml. Three days after initiating the culture, 100 µl of recombinant IL-2 (rIL-2, 1 µg / ml) was added to each well. After 4 days cell culture, 200 µl of cells (2.5 × 10⁶ cells / well) in duplicate were cultured in 96-wells overnight by adding 20 µl of 0.05 mCi / ml [methyl-³H] thymidine. The cultures were investigated for primary proliferation responses as measured by ³H-thymidine uptake. The results were expressed as counts per minute (cpm) (see Publication II).

3.11 Target tissue histology

To visualize roughly typical pathological findings in asthma mucus production was evaluated by histological staining. The lungs and hearts were removed en bloc and inflated with a similar volume of 10% buffered formalin (600 µl) at a constant pressure and then immediately soaked in the same solution for approximately 24 h, and thereafter embedded in paraffin [61]. Lung tissue sections (4 µm) were stained with haematoxylin and eosin (HE) and Periodic acid-schiff (PAS) (see Publication I, II and III).

3.12 Major basic protein (MBP) evaluation

Immunohistochemical staining of sectioned lung tissue was performed with rabbit polyclonal antibody against murine MBP. Pictures were acquired with LEICA DMLB light microscope and LEICA DC 300 camera, later on, imported into Adobe Photoshop 7 (see Publication I and II).

3.13 Morphometric analysis of airway thickness.

Bronchi with a diameter less than 250 µm presenting a smooth circular and oval profile were selected. Bronchial measurements were made at 250 × magnification using a LEICA
Q500 MC Qwin system as schematically illustrated in Publication I, Fig.1. Five mice from each group were enrolled in this measurement. The differences between individual groups were tested by one way ANOVA statistical analysis for multiple comparisons.

### 3.14 Non-invasive whole body plethysmographic recordings

To monitor non-invasively the bronchial responses after TDI and allergen sensitization we used whole body plethysmographic equipment (EMKA). With this non-invasive method the airway variable that best describes mouse asthma is Penh (enhanced pause in the breathing pattern). Using nebulized saline as baseline, we assayed increase in Penh by increasing concentrations of acetyl-β-methylcholine chloride (methacholine, 5, 10, 20 and 40 mg/ml) and compared the results between the study groups. The measurements were performed for 1 min by 4 min intervals for data collection, the degrees of bronchial hypersensitivity responses induced by allergen exposure could be assessed sequentially. The equipment allowed to simultaneously record maximum 4 groups of mice. Recordings were done 24 h before the first intranasal challenge and 24 h after the third intranasal challenge (see Publication III).
4. SUMMARY OF RESULTS FROM THE PUBLICATIONS

4.1 Publication I

The hypothesis of pathogenesis in TDI-induced asthma is still controversial, both Th1 and Th2 dominant immune responses have been reported. In order to address this issue, we developed a murine model of TDI-induced asthma by subcutaneous sensitization followed by intranasal challenge. Inflammatory responses to the OVA and OVA peptide 323-339 by intraperitoneal sensitization and intranasal challenge were also investigated as positive controls.

Our results demonstrated a neutrophil-dominant inflammation with infiltration of a few eosinophils in the peri-bronchial and peri-vascular regions of the lung in the TDI asthma model. This was accompanied by hyperplasia / hypertrophy of cells lining the airways and mucus production as shown by HE staining. Positive immunohistochemical MBP staining in the parenchyma was also shown. Th2 cytokine IL-4 and IgE antibody production were significantly increased 5 days after last challenge while IFN-γ level was below the detection limit. In contrast, eosinophil-dominant inflammation was observed and typical Th2 responses were triggered in OVA and OVA 323-339 mice. Conclusion: the clear elevation of IL-4 and total serum IgE could allow to conclude a possible Th2-like dominated allergic response in TDI-exposed BALB/c mouse model but with a neutrophil-dominant pulmonary inflammation.

