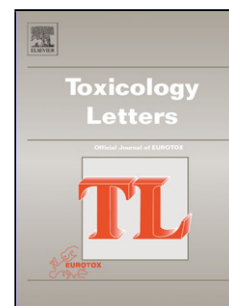


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Thymic stromal lymphopoietin (TSLP) and Toluene-diisocyanate-induced airway inflammation: alleviation by TSLP neutralizing antibody

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Highlights:

- Toluene-diisocyanate (TDI) is the most common cause of occupational asthma.
- Th1/Th2 cells responses and Th17/Treg cells imbalance are involved in the

important pathological mechanisms of asthma.

- Thymic stromal lymphopoietin (TSLP) plays an important role in the development of TDI-induced asthma.
- TSLP neutralizing antibody (TSLP-Ab) can effectively alleviate airway inflammation of TDI-induced asthma.

Abstract

Toluene-diisocyanate (TDI) is mainly used in the manufacturing process of polyurethane foams, and is a potent inducer of occupational asthma characterized by airway inflammation and airway hyperreactivity. Thymic stromal lymphopoietin (TSLP) plays an important role in the development of asthma, and correlating with the differentiation of Th2 and Th17 cells. However, the role of TSLP in TDI-induced asthma remains unclear. In this study, 96 TDI-exposed workers as well as a mouse model of TDI-induced asthma were investigated. The air exposure assessment result of TDI in the workplace showed that workers were exposed to inhalation of a very high concentration of TDI, approximately 8 times the recommended level, leading to a decrease in pulmonary function and an increase in inflammatory cells, as well as TSLP and IgE levels in the supernatant of sputum obtained from exposed workers. In order to further investigate the role of TSLP in the pathogenesis of TDI-induced asthma, a mouse model of TDI-induced asthma was also employed. Histopathological analysis of mouse lung and bronchus showed an obvious infiltration of inflammatory cells around the bronchus. The levels of inflammatory cells, IFN- γ , IL-4 and IL-17 in

bronchoalveolar lavage fluid (BALF), the expression levels of TSLP protein and ROR- γ t and IL-17 mRNA in mouse lung tissues were also significantly increased. However, after treatment with TSLP neutralizing antibody (TSLP-Ab), the degree of pulmonary and bronchial inflammation in mice was significantly alleviated, and the levels of inflammatory cells, IFN- γ , IL-4 and IL-17 in BALF, and the expression levels of ROR- γ t and IL-17 mRNA in lung tissue were significantly decreased. Our data shows that TSLP plays an important role in the pathogenesis of TDI-induced asthma, and that TSLP-Ab can effectively alleviate TDI-induced airway inflammation of asthma.

Keywords: Toluene-diisocyanate (TDI); Asthma; Inflammation; Thymic stromal lymphopoietin (TSLP); Interleukin-17 (IL-17)

1. Introduction

Occupational asthma (OA), which accounts for approximately 10% of all adult-onset asthma, is a chronic airway inflammatory disease characterized by chronic airway inflammation, airway hyper-reactivity and airway remodeling (Gautrin et al., 2003; Lummus et al., 2011; Racine et al., 2017). Diisocyanates remain one of the most commonly reported causes of occupational asthma (Johnson et al., 2005; Stocks et al., 2015). Toluene-diisocyanate (TDI), a member of the diisocyanate family, is an important industrial material and is widely used in the synthesis of polyurethane soft foam plastics, coatings, rubber, adhesives and other materials (Klees and Ott, 1999). The main effects of TDI are its stimulatory and sensitizing properties (Pauluhn 2014). Inhalation of high concentrations of TDI can cause throat irritation, airway

inflammation and lung edema. Long term exposure to TDI can cause bronchial asthma, hypersensitivity pneumonitis, and an accelerated rate of decline in lung function (Ott et al., 2003; Pham et al., 2017). Workers exposed to TDI have a significantly higher propensity to develop OA (Sharifi et al., 2013). With the extensive use of TDI in decorative materials and furniture manufacturing, the contact population is not only limited to factory workers but also to those exposed to TDI fumes. Occupational and environmental exposures of TDI are already a serious public health problem.

Evidence from human and animal model studies suggests that TDI-induced asthma is characterized by Th2-dominated airway inflammation along with neutrophil and eosinophil infiltration into the airways (Del Prete, et al., 1993; Ban et al., 2006; Schupp and Collins, 2012). Th2 cells and their cytokines (IL-4, IL-5, and IL-13) play a key role in the induction and pathogenesis of asthma (Swierczyńska-Machura and Pałczyński, 2012). Thymic stromal lymphopoietin (TSLP) is a member of the IL-2 cytokine family and is an IL-7-like cytokine, mainly secreted by epithelial cells, keratinocytes, stromal cells, mast cells, basophils, and macrophages (Friend et al., 1994; Roan et al., 2012; Ying et al., 2008). TSLP can differentiate naive CD4⁺ T lymphocytes into Th2 cells and produce IL-4, IL-5, IL-13, while reducing the expression of Th1 related interferon- γ (IFN- γ) (Soumelis, et al., 2002). In previous studies, TSLP expression has been shown to be increased in asthmatic airways and correlates with the expression of Th2-attracting chemokines and disease severity, suggesting that TSLP is involved in the pathogenesis of human asthma (Byer 2014; Biagini Myers et al., 2014; Hirose et al., 2017; Liu et al., 2018).

However, several reports suggest that the pathological mechanism of OA is also due to the involvement of Th17 cells which have been shown to mediate pathogenic inflammatory responses (Zhou et al., 2017; Wisniewski and Borish, 2011). Recent studies showed that Th17 cells play an important role in chronic inflammatory diseases by producing IL-17/IL-17A, IL-17F, IL-6, TNF- α , and IL-22, which mediate protection against extracellular microbes, linked to the development of autoimmune diseases in vivo. In animal models, IL-17A has been shown to exacerbate Th2 cell-mediated eosinophilic airway inflammation and hyper-responsiveness (Hasegawa et al., 2017; Wilson et al., 2009). In patients, IL-17 has been implicated in severe forms of asthma (Alyasin et al., 2013; Hasegawa et al., 2017). Some studies have also reported that TSLP and IL-17A are closely related to the development of asthma (Yadava et al., 2014; Qin et al., 2018). It is also reported that TSLP can promote differentiation of Th17 cells under certain pathological conditions (Tanaka et al., 2009; Lee et al., 2013).

In the present study, we examined the sputum levels of TSLP, IL-17 and IgE in TDI-exposed workers to determine whether the levels of these cytokines were associated with TDI exposure. TSLP neutralizing antibody (TSLP-Ab) was used in an animal model to evaluate the functional link between TSLP expression and TDI-induced asthma.

2. Materials and methods

2.1. Study subjects

96 exposed workers were recruited from a TDI production factory in North China,

and 88 non-exposed controls were recruited from the same residential areas. These controls were not exposed to TDI or other occupational environmental hazards. All subjects with a prior history of immune-related diseases or drug allergies were excluded. Trained interviewers used a pre-tested epidemiologic questionnaire to collect personal data from the participants regarding demographics (e.g., age and gender), previous work history and individual and family history of immune-related diseases. All subjects were required to sign informed consent forms.

After each subject was interviewed, sputum was collected for cells and supernatant isolation. This study was approved by the Research Ethic Committee of Shandong Academy of Occupational Health and Occupational Medicine, Jinan, China. All participants were also required to complete a questionnaire which included questions on BMI, age, drinking, smoking, working age and other occupational exposures.

2.2. Air sampling and TDI exposure assessment

The extent of TDI exposure was assessed in a TDI production and packaging workshop. Prior to the collection of air samples, the absorption solution was prepared by adding 35 ml hydrochloric acid ($\rho_{20}=1.18$ g/ml) and 22 ml glacial acetic acid to 600 ml water, and then diluting to 1 L with water. On each of the three sampling days, a total of 180 L of personal air samples were collected in the breathing zone of workers using aspirators at a calibrated flow rate of 3 L/min. After each shift, air samples were placed in air-tight containers, immediately sent to the laboratory and stored at 4 °C until the analysis. The air samples were analyzed for TDI content by gas chromatography

technique using a gas chromatograph (7890A, Agilent Technologies, CA, USA) with a mass selective detector (5975C, Agilent Technologies, CA, USA). The protocol was performed according to the methods for determination of isocyanates in the air of the workplace (GBZ/T 160.67-2004, National Occupational Health Standards of the People's Republic of China). The time-weighted average (TWA) concentration of 8 h was calculated according to the investigation of various types of work hours.

2.3. Pulmonary function measurement

The pulmonary function of the exposed workers and non-exposed controls in this study was measured using a digital spirometer (Pony FX, COSMED Inc., Italy). The parameters measured here were forced vital capacity (FVC), forced expiratory volume in one second ($FEV_{1.0}$) and $FEV_{1.0}/FVC$. The measurements were taken with the subjects sitting upright on a chair with the nose blocked with a clip while slightly biting on the mouthpiece. After taking 3-4 tidal breaths, the subjects inhaled and exhaled quickly and deeply, then held their breaths for 6 seconds. The measurements were performed 3 times with one-minute rests in between tests and the mean value was used for the analysis. Predicted values based on sex, age, and height for FVC, $FEV_{1.0}$ and $FEV_{1.0}/FVC$ were computed using regression equations of Crapo et al., (1981).

2.4. Sputum collection and analysis

Briefly, sputum volumes were recorded after the subjects inhaled 3%-5% hypertonic saline using an ultrasonic atomizer (402B, Yuwell, Jiangsu, China).

Mucocellular clumps were dispersed with 0.1% dithiothreitol (Sputolysin, Merck Millipore, Darmstadt, Germany) and filtered before a total cell count was performed. Cells were obtained following centrifugation at $300 \times g$ for 15 min. After centrifugation, the supernatant was harvested and stored at -80°C for TSLP, IL-17 and IgE measurement. 200 μl of resuspended cells were mixed with 800 μl PBS. 20 μl of this resuspension was added to 20 μl 0.4% chlorophenolan to determine total cell numbers. The sediment glass slides were stained using Diff-Quick Stain Kit (Solarbio, Beijing, China) according to manufacturer's protocol. The sputum cell smear was observed under an optical microscope (Olympus BX41, Olympus Corporation, Tokyo, Japan) and the percentage of neutrophil, eosnophils, monocyte, basophil and lymphocyte was determined.

2.5. Determination of TSLP, IL-17 and IgE levels in sputum

The levels of TSLP, IL-17 and IgE in sputum supernatant were determined using commercial ELISA kits (eBioscience, MA, USA) according to manufacturer's instructions. The absorbance of samples was read at 450nm on a microplate reader (SpectraMax190, Molecular Devices, CA, USA).

2.6. Animals and treatments

Specific-pathogen-free (SPF) male BALB/c mice, 6-8 weeks old, were purchased from the Experimental Animal Centre of Shandong University. The mice were housed in a SPF house (temperature $23 \pm 2^{\circ}\text{C}$, humidity range 40-70%, 12 h light/dark cycle).

Food and UV-sterilized tap water were provided *ad libitum*. All procedures were conducted in strict accordance with the Guidelines for Animal Experiments published by the Ministry of Science and Technology of the People's Republic of China (GB14924.2-2001, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Shandong Academy of Occupational Health and Occupational Medicine.

After one week of acclimatization, mice were randomly divided into three groups with 6 in each: AOO group (solvent control group), TDI group and TSLP-Ab group. The TDI-induced asthma model was prepared by a modification of previous methods (Liang et al., 2015; Hirota et al., 2012). The vehicle solution (AOO) used to dissolve TDI consisted of a mixture of 2 volumes of acetone (Sigma-Aldrich, Shanghai, China), 3 volumes of olive oil for dermal sensitization, 1 volume of acetone and 4 volumes of olive oil for airway challenge. Male BALB/c mice had the dorsum skin on both ears sensitized with 0.3% TDI (20 μ l per ear) on days 1 and 8. On days 15, 18 and 21, mice were placed in individual cylindrical chambers and challenged through the airways with 0.01% TDI dissolved in acetone/olive oil (1:4) by means of compressed air nebulization (NE-C28, Omron, Tokyo, Japan) for 3 h each time. Mice in the control group were sensitized and challenged by using the same procedures with the same amount of AOO without TDI. The TSLP-Ab group was administered by a intraperitoneal injection of 15 mg/kg TSLP neutralizing antibody (R&D Systems Inc., MN, USA) 30 min prior to challenging with TDI on days 15, 18 and 21. The AOO and TSLP group mice were also treated with the same amount of solvent.

2.7. Airway hyper-responsiveness (AHR)

Airway hyper-responsiveness (AHR) to methacholine (Sigma-Aldrich, MO, USA) was assessed 24 h after the third challenge with TDI. Briefly, the mice were placed in a plethysmographic chamber (PLY 310, EMMS, Hampshire, UK) and challenged with vehicle (sterile saline) followed by increasing concentrations of methacholine (1.25, 2.5, 5, and 10 mg/mL) for successive periods of 3 min, followed by 4 min recovery after each treatment, using an ultrasonic nebulizer. Aerosols were generated with a matching ultrasonic nebulizer and then released into the main chamber for 3 min. Pressure fluctuations caused by breathing of the mice were continuously monitored for 4 min after each nebulization and these pressure fluctuations were subsequently quantified by measuring enhanced pause (Penh). Penh is a parameter derived from analysis of the respiratory pattern, and increases in Penh are related to an increase in airway resistance (Lundblad, et al., 2002).

2.8. Behavioural changes in mice after exposure

Behavioural changes were monitored after treatment by characteristics that included major symptoms of asthma such as irritability, shortness of breath, nodding to facilitate breathing, arching back, fore limb contraction and lip purpura.

2.9. Analysis of bronchoalveolar lavage fluid (BALF) from mice

Bronchoalveolar lavage fluid (BALF) was collected from mice 24 h after the third challenge. 10% chloral hydrate (0.15 ml/20 g) of anesthesia was administered via

intraperitoneal injection. An 18 G blunt cannula needle was inserted slowly into the trachea and 0.3 ml saline was injected into the lungs twice. The fluid was allowed to remain in the lungs for 1 min and a total of 0.4 ml of BALF was collected. BALF was centrifuged for 10 min at $300 \times g$ and cells were resuspended in 0.3 ml saline. An animal blood analyzer (Drew Scientific Inc., ML, USA) was used to determine the total number and types of inflammatory cells. The levels of IFN- γ , IL-4 and IL-17 in BALF were measured using the ELISA kits (eBioscience, CA, USA) according to the manufacturer's instruction.

2.10. Histopathological examination of lung and bronchus from mice

The left lungs and bronchus from mice were harvested 24 h after the third challenge, fixed in 4% neutral formalin and embedded in paraffin blocks. 5 μ m sections were made and the tissues were stained with hematoxylin and eosin (H&E).