4.2 Publication II

It has been reported that OVA peptide 323-339 was responsible for 25–35% of the BALB/c T-cell response to the intact OVA. Further, it is highly probable that OVA 323-339 could be closely related to the peptide naturally created by the APCs during OVA processing. Moreover, Janssen et al. demonstrated that the immune-dominant epitope of OVA, e.g. OVA 323-339, accounts for 50% of the OVA-specific B-cell response and 60–
70% of the OVA-specific T-cell response. So far little information has been acquired concerning whether OVA 323-339 could develop the same \textit{in vivo} immune response as OVA did in allergen-treated mouse.

This part of our study aimed to investigate whether OVA 323-339 was the major antigen of OVA and could be regarded as a key peptide in the development of OVA-related asthma. Mice were treated with OVA and peptide OVA 323-339 equally as in publication I. Splenocytes from sacrificed mice were isolated and cultured under stimulus of each allergen or medium.

When evaluated by hematoxylin/eosin and MBP immunohistochemical staining, OVA and OVA 323-339 induced similar lung inflammation. Significant increase of serum total IgE and OVA-specific IgE were observed in OVA mice when compared to saline control, but no significant increase of OVA 323-339-specific IgE was observed in OVA mice. Interestingly, OVA 323-339 mice showed both higher serum levels of OVA-specific IgE and OVA 323-339-specific IgE. In addition, increased serum IL-4 and lower IFN-\gamma were obtained in OVA323-339 mice as well as in OVA mice. The proliferative response to OVA was found in cultured splenocytes of both OVA and OVA 323-339 mice, while the similar proliferative response to OVA 323-339 was only observed in the splenocytes of OVA 323-339-sensitized and challenged mice. Conclusion: OVA 323-339 induced a Th2-like response in the mouse model similar to OVA. However, unlike OVA, OVA 323-339 had in vitro a clearly limited immunogenic potency to activate OVA-sensitized and challenged mice splenocytes.

4.3 Publication III

The mechanism responsible for TDI-induced asthma is not fully understood and the determinant for exposure remains far from clear. There are several possibilities for the subjects to become sensitized in the TDI industry and thereby finally develop into asthma. Skin sensitization is believed to be one of most important sensitization routes besides inhalation. Therefore type I allergy model was used to study the mechanism of bronchial hyperreactivity in TDI-induced asthma. This could better mimic the development and possible mechanisms of occupationally related asthma. In this context, we developed an
improved TDI asthma mouse model to validate the phenotype of pulmonary inflammation and immune responses. Mice were sensitized with TDI or OVA epicutaneously once a week for 3 weeks, and were then given intranasal challenge every other day for a total of 3 times. The control mice were treated similarly. On the days before the first and after the last intranasal challenge, pulmonary functions were tested by plethysmography system. TDI-exposed mice exhibited a neutrophil-dominant pulmonary inflammation, in contrast to the eosinophil-dominant inflammation presented in OVA mice. Similar results were observed in the BALF differential cell counts. Both TDI and OVA mice had significantly increased AHR when evaluated by Penh, compared to respective controls. Augmented serum levels of IgG1- and IgE- antibodies were observed in TDI- and OVA-exposed groups. Moreover, both groups displayed elevated serum IL-5 protein levels. However, OVA group showed significantly increased IL-1β protein. Conclusion: Increased TDI-specific IgE- and IgG1- antibodies may play important roles in the development of TDI-induced asthma mouse model like OVA-specific IgE- and IgG1-antibodies did in OVA mice, the TDI-induced neutrophil-dominant pulmonary inflammation, however, was clearly different from the response in OVA mice.