2.11. Western blot analysis

Lung tissues were homogenized using a mortar and pestle and proteins were extracted with RIPA lysis buffer (Boster, Wuhan, China) containing phenylmethylsulfonyl fluoride (PMSF) (Boster, Wuhan, China). 20 μ g of protein was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred onto a nitrocellulose membrane. Membrane-bound proteins were blocked with 5% milk for 60 min. The membrane was incubated with a mouse antibody against TSLP (1:500, R&D Systems Inc., MN, USA) overnight at 4 °C. The

membrane was incubated with horse radish peroxidase-labeled anti-mouse secondary antibody (1:1000, Servicebio, Wuhan, China) for 1 h at room temperature the following day. Chemiluminescent HRP substrate (Servicebio, Wuhan, China) was used to visualize the protein bands using an imaging instrument (C-DiGit™ Blot Scanner, LI-COR Biosciences, NE, USA), and the protein bands were quantitated using its Image Studio version 5.2.5.

2.12. Quantitative analysis of Foxp3, ROR- γ t and IL-17 mRNA expression of lung tissues by real-time PCR

Total RNA was extracted from mice lung tissues using Trizol Reagent (Thermo Fisher, MA, USA). 1 μ g of total RNA from each sample was used to synthesize cDNA using the FastQuant RT kit (TIANGEN, Beijing, China), then amplified using SYBR Green qPCR Master Mix (Roche, Basel, Switzerland). RT-PCR was performed on the Roche LightCycler® system (Roche, Basel, Switzerland) under the following conditions: one cycle at 95°C for 5 min, 40 amplification cycles (95°C for 15s, 60°C for 20s, and 72°C for 15s). Sample fluorescence was measured during the annealing step and fluorescence data was continuously collected to obtain the dissociation curve. Fluorescence signal was plotted *versus* Ct (threshold cycle) based on dRn (baseline-corrected, reference dye-normalized fluorescence) to obtain a standard curve and to measure initial template quantity. The average Δ - Δ cycle threshold of different samples was analyzed to calculate the relative mRNA levels using GAPDH mRNA as the control. Fluorescence data were analyzed using GraphPad Prism v5.0 Software (GraphPad, CA,

USA). The forward and reverse primers used for each gene are shown in Table 1.

Table 1. Primers used for qRT-PCR in this study

Gene	Forward (5'-3')	Reverse (5'-3')
<i>TSLP</i>	ATTTGCCCCGGAGAACAAGAGA	TTGGACTTCTTGTGCCATTTCC
<i>Foxp3</i>	CTCGCATGTTTCGCCTACTTC	CATCTACGGTCCCACTGCT
<i>RORγt</i>	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT
<i>IL-17</i>	TTTAACTCCCTTGCGCAAAA	CTTTCCTCCGCATTGACAC
<i>GAPDH</i>	ACTTGAAGGGTGGAGCCAAA	GTGGTCATGAGCCCTTCCAC

2.13. Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (IBM Corporation, IL, USA). All data (except for the basic information of workers) were expressed as mean $\pm$ standard deviation (SD). Group comparisons were performed by one-way analysis of variance (ANOVA), the student's t test was performed between exposed and control worker groups, and χ^2 test was used for the comparison of smoking and drinking. The data of the protein and mRNA levels in the animal experiment were normalized, and non-parametric analysis (Mann-Whitney test) was performed. P values < 0.05 were considered as significant differences between groups.

3. Results

3.1. Study population

In this cross-sectional study, we enrolled 96 exposed workers and 88 non-exposed controls. The characteristics of the total 184 participants are shown in Table 2. All of the workers in this study were males. The exposed workers and controls were matched

by age, BMI, smoking and drinking habits.

Table 2. The basic information of TDI-exposed workers and control workers

	Exposed group (n=96)	Control group (n=88)	<i>p</i> value
Age (years), means (range)	39.0 (29~48)	37.2 (26~50)	0.053 ^a
BMI (kg/m ²), means (range)	25.2 (16.59~32.00)	23.52 (16.96~33.52)	0.248 ^a
Smoking			0.385 ^b
Yes (%)	33 (0.34)	28 (0.32)	
NO (%)	63 (0.66)	60 (0.68)	
Drinking			0.742 ^b
Yes (%)	28 (0.29)	30 (0.34)	
No (%)	68 (0.71)	58 (0.66)	
TDI exposure time (years) median (range)	8.6 (3~19)	—	—

^a stands for t test, ^b stands for χ^2 test

3.2. Air exposure assessment

The exposed workers had an average exposure level of 0.81 mg/m³ (range: 0.41 to 1.22 mg/m³), while the level of TDI in the control factory workers were below the limit of detection. The level of TDI in the TDI production factory workers exceeded the recommended time-weighted average (TWA) in China (0.1 mg/m³). This confirms that workers are exposed to high concentrations of TDI, approximately 8 times above the recommended limit.

3.3. Pulmonary function in exposed and control groups

As shown in Fig. 1, the values of FVC (% of predicted) (Fig. 1A), FEV_{1.0} (% of predicted) (Fig. 1B) and FEV_{1.0}/FVC (%) (Fig. 1C) in the exposed group were

significantly lower ($p < 0.05$) than those in the control group. This indicates that long term exposure to high concentrations of TDI can lead to pulmonary function damage.

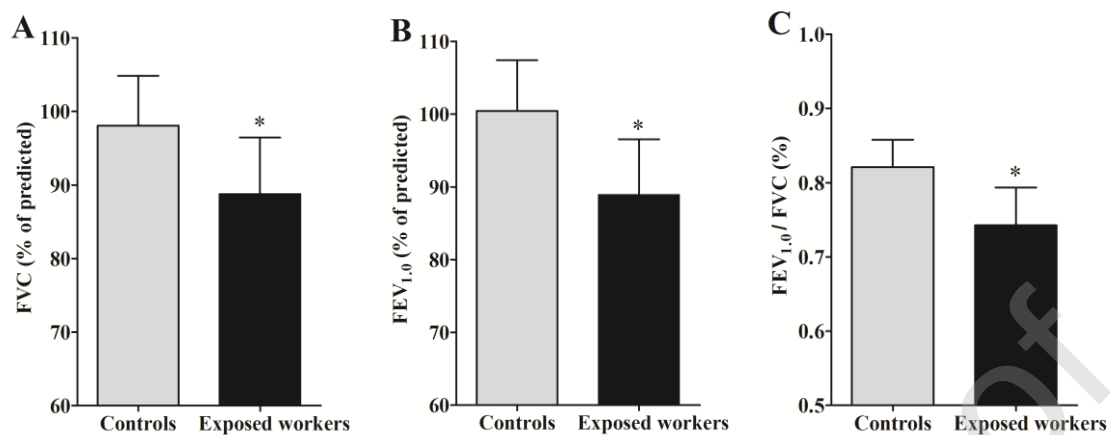


Fig. 1 A comparison of pulmonary function between the two groups of workers. Pulmonary function indexes of (A) FVC (% of predicted), (B) FEV_{1.0} (% of predicted) and (C) FEV_{1.0}/FVC (%) were measured and analyzed. Data are plotted as the mean $\pm$ SD. The student's t test was performed. * $p < 0.05$, compared to the control group.

3.4. Analysis of sputum cells from exposed and control workers

Sputum cells from the two groups were collected and these included squamous cells, respiratory epithelial cells, neutrophils, eosinophils, basophils, monocytes and leukomonocytes. The number of different cells types were analyzed and the results are shown in Fig. 2. Significantly higher levels ($p < 0.05$) of neutrophils and eosinophils were observed in sputum obtained from the exposed workers group than those from the control group. In contrast, decreased levels of leukomonocytes were detected in the exposed workers ($p < 0.05$) as compared to controls. No significant differences were detected in the levels of basophils between both groups.