Dermal sensitization on its own could initiate pathophysiology of altered airway functions and, surprisingly, challenge with TDI after sensitization did not exacerbate bronchial hyper-reactivity. This result stresses the importance of dermal sensitization in the development of TDI-induced asthma.
5. GENERAL DISCUSSION

5.1 Immune and inflammatory responses in the TDI-induced asthma model

Understanding the pathogenesis of occupational asthma is a crucial step toward optimal prevention and management of the condition. In this respect, identifying the structural and biological airway characteristics that determine the potential for inducing airway sensitization is fundamental to the implementation of primary preventive strategies [23]. To our knowledge, there are only a few mouse models of TDI-induced asthma which can well describe the pathophysiology in the development of TDI-induced asthma up to now. Hitherto, the pathogenesis of occupational asthma caused by LMW agents, such as TDI still remains largely unknown [23]. So it is of interest to study the development of asthma by means of animal models, exploring the contradictory inflammatory phenotypes and immune responses postulated [6]. Clinically, assessment methodology for skin exposure is not as well developed as it is for exposure by inhalation. Isocyanate skin exposure has been documented in auto body shops and among spray painters using polyurethane products, despite the use of standard personal protective equipment, even in settings where airborne concentrations are minimal [62-64]. Further studies are needed to investigate the role of skin exposure at workplace in the mechanism of sensitization and the development of OA [17].

The immune system must achieve a balance of sensitivity in order to respond to foreign antigens without responding to the antigens of the host itself. When the immune system responds to very low levels of antigen that it usually should not respond to, an immunological hypersensitivity response occurs. Immunological hypersensitivity is defined as the cause of allergy, which in the airways commonly refers to conditions or reactions associated with an IgE antibody-mediated response following antigenic exposure [65].

Binding of specific IgE antibodies to hapten conjugated with human serum albumin has been reported in a low proportion of workers with ascertained OA caused by most LMW agents, including diisocyanates (0–39%). The presence of IgE antibodies specific to LMW
agents conjugates generally shows a very high specificity for OA, although they can be found in asymptomatic exposed workers as well [23].

In the mouse IgE test, dermal exposure to a test chemical is followed by measurement of total serum IgE antibody level. An increase in total IgE is thought to imply that the chemical is a respiratory sensitizer [25]. The detection of Th2-type cytokines released from CD4 lymphocytes during airway sensitization of an animal to a chemical is considered to be indicative of respiratory sensitization. IL-4, IL-5, IL-10 and IL-13 cause class switching to IgE production by B lymphocytes [25, 66] and the inhibition of IFN-\(\gamma\) production. Cytokine profiling distinguishes Th2 responses, believed to be an integral mechanism in respiratory sensitization from Th1 responses which predominate in skin sensitization, known to involve delayed hypersensitivity (type IV) [25].

In our study, from both epicutaneous and subcutaneous TDI sensitization models, significantly increased serum IgE levels were observed (Publication I, Publication II) in company with increased serum IL-4 (Publication I) and IL-5 (Publication III). In contrast, we did not find any increase in IFN-\(\gamma\).

Allergic diseases including allergic asthma manifest many forms, for in instance, they can be defined as adverse health effects resulting from the stimulation of a specific immune response [67]. For example, selective type II (Th2) allergic responses such as respiratory hypersensitivity can be induced in susceptible persons after airway exposure to a chemical allergen. A variety of chemicals including various diisocyanates, acid anhydrides, and reactive dyes, can cause allergic sensitization of the respiratory tract associated with occupational asthma and other symptoms [6, 68-74]. Unlike typical protein allergens that cause asthma, diisocyanates are extremely reactive compounds and great uncertainty exists regarding the carrier proteins for these chemical haptens \textit{in vivo} [1].

The difference between the airway inflammation of diisocyanate asthmatic patients and common atopic asthmatic patients may reflect differences in the immune mechanisms central to pathogenesis. The most striking difference in the limited number of diisocyanate patients studied is the relative increase in neutrophil influx and IL-8 production [45, 75-76].

So far, quite limited animal models of diisocyanate asthma have been applied. Most of the studies, commonly based on guinea pigs, have demonstrated the acute inhalational toxicity and immunogenicity of diisocyanates, and the features characteristic of human asthma,
including airway inflammation, airway hyperresponsiveness, and mucus hypersecretion following respiratory tract exposure and sensitization [1-2, 77-78].