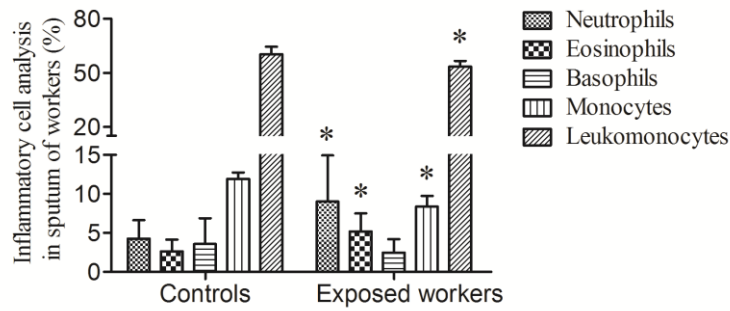


Fig. 2 Analysis of inflammatory cells in sputum of workers. Cells collected from induced sputum of workers were enumerated and the percentage of neutrophils, eosinophils, basophils, monocytes and leukomonocytes were plotted. Data are represented as the mean $\pm$ SD. The student's t test was performed. * $p < 0.05$, compared to the control group.

3.5. The levels of TSLP, IL-17 and IgE in sputum supernatant

To further explore inflammatory changes in the sputum of subjects from both groups, levels of TSLP, IL-17 and IgE were analyzed using ELISA. Results showed a significant increase ($p < 0.05$) in the levels of TSLP and IgE in the exposed group as compared to the control group (Fig. 3A, C). No significant changes ($p > 0.05$) in IL-17 were detected between the two groups (Fig. 3B).

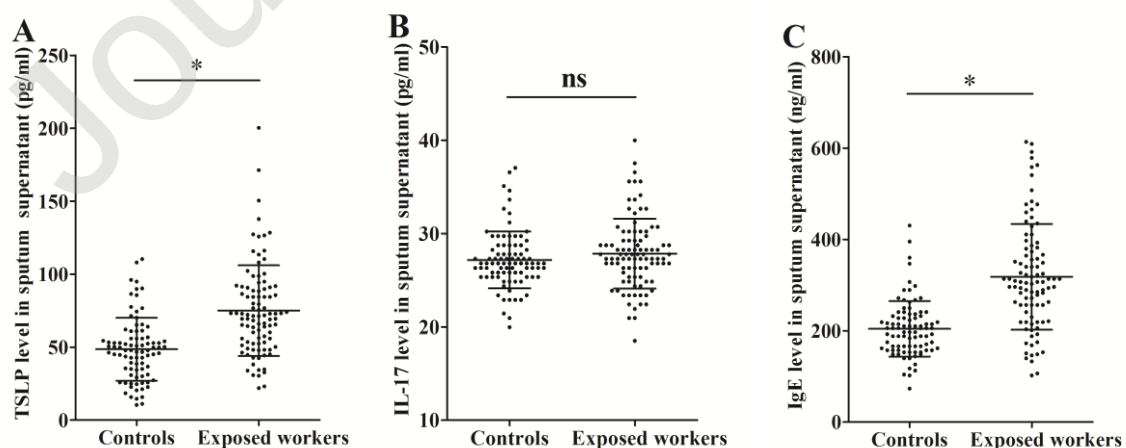


Fig. 3. Levels of TSLP, IL-17 and IgE in sputum supernatant. Total levels of (A) TSLP, (B) IL-17 and (C) IgE in sputum supernatant from control and exposed workers as measured by ELISA. Data are plotted as the mean $\pm$ SD. The student's t test was performed. * $p < 0.05$, compared to the control group. Ns: non-significant.

3.6. Airway hyper-reactivity (AHR) analysis of mice

A mouse model of TDI-induced asthma was also used in our study. We randomly divided 18 mice into three groups of 6 each: TDI group, TSLP neutralizing antibody (TSLP-Ab) group and control group (AOO group). Airway reactivity for all mice was assessed 24 h after the last challenge. Results showed that the Penh value of TDI-induced asthmatic mice exhibited a significant increase ($p < 0.05$) in AHR compared to those in the AOO group when challenged with methacholine (1.25, 2.5, 5.0 and 10.0 mg/ml). This hyper-responsiveness significantly alleviated ($p < 0.05$) following TSLP-Ab treatment (Fig. 4).

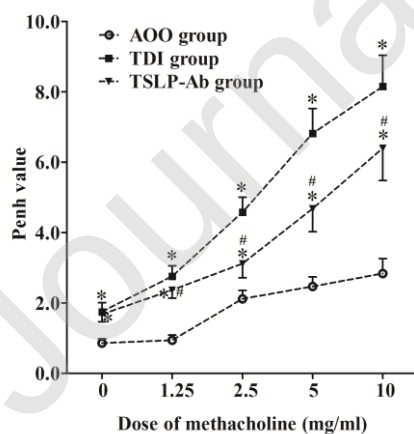


Fig. 4. Mouse airway hyper-reactivity (AHR) analysis. AHR to methacholine was assessed 24 h after the third challenge. Penh values were measured after increasing doses of methacholine, as described in Materials and Methods. Data are plotted as the

mean $\pm$ SD ($n = 6$ per group). One-way analysis of variance (ANOVA) test was performed. * $p < 0.05$, compared to the AOO group; # $p < 0.05$, compared to the TDI group. The behavior of animals was monitored for the major changes of asthma as outlined in section 2.8.

3.7. Analysis of inflammatory cells in BALF of TDI-induced asthma mouse model

Analysis of the total number and types of inflammatory cells was performed in BALF obtained from mice 24 hours after treatment (Fig 5). We observed a significant increase ($p < 0.05$) in the number of neutrophils, eosinophils, basophils, monocytes and leukomonocytes in the BALF of the TDI-exposed mice as compared to those in the control group. In the TSLP-Ab group, the number of neutrophils, eosinophils and leukomonocytes were higher ($p < 0.05$) than that of the control group but lower ($p < 0.05$) than that in the TDI group. Compared with the TDI group, the number of basophils and monocytes in the TSLP-Ab group were decreased, however it was not found to be statistically significant ($p > 0.05$).

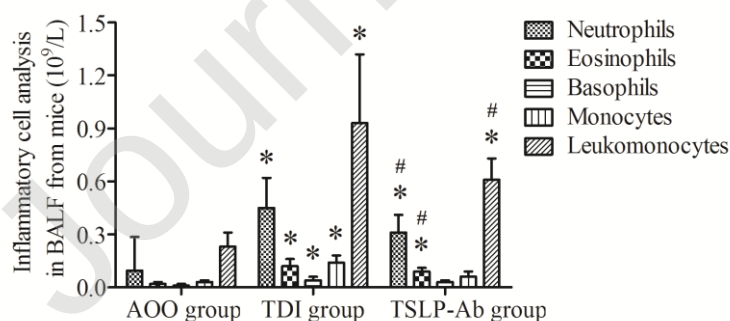


Fig. 5. Analysis of inflammatory cells in BALF from TDI-treated mice. The total number and types of inflammatory cells including neutrophils, eosinophils, basophils, monocytes and leukomonocytes were determined. Data are plotted as the mean $\pm$ SD

(n = 6 per group). One-way analysis of variance (ANOVA) test was performed. * $p < 0.05$, compared to the AOO group; # $p < 0.05$, compared to the TDI group.