More mechanistic studies have been limited by the lack of widely accepted animal models that replicate human diisocyanate asthma. Mouse models are well known to offer numerous advantages, including the availability of genetically manipulated strains and a wide array of diagnostic molecular tools. However, it has been difficult to evoke diisocyanate-induced airway inflammation in mice, potentially related to poor delivery of the highly reactive diisocyanates to the lower airways, as well as the difficulties measuring airway hyperresponsiveness. In addition, these present models vary greatly in regard to mice strain, the type of diisocyanates used, the route, timing, and dosing of diisocyanate sensitization and challenge, and therefore the subsequent differential inflammatory and physiologic responses.

In our study, we assessed the recruitment of neutrophils, eosinophils, macrophages and lymphocytes in BALF. A significant increase in BALF neutrophils was found in TDI-sensitized and -challenged mice, but we failed to show macrophage-derived, increased IL-8, G-CSF or MIP-2 levels in serum, nor indication of increase VGEF production, latter of which is related to bronchial asthma (Publication III). However, consistent neutrophil-dominant inflammatory type was also observed in both epicutaneously and subcutaneously sensitized TDI mice models. The mechanisms for the infiltration of neutrophils are still uncertain and may be partially due to the direct stimulation to the bone marrow or the non-specific effect of the allergen like toxic response.

The mechanisms of absence of immune responses may vary and involve complex immunological mechanisms including absent or insufficient co-stimulatory signals from antigen presenting cells.

5.2 OVA peptide 323-339 and OVA-induced asthma

Egg is an essential ingredient in human and animal food and it is very difficult to totally eliminate its exposure [79]. OVA, which constitutes approximately 58% (w/w) of the entire hen’s egg white extract, is the most dominant ingredient of the five major allergens of egg white and is universally used as the main allergen in establishing different animal models of asthma, food and dermal allergy. The complete amino acid sequence of OVA has been
elucidated. OVA’s allergenic epitopes were mainly determined by the primary structure and are dependent on a certain peptide chain length [59-60, 80]. Among many egg white proteins, OVA 323-339 as well as intact OVA, was reported to encompass B cell epitopes which were recognized by specific IgE antibodies. The OVA peptide 323-339 sequence was also demonstrated to include CD4+ T cell epitopes, which were restricted by the MHC class I-Ad molecule in mice [81-83], and was considered to encompass at least one B cell epitope [84-85]. Furthermore, the OVA specific T cell line from hen’s egg allergic patients was able to recognize OVA 323-339 presented by HLA-DR10 [81, 86].

OVA is widely used as a model protein in structural as well as immunological studies[79]. Murine models have been broadly employed to study atopic asthma. Mouse ovalbumin models have successfully duplicated many features of atopic asthma: eosinophilic airway inflammation, Th2 T cell responses, and airway hyperresponsiveness, and have greatly contributed to our understanding of the immune mechanisms in atopic asthma [1, 10].

OVA 323-339 has been used extensively to study the nature of class II MHC-peptide binding and T cell activation [87-91]. It has been reported that OVA peptide 323-339 was responsible for 25-35% of the BALB/c T-cell response to the intact OVA, and it is highly probable that OVA 323-339 was closely related to the naturally created peptide by the APCs during OVA processing [59, 82, 85, 92]. However, Janssen et al. demonstrated that the immunodominant epitope of OVA, e.g. OVA 323-339, accounts for 50% of the OVA-specific B cell response and 60-70% of the OVA-specific T cell response [93]. So far, there is only a small amount of available information on in vivo immune responses in OVA 323-339 treated animals. Kearney et al. reported that systemic injection of OVA or OVA 323-339 in a T cell transfer model induced a rapid and strong, but transient DO11.10 T cell proliferation [94]. They also described that the induction of more local and less transient responses by the subcutaneous administration of OVA 323-339 in Freund’s Complete Adjuvant, resulting in a significantly enhanced T cell response upon in vitro stimulation with OVA 323-339 [94-95]. Knowledge on the immunogenicity of allergens is important and mandatory for establishing modern therapeutic methods for the treatment of allergy.

In our study, OVA-treated mice illustrated an increased total and OVA-specific IgE production in serum, while OVA 323-339 mice showed a significant increase in the production of OVA 323-339-specific IgE. Interestingly OVA-specific IgE also was detected in OVA 323-339 sensitized mice, which was consistent with the data from Renz et al [96].
But, by ELISA detection using OVA 323-339 conjugated to BSA we did not find increased OVA323-339-specific IgE in OVA sensitized mice. In contrast, Renz and his colleagues found 50% and 80% reduction in OVA-specific IgE from the serum samples of OVA-sensitized and OVA 323-339 sensitized mice after pre-incubation with OVA 323-339. This could be explained by the fact that in vivo OVA 323-339 might bind to carrier protein and acquire weak immunogenicity for antibody production (low total IgE in OVA 323-339 sensitized mice). Furthermore, the low detectable concentration of anti-OVA323-339 IgE in OVA sensitized mice might imply that the OVA amino acid sequence 323-339 may not be as strong immunogen as intact OVA.

Very low specific-IgG\textsubscript{1} to OVA and OVA 323-339 were detected as well as IgG\textsubscript{2A} in serum and BALF suggested IgE-mediated response may dominate in the immune response triggered by OVA and its peptide.

Increased IL-5 but not IL-4 indicated that the dermal sensitization route with OVA may activate the Th2 response by different cytokines rather than that of intraperitoneal sensitization route (IL-4 dominant activation). Lower IFN-\textgamma{} in serum can distinguish a Th2 immune response to both OVA and OVA 323-339 from a Th1 cell-mediated reaction.

5.3 Immunoglobulin G and occupational asthma

A high level of specific IgG-antibodies to diisocyanates has been shown to be closely correlated with the level of workplace exposure, and is more sensitive, but less specific than IgE in identifying occupational asthma [23, 97].

Guinea pig models historically used to study TDI-induced asthma, display both immediate and late-onset airway responses as well as dose-dependent IgG-antibody-specific responses [33, 77, 98]. Specific IgG antibodies have at least been shown to reflect exposure, although IgG antibodies do not reflect airway hypersensitivity [33].

In our intraperitoneal study, neither OVA nor OVA 323-339 induced an increased production of IgG\textsubscript{1}- and IgG\textsubscript{2A}-antibodies. However, in the epicutaneous study, both OVA and TDI mice demonstrated that increased IgG\textsubscript{1}–antibodies paralleled IgG\textsubscript{2A}, which may suggest an immune response specific to the epicutaneous sensitization route (sensitization route-dependent phenomenon) or there was an immune tolerance that occurred.
5.4 Proliferation responses to OVA and OVA peptide 323-339 (Publication I)

In the mouse model of intraperitoneal sensitization and intranasal challenge, splenocyte stimulation with OVA induced an increased proliferative response as observed in both OVA and OVA 323-339-sensitized and challenged mice, while proliferation to OVA 323-339 only occurred in splenocytes of the OVA 323-339 mice and not in the OVA mice. Accordingly, cells stimulated with control culture medium demonstrated very low thymidine incorporation when compared with the responses to OVA and OVA 323-339 stimulation. Our presented proliferative results are consistent with the results of Janssen et al. who demonstrated that OVA 323-339 induced a proliferative response in cell cultures from OVA-challenged animals [99].