3.8. IFN- γ , IL-4 and IL-17 levels in the BALF of TDI-induced asthma mouse model

Levels of IFN- γ , IL-4 and IL-17 were also analyzed in BALF from mice using ELISA. Results showed that IFN- γ (Fig. 6A), IL-4 (Fig. 6B) and IL-17 (Fig. 6C) were highly expressed in the BALF of TDI-exposed mice as compared to the control group. These were significantly reduced in the TSLP-Ab group as compared to the TDI group (Fig. 6).

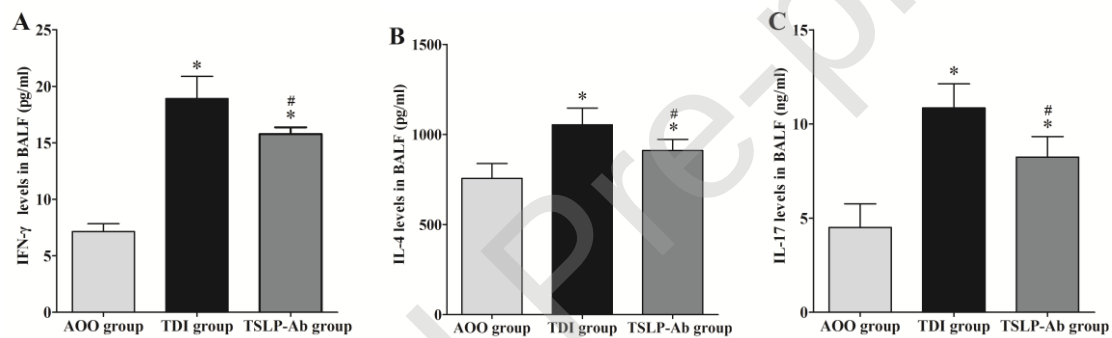


Fig. 6. IFN- γ , IL-4 and IL-17 levels in the BALF of mice. (A) IFN- γ , (B) IL-4 and (C) IL-17 levels were measured by ELISA. Data are plotted as the mean $\pm$ SD (n = 6 per group). One-way analysis of variance (ANOVA) test was performed. * $p < 0.05$, compared with AOO group; # $p < 0.05$, compared to TDI group.

3.9. The pathology of lung and bronchus from mice

Histopathology of lung and bronchus sections from mice from the various groups correlated to results observed with the analysis performed on the respective BALF samples. H&E staining revealed that lung and bronchus in the TDI group exhibited

typical pathological features of TDI-induced airway disease as compared to that from the control group (Fig. 7A, D). An infiltration of inflammatory cells was observed around the bronchus of TDI-exposed mice. We also a thickening of the lining of the airway epithelium and an increase in bronchial epithelial cell proliferation and bronchial mucus secretion in TDI group (Fig. 7B, E). In the TSLP-Ab group, these symptoms were alleviated. A reduction in the level of inflammatory cell infiltration around the peribronchial area, the proliferation of bronchial epithelial cells and bronchial wall thickness was observed (Fig. 7C, F).

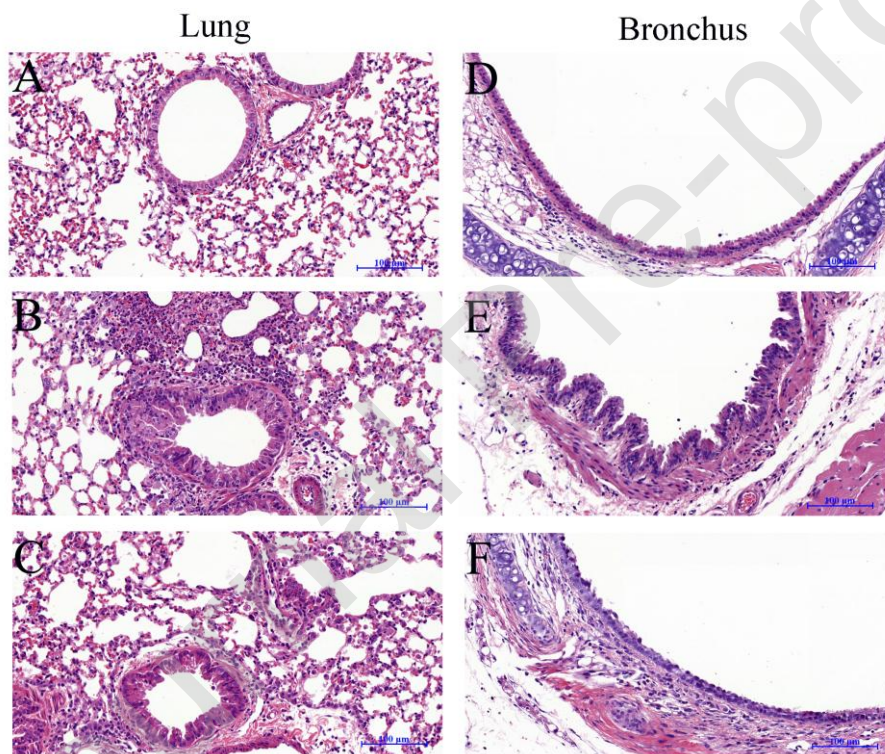


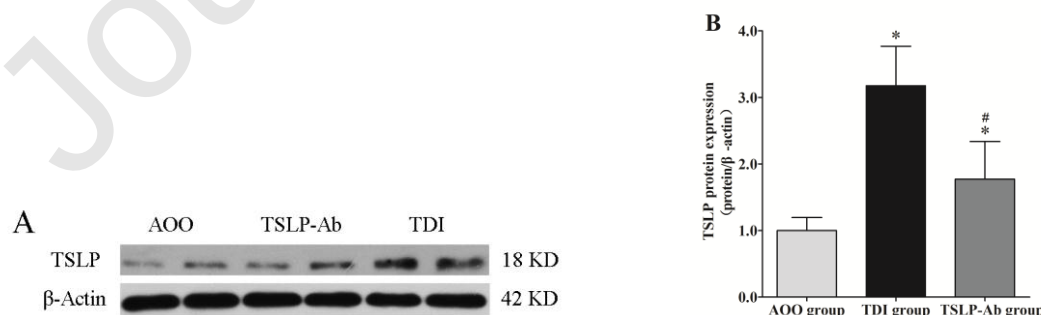
Fig. 7. Histopathology of the lung and bronchus sections from mice. The lung and bronchus tissue slices were fixed with formaldehyde and stained by hematoxylin and eosin (H&E), and then examined and imaged with optical microscopy (200 × mag.). The representative histology image from (A, D) AOO group shows open clear pulmonary alveoli and bronchus in the absence of inflammatory cells, (B, E) TDI group

depicts inflammatory cell infiltration and a marked increase in the bronchial wall thickness, (C, F) TSLP-Ab group shows an alleviation of inflammatory cell infiltration and bronchial wall thickness.

3.10. Expression of TSLP protein and IL-17, ROR- γ t and Foxp3 mRNA levels in lung

Levels of TSLP in lung tissue of mice from all three groups were also analyzed. Western blot results showed that TSLP was highly expressed in the TDI-exposed group as compared to the control group. This expression decreased following TSLP-Ab treatment (Fig. 8A, B).

We also analyzed the levels of IL-17, ROR- γ t and Foxp3 mRNA in lung tissue of mice from all three groups. The result showed that the levels of IL-17 and ROR- γ t mRNA were highly expressed in the TDI-exposed group as compared to the control group (Fig. 8C, D). In contrast, expression of IL-17 and ROR- γ t in the TSLP-Ab group was reduced as compared to the TDI-exposed group (Fig. 8C, D). mRNA levels of Foxp3 remained unchanged in the three groups (Fig. 8E). These data suggest that an imbalance in Th17/Treg cells may play an important role in TDI-induced asthma and that treatment with TSLP-Ab could potentially rescue this.



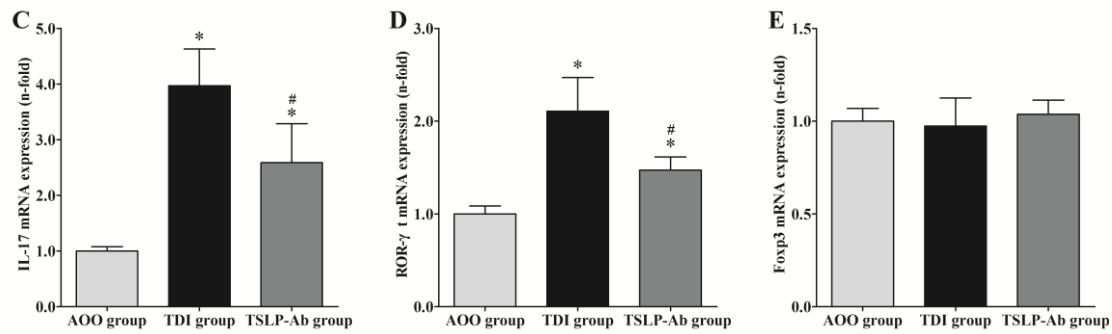


Fig. 8. Expression levels of TSLP protein, Foxp3, ROR- γ t and IL-17 mRNA in lung tissue. (A) TSLP levels were determined by western blot and (B) quantitated by the intensity of bands. mRNA expression levels of (C) IL-17, (D) ROR- γ t and (E) Foxp3 as measured by real-time PCR. The data were normalized, and plotted as mean $\pm$ SD (n = 6 per group). Non-parametric analysis (Mann-Whitney test) was performed. * p < 0.05, compared to the AAO group; # p < 0.05, compared to the TDI group.

4. Discussion

Here we studied the effects of TDI-induced asthma in exposed workers as well as in a mouse model of TDI-induced asthma. We observed that these workers were exposed to high levels of TDI, approximately 8 times the recommended limit (TWA: 0.1mg/m³). Several reports have showed that the main biological effects of TDI are its stimulation and sensitization abilities which can reduce pulmonary function and cause asthma (Wegman et al., 1977; Talini et al., 2013; Pauluhn 2014). TDI exposure can also cause respiratory symptoms such as coughing, shortness of breath, airway edema and neutrophilic bronchitis (Thompson et al., 1987; Clark et al., 1998; Pala et al., 2011). Our results showed that the exposed group had decreased values of FEV_{1.0} (% of predicted) and FEV_{1.0}/FVC (%) as compared to those in the control group, indicating

that TDI has a negative effect on pulmonary function. This demonstrated that the exposed workers were constantly engaged in an environment with high concentrations of TDI and therefore had a greater propensity to develop TDI-induced asthma.

In this study, we collected sputum from all participants and performed a total cell count. We found highly increased levels of neutrophils and eosinophils but a decrease in leukomonocytes in sputum from exposed workers as compared to the control group. In humans, asthma, atopic dermatitis and allergic rhinitis form a triad of common atopic IgE-dependent diseases (Kay, 2001). We have shown in this study that IgE is highly expressed in the exposed group as compared to the control group. Published works have shown that the cytokine TSLP may play a critical role in initiating the pathogenesis of allergic diseases (Omori-Miyake and Ziegler, 2012; Segawa and Hirasawa, 2014). Here we showed that sputum supernatant from the exposed group had a higher expression of TSLP as compared to controls. Induced sputum supernatant TSLP and TNF- α levels were significantly higher in asthmatic patients and their levels depend on asthma severity (Berraïes et al., 2016). All these data demonstrated the occurrence of airway inflammation in exposed workers, indicating that TDI exposure can cause airway and lung inflammation. Our data also point to a role for TSLP in the development of TDI-induced asthma.

In this study, we also established a mouse model of TDI-induced asthma. The methodology used here was largely based on De Vooght et al.'s (2009) study with some modifications. Mice were challenged with 0.01% TDI through the airways. We monitored the behavioural changes of the mice measured the airway hyper-

responsiveness, analyzed the inflammatory cells in BALF, determined the levels of IL-4 and IFN- γ and performed histopathological examination on lung tissues to confirm the successful establishment of a mouse model with TDI-induced asthma. It was reported that one of the important pathological mechanisms of asthma is the immune imbalance of Th17/Treg cells (Chen et al., 2011; Liljevald et al., 2016). Treg cells have anti-inflammatory effects and maintain immune tolerance function, however, they are also capable of inhibiting the differentiation and development of Th17 cells (Girtsman et al., 2010). The transcription factor Foxp3 regulates the differentiation of Treg cells (Schmidt et al., 2016). When the initial T cell is stimulated by exogenous antigen, it differentiates into Th17/Treg cells and this process is determined by the expression of ROR γ t and Foxp3. ROR γ t is the key transcription factor controlling the differentiation of Th17 cells (Liljevald et al., 2016), Foxp3 is a specific transcription factor of Treg cell whose activity is critical for Treg development and function, as well as ROR γ t for Th17 cells (Chen et al., 2011). At present, the role of Th17/Treg balance in the pathogenesis of TDI asthma remains unclear. In this study, we showed that IL-17 mRNA was significantly increased in the lung tissue of mice in the TDI-exposed group compared to the control group. The mRNA expression of its regulatory factor, ROR γ t was also increased. However, the mRNA expression level of Treg cells transcription factor Foxp3 decreased. These results suggest that a Th17/Treg cell immune imbalance is involved in the pathogenesis of TDI-induced asthma. In contrast, there were no significant changes in IL-17 levels in TDI-exposed factory workers. This could be because IL17 is primarily expressed in acute asthma attacks (Wang et al., 2016).

Here, the expression of TSLP in the sputum supernatant of TDI-exposed workers was significantly higher than that in the control group. Moreover, the level of TSLP in the BALF was also significantly higher in the mouse mode of TDI-induced asthma than that in the control, suggesting that TSLP is indeed closely linked to asthma and is highly expressed in TDI-induced asthma. After intervention with TSLP-Ab, the asthmatic symptoms of TDI-exposed mice were alleviated significantly and the expressions of ROR γ t and IL-17a mRNA were also significantly reduced. TSLP may promote the differentiation of Th17 cells and IL-17 release to regulate TDI-induced asthma. This result is consistent with findings in the ovalbumin asthma mouse model showing that TSLP can promote Th17 cell differentiation, IL-17 release, and aggravate airway inflammation (De Vooght et al., 2009). It was also reported that TSLP-activated dendritic cells (TSLP-DCs) promoted the conversion of naive CD4⁺ T lymphocytes to Foxp3⁺ regulatory T lymphocytes (Tregs) in human thymus and mouse model (Watanabe et al., 2005; Besin et al., 2008). In BALF taken from asthmatic children, the level of Th17/Treg is lower than that of normal children and it was shown that Treg cells lose their ability to inhibit Th17 cells. This was rescued after treatment with glucocorticoids (Hartl et al., 2007). As compared to mild asthma, a reduction in the expression of Foxp3 and proliferation rate of Treg cells are observed in moderate and severe asthma. This may be due to an increase in adaptability caused by increased inflammation with asthma progression (Lee et al., 2007). Asthma inhibits Treg cells both quantitatively and functionally and TSLP may be a key factor in promoting early proliferation of Treg cells.