The proliferative response assay demonstrated that OVA 323-339 in vitro stimulation did lead to splenocyte proliferation and activation in cultivated cells. This could imply that small peptides such as OVA 323-339 can play their role by being presented and expressed on the membrane surface of activated splenocytes in the way same as in OVA mice. This is also consistent with previous ideas: OVA and OVA 323-339 encompassed CD4+ T cell epitopes, which were restricted by the MHC class I-Ad molecule in mice [81-83], and was considered to share at least one B cell epitope [84-85].

5.5 Airway Hyper-Responsiveness and Penh (Publication III)

Various alternative approaches for assessing AHR have been reported. Non-invasive methods based on single- or dual-chamber plethysmography appear to have much merit [100-101]. A whole-body plethysmography technique, which has gained considerable popularity, can be performed in un-anaesthetized and unrestrained mice by means of measurement of the dimensionless parameter known as enhanced Pause (Penh). It is the measured length of time between inspiratory and expiratory air and describes breathing difficulties [102]. Unfortunately this technique has limitations in models associated with extensive parenchymal disease, in which abnormalities may correlate poorly with airway and
tissue mechanical properties [103]. The method has therefore been the subject of extensive criticism [104-106] and has fallen into disfavour, even though many of the criticisms in part can be answered [107]. Nevertheless, as a pulmonary function parameter, Penh may still have value in screening procedure and certainly proved to be useful for assessing AHR in our low-level challenge model, in which parenchymal disease is not considered to be a confounding factor [108].

The ventilatory function is a composite index of airway obstruction [109]. Penh, as a parameter evaluating ventilator function by unrestrained whole body plethysmograph, can assess the changes in the ventilatory patterns and partially reflect degree of bronchial obstruction [110]. In our study, significant increase in Penh after intranasal challenges (compared to controls, TDI $p = 0.0018$ and OVA $p = 0.0208$) suggested an asthmatic response took place in the airways. The increased Penh before intranasal challenge (TDI $p = 0.077$ and OVA $p = 0.0315$ vs their controls respectively) indicated that this asthmatic response seems to start after repeated epicutaneous sensitizations instead of after intranasal challenge. This phenomenon implied that AHR may have been triggered earlier than the first intranasal challenge, namely initiated by epicutaneous TDI sensitization. The subsequent intranasal challenge may have induced tolerance as suggested by the increased serum IgG$_{2A}$ immunoglobulin level. This finding was somehow similar to that of Vanoirbeek and his colleagues [110]: with the increase of challenge by times, the AHR decreased, possibly caused by induction of tolerance.
6. CONCLUSIONS

1) TDI can in BALB/c mice induce a similar Th2-like immune response and AHR but a more pronounced neutrophil-dominant bronchial inflammation, rather than the eosinophilic inflammation in response to OVA / OVA peptide 323-339.

2) OVA peptide 323-339 can both in vivo and in vitro express the major immunogenicity of OVA and induce a typical Th2 immune response in the BALB/c mouse model. In vivo, amino acid sequence 323-339 of intact OVA is not well expressed as a dominant antigen epitope.

3) Different sensitization routes do not change the phenotypes of both the TDI- and OVA-induced responses in the BALB/c mouse model.

4) Our results from the BALB/c mouse model may not allow characterizing the role of IgE in the airway responses to TDI.

5) Intranasal challenge with TDI after epicutaneous sensitization did not exacerbate bronchial hyper-reactivity.
7. FUTURE RESEARCH PERSPECTIVES

1) To investigate whether the use of anti-IgE-antibodies can inhibit the release of inflammatory mediators and to investigate further the role of IgE in the development of TDI-induced asthma both in vivo and in vitro.

2) To establish allergen or chemical-conjugate sensitized T cell and B cell lines (splenocyte) and to investigate further the potential role of allergens and chemical-conjugate in T cell activation, the secretion of cytokines and antibodies in vitro.

3) Further studies should be done on the mechanisms leading to the neutrophil infiltration into the target airway tissue.

4) It would be of interest to study the cross-reactivity between different isocyanates such as HDI, MDI and TDI using the established mouse models.
8. SOURCE OF DATA