In conclusion, our results suggest that TSLP plays an important role in the pathogenesis of asthma. After intraperitoneal injection of TSLP-Ab in our mouse model, the symptoms of asthma as well as airway inflammation were alleviated. This therapeutic effect appears to be related to the inhibition of Th17 cell differentiation and the reduction of IL-17 release. Hence, our study shows that TDI-induced asthma could potentially be prevented or treated by inhibiting the expression of TSLP molecules or binding to its receptors. Further studies are required to explore the therapeutic mechanisms of TDI-induced asthma by inhibiting TSLP expression.

Conflict of interest

All authors declare that there are no conflicts of interest.

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References

Alyasin, S., Karimi, M.H., Amin, R., Babaei, M., Darougar, S., 2013. Interleukin-17 gene expression and serum levels in children with severe asthma. Iran J Immunol

10, 177-185.

Ban, M., Morel, G., Langonne, I., Huguet, N., Pepin, E., Binet, S., 2006. TDI can induce respiratory allergy with Th2-dominated response in mice. *Toxicology* 218, 39-47.

Berraïes, A., Hamdi, B., Ammar, J., Hamzaoui, K., Hamzaoui, A., 2016. Increased expression of thymic stromal lymphopoietin in induced sputum from asthmatic children. *Immunol Lett* 178, 85-91.

Besin, G., Gaudreau, S., Ménard, M., Guindi, C., Dupuis, G., Amrani, A., 2008. Thymic stromal lymphopoietin and thymic stromal lymphopoietin-conditioned dendritic cells induce regulatory T-cell differentiation and protection of NOD mice against diabetes. *Diabetes* 57, 2107-2117.

Biagini Myers, J.M., Martin, L.J., Kovacic, M.B., Mersha, T.B., He, H., Pilipenko, V., Lindsey, M.A., Ericksen, M.B., Bernstein, D.I., LeMasters, G.K., Lockey, J.E., Khurana Hershey, G.K., 2014. Epistasis between serine protease inhibitor Kazal-type 5 (SPINK5) and thymic stromal lymphopoietin (TSLP) genes contributes to childhood asthma. *J Allergy Clin Immunol* 134, 891-899 e893.

Byers, D.E., 2014. Defining the roles of IL-33, thymic stromal lymphopoietin, and IL-25 in human asthma. *Am J Respir Crit Care Med* 190, 715-716.

Chen, Z., Lin, F., Gao, Y., Li, Z., Zhang, J., Xing, Y., Deng, Z., Yao, Z., Tsun, A., Li, B., 2011. FOXP3 and ROR γ mat: transcriptional regulation of Treg and Th17. *Int Immunopharmacol* 11, 536-542.

Clark, R.L., Bugler, J., McDermott, M., Hill, I.D., Allport, D.C., Chamberlain, J.D.,

1998. An epidemiology study of lung function changes of toluene diisocyanate foam workers in the United Kingdom. *Int Arch Occup Environ Health* 71, 169-179.
- Crapo, R.O., Morris, A.H., Gardner, R.M., 1981. Reference spirometric values using techniques and equipment that meet ATS recommendations. *Am Rev Respir Dis* 123, 659-664.
- De Vooght, V., Vanoirbeek, J.A., Haenen, S., Verbeken, E., Nemery, B., Hoet, P.H., 2009. Oropharyngeal aspiration: an alternative route for challenging in a mouse model of chemical-induced asthma. *Toxicology* 259, 84-89.
- Del Prete, G.F., De Carli, M., D'Elia, M.M., Maestrelli, P., Ricci, M., Fabbri, L., Romagnani, S., 1993. Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol* 23, 1445-1449.
- Friend, S.L., Hosier, S., Nelson, A., Foxworthe, D., Williams, D.E., Farr, A., 1994. A thymic stromal cell line supports in vitro development of surface IgM⁺ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp Hematol* 22, 321-328.
- Gautrin, D., Newman-Taylor, A.J., Nordman, H., Malo, J.L., 2003. Controversies in epidemiology of occupational asthma. *Eur Respir J* 22, 551-559.
- Girtsman, T., Jaffar, Z., Ferrini, M., Shaw, P., Roberts, K., 2010. Natural Foxp3(+) regulatory T cells inhibit Th2 polarization but are biased toward suppression of Th17-driven lung inflammation. *J Leukoc Biol* 88, 537-546.

- Hartl, D., Koller, B., Mehlhorn, A.T., Reinhardt, D., Nicolai, T., Schendel, D.J., Gries, M., Krauss-Etschmann, S., 2007. Quantitative and functional impairment of pulmonary CD4⁺CD25^{hi} regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 119, 1258-1266.
- Hasegawa, T., Uga, H., Mori, A., Kurata, H., 2017. Increased serum IL-17A and Th2 cytokine levels in patients with severe uncontrolled asthma. *Eur Cytokine Netw* 28, 8-18.
- Hirose, K., Iwata, A., Tamachi, T., Nakajima, H., 2017. Allergic airway inflammation: key players beyond the Th2 cell pathway. *Immunol Rev* 278, 145-161.
- Hirota, J.A., Hirota, S.A., Warner, S.M., Stefanowicz, D., Shaheen, F., Beck, P.L., Macdonald, J.A., Hackett, T.L., Sin, D.D., Van Eeden, S., Knight, D.A., 2012. The airway epithelium nucleotide-binding domain and leucine-rich repeat protein 3 inflammasome is activated by urban particulate matter. *J Allergy Clin Immunol* 129, 1116-1125 e1116.
- Johnson, V.J., Yucesoy, B., Luster, M.I., 2005. Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* 116, 851-858.
- Kay, A.B., 2001. Allergy and allergic diseases. First of two parts. *N Engl J Med* 344, 30-37.
- Klees, J.E., Ott, M.G., 1999. Diisocyanates in polyurethane plastics applications. *Occup Med* 14, 759-776.
- Lee, H.C., Sung, S.S., Krueger, P.D., Jo, Y.A., Rosen, H.R., Ziegler, S.F., Hahn, Y.S.,

2013. Hepatitis C virus promotes T-helper (Th)17 responses through thymic stromal lymphopoietin production by infected hepatocytes. *Hepatology* 57, 1314-1324.
- Lee, J.H., Yu, H.H., Wang, L.C., Yang, Y.H., Lin, Y.T., Chiang, B.L., 2007. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. *Clin Exp Immunol* 148, 53-63.
- Liang, J., Zhao, H., Yao, L., Tang, H., Dong, H., Wu, Y., Liu, L., Zou, F., Cai, S., 2015. Phosphatidylinositol 3-kinases pathway mediates lung caspase-1 activation and high mobility group box 1 production in a toluene-diisocyanate induced murine asthma model. *Toxicol Lett* 236, 25-33.
- Liljevald, M., Rehnberg, M., Soderberg, M., Ramnegard, M., Borjesson, J., Luciani, D., Krutrok, N., Branden, L., Johansson, C., Xu, X., Bjursell, M., Sjogren, A.K., Hornberg, J., Andersson, U., Keeling, D., Jirholt, J., 2016. Retinoid-related orphan receptor gamma (RORgamma) adult induced knockout mice develop lymphoblastic lymphoma. *Autoimmun Rev* 15, 1062-1070.
- Liu, S., Verma, M., Michalec, L., Liu, W., Sripada, A., Rollins, D., Good, J., Ito, Y., Chu, H., Gorska, M.M., Martin, R.J., Alam, R., 2018. Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: The role of thymic stromal lymphopoietin. *J Allergy Clin Immunol* 141, 257-268 e256.
- Lummus, Z.L., Wisnewski, A.V., Bernstein, D.I., 2011. Pathogenesis and disease mechanisms of occupational asthma. *Immunol Allergy Clin North Am* 31, 699-716, vi.

- Lundblad, L.K., Irvin, C.G., Adler, A., Bates, J.H., 2002. A reevaluation of the validity of unrestrained plethysmography in mice. *J Appl Physiol* (1985) 93, 1198-1207.
- Omori-Miyake, M., Ziegler, S.F., 2012. Mouse models of allergic diseases: TSLP and its functional roles. *Allergol Int* 61, 27-34.
- Ott, M.G., Diller, W.F., Jolly, A.T., 2003. Respiratory effects of toluene diisocyanate in the workplace: a discussion of exposure-response relationships. *Crit Rev Toxicol* 33, 1-59.
- Pala, G., Pignatti, P., Moscato, G., 2011. Occupational exposure to toluene diisocyanate and neutrophilic bronchitis without asthma. *Clin Toxicol (Phila)* 49, 506-507.
- Pauluhn, J., 2014. Development of a respiratory sensitization/elicitation protocol of toluene diisocyanate (TDI) in Brown Norway rats to derive an elicitation-based occupational exposure level. *Toxicology* 319, 10-22.
- Pham, D.L., Trinh, T.H., Ban, G.Y., Kim, S.H., Park, H.S., 2017. Epithelial folliculin is involved in airway inflammation in workers exposed to toluene diisocyanate. *Exp Mol Med* 49, e395.
- Qin, W., Deng, T., Cui, H., Zhang, Q., Liu, X., Yang, X., Chen, M., 2018. Exposure to diisodecyl phthalate exacerbated Th2 and Th17-mediated asthma through aggravating oxidative stress and the activation of p38 MAPK. *Food Chem Toxicol* 114, 78-87.
- Racine, G., Castano, R., Cartier, A., Lemiere, C., 2017. Diagnostic Accuracy of Inflammatory Markers for Diagnosing Occupational Asthma. *J Allergy Clin*

Immunol Pract 5, 1371-1377 e1371.

Roan, F., Bell, B.D., Stoklasek, T.A., Kitajima, M., Han, H., Ziegler, S.F., 2012. The multiple facets of thymic stromal lymphopoietin (TSLP) during allergic inflammation and beyond. *J Leukoc Biol* 91, 877-886.

Schmidt, A., Elias, S., Joshi, R.N., Tegner, J., 2016. In Vitro Differentiation of Human CD4+FOXP3+ Induced Regulatory T Cells (iTregs) from Naive CD4+ T Cells Using a TGF-beta-containing Protocol. *J Vis Exp*.

Schupp, T., Collins, M.A., 2012. Toluene diisocyanate (TDI) airway effects and dose-responses in different animal models. *EXCLI J* 11, 416-435.

Segawa, R., Hirasawa, N., 2014. Exacerbation of allergic diseases by chemicals: role of TSLP. *J Pharmacol Sci* 124, 301-306.

Sharifi, L., Karimi, A., Shokouhi Shoormasti, R., Miri, S., Heydar Nazhad, H., Bokaie, S., Fazlollahi, M.R., Sadeghniiat Haghighi, K., Pourpak, Z., Moin, M., 2013. Asthma Symptoms and Specific IgE Levels among Toluene Diisocyanate (TDI) Exposed Workers in Tehran, Iran. *Iran J Public Health* 42, 397-401.

Soumelis, V., Reche, P.A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gorman, D., Zurawski, S., Abrams, J., Menon, S., McClanahan, T., de Waal-Malefyt Rd, R., Bazan, F., Kastelein, R.A., Liu, Y.J., 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 3, 673-680.

Stocks, S.J., Jones, K., Piney, M., Agius, R.M., 2015. Isocyanate exposure and asthma in the UK vehicle repair industry. *Occup Med (Lond)* 65, 713-718.

- Swierczyńska-Machura, D., Pałczyński, C., 2012. [Pathogenetic and clinical aspects of diisocyanate asthma]. *Med Pr* 63, 97-103.
- Talini, D., Novelli, F., Bacci, E., Costa, F., Dente, F.L., Di Franco, A., Malagrino, L., Vagaggini, B., Paggiaro, P., 2013. Mild improvement in symptoms and pulmonary function in a long-term follow-up of patients with toluene diisocyanate-induced asthma. *Int Arch Allergy Immunol* 161, 189-194.
- Tanaka, J., Watanabe, N., Kido, M., Saga, K., Akamatsu, T., Nishio, A., Chiba, T., 2009. Human TSLP and TLR3 ligands promote differentiation of Th17 cells with a central memory phenotype under Th2-polarizing conditions. *Clin Exp Allergy* 39, 89-100.
- Thompson, J.E., Scypinski, L.A., Gordon, T., Sheppard, D., 1987. Tachykinins mediate the acute increase in airway responsiveness caused by toluene diisocyanate in guinea pigs. *Am Rev Respir Dis* 136, 43-49.
- Wang, Z.H., Li, X.M., Wang, Y.S., Guo, Z.Y., 2016. Changes in the Levels of Interleukin-17 Between Atopic and Non-atopic Children with Mycoplasma pneumoniae Pneumonia. *Inflammation* 39, 1871-1875.
- Watanabe, N., Wang, Y.H., Lee, H.K., Ito, T., Wang, Y.H., Cao, W., Liu, Y.J., 2005. Hassall's corpuscles instruct dendritic cells to induce CD4⁺CD25⁺ regulatory T cells in human thymus. *Nature* 436, 1181-1185.
- Wegman, D.H., Peters, J.M., Pagnotto, L., Fine, L.J., 1977. Chronic pulmonary function loss from exposure to toluene diisocyanate. *Br J Ind Med* 34, 196-200.
- Wilson, R.H., Whitehead, G.S., Nakano, H., Free, M.E., Kolls, J.K., Cook, D.N., 2009.

Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. *Am J Respir Crit Care Med* 180, 720-730.

Wisniewski, J.A., Borish, L., 2011. Novel cytokines and cytokine-producing T cells in allergic disorders. *Allergy Asthma Proc* 32, 83-94.

Yadava, K., Massacand, J., Mosconi, I., Nicod, L.P., Harris, N.L., Marsland, B.J., 2014. Thymic stromal lymphopoietin plays divergent roles in murine models of atopic and nonatopic airway inflammation. *Allergy* 69, 1333-1342.

Ye, Z., Huang, Y., Liu, D., Chen, X., Wang, D., Huang, D., Zhao, L., Xiao, X., 2012. Obesity induced by neonatal overfeeding worsens airway hyperresponsiveness and inflammation. *PLoS One* 7, e47013.

Ying, S., O'Connor, B., Ratoff, J., Meng, Q., Fang, C., Cousins, D., Zhang, G., Gu, S., Gao, Z., Shamji, B., Edwards, M.J., Lee, T.H., Corrigan, C.J., 2008. Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol* 181, 2790-2798.

Zhou, T., Huang, X., Zhou, Y., Ma, J., Zhou, M., Liu, Y., Xiao, L., Yuan, J., Xie, J., Chen, W., 2017. Associations between Th17-related inflammatory cytokines and asthma in adults: A Case-Control Study. *Sci Rep* 7, 15502.