



Research article

Significant upregulation of cytokine secretion from T helper type 9 and 17 cells in a NC/Nga mouse model of ambient chemical exposure-induced respiratory allergy



Risako Nishino, Tomoki Fukuyama ^{*}, Yuko Watanabe, Yoshimi Kurosawa, Tadashi Kosaka, Takanori Harada

Toxicology Division, Institute of Environmental Toxicology, Uchimoriya-machi 4321, Joso-shi, Ibaraki 303-0043, Japan

ARTICLE INFO

Article history:

Received 18 December 2015

Received in revised form 18 February 2016

Accepted 11 April 2016

Available online 14 April 2016

Keywords:

Respiratory allergy

NC/Nga mice

Particulate matter

Trimellitic anhydride

Helper T cell

ABSTRACT

It has been reported that ambient chemical exposure is closely associated with respiratory allergies. We attempted to develop an original protocol for detecting ambient chemical exposure-induced respiratory allergy in different strains of mice. In the process of comparing allergic potency of these mice, we observed that NC/Nga mice showed significant upregulation of respiratory allergic symptoms as well as specific type of cytokine secretions. The main purpose of this study was to investigate the mechanism underlying these phenomena in NC/Nga mice in comparison with BALB/c mice. For the model of respiratory allergy, female BALB/c and NC/Nga mice were sensitized and challenged with trimellitic anhydride. Clinical observation, IgE and immunocyte counts, and cytokine profile in the serum, lymph nodes, and bronchoalveolar lavage fluid were recorded. We also monitored the expression of genes encoding pro-inflammatory cytokines in the lung. We found that worsening of respiratory status was noted only in NC/Nga mice, whereas Th2 reactions were significantly increased in BALB/c mice compared with NC/Nga mice. In contrast, the levels of Th9 and Th17-derived cytokines in NC/Nga mice were significantly higher than those in BALB/c mice. Thus, Th9 and Th17 may be involved in the aggravation of respiratory allergic symptoms induced by ambient chemicals.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

It has been reported that exposure to ambient chemicals including particulate matter (PM) was closely associated with respiratory health and diseases including asthma and chronic obstructive pulmonary disease (Loftus, Yost, Sampson, Arias, Torres, Vasquez, Bhatti, & Karr 2015; McCormack, Belli, Kaji, Matsui, Brigham, Peng, Sellers, Williams, Diette, Breyse, & Hansel 2015). Especially, PM with an aerodynamic size $\leq 2.5 \mu\text{m}$ (PM 2.5) or $2.5\text{--}10 \mu\text{m}$ (PM 2.5–10) can easily penetrate the pulmonary alveoli and act as a respiratory allergen or adjuvant. According to the previous reports, PM 2.5–10 induced-toxicity typically results in an inflammatory response involving influx of neutrophils into the lungs (Li, Gilmour, Donaldson, & MacNee 1997) and upregulation of inflammatory cytokines including interleukin (IL)-8, IL-6, and tumor necrosis factor (TNF) α (Becker, Soukup, Gilmour, & Devlin 1996; Carter, Ghio, Samet, & Devlin 1997). However, the exact mechanisms of functional allergic response and pro-inflammatory action of these

ambient chemicals are still not fully understood. One of the objectives of this project was to elucidate the mechanisms of functional and pro-inflammatory potential of ambient chemicals, using our original in vivo detection method for respiratory allergy.

Although the risk of ambient chemical allergens has been a topic of interest since a long time, there are still limited options to detect the respiratory allergenicity of these chemicals. Serum immunoglobulin E (IgE) test and cytokine fingerprinting are well known methods for detecting the respiratory allergenicity of ambient chemicals. Serum IgE test has been reported by Dearman, Basketter, & Kimber (1992) and is based on the increased levels of serum IgE due to chemical allergen exposure. Mice were sensitized dermally with chemical allergen and total serum IgE levels were measured at 14 days after the first chemical sensitization. On the other hand, cytokine fingerprinting focused on the secretion profile of cytokines produced by helper T cell type 1 (Th1) or Th2 followed by chemical allergen exposure. Respiratory allergens tend to induce the secretion of Th2 type cytokines such as IL-4 and -13, whereas contact allergens induce Th1 cytokine secretions such as interferon (IFN)- γ and TNF α (Dearman, Betts, Humphreys, Flanagan, Gilmour, Basketter, & Kimber 2003a). Both methods proved easy to detect the respiratory allergenicity potential of ambient chemical allergens within a short period; however, there were many false-positive as well as false-negative outcomes because in both methods, the chemical is applied only dermally even though the

Abbreviations: BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IFN, interferon; IL, interleukin; LN, lymph node; PM, particulate matter; Th, helper T cell; TMA, trimellitic anhydride; TNF, tumor necrosis factor.

^{*} Corresponding author.

E-mail address: fukuyama@iet.or.jp (T. Fukuyama).

objective is to detect respiratory allergenicity (Dearman, Skinner, Humphreys, & Kimber 2003b). Therefore, the primary objective in our project is to develop an original detection protocol focused on the respiratory tract as the target organ of ambient chemical allergen-induced respiratory allergy.

To achieve our aims and demonstrate the usefulness of our protocol, we previously validated our original protocol in mice with trimellitic anhydride (TMA) and glutaraldehyde as a positive control substance for ambient chemical exposure-induced respiratory allergy (Fukuyama, Ueda, Hayashi, Tajima, Shuto, Saito, Harada, & Kosaka 2008; Fukuyama, Tajima, Ueda, Hayashi, Shutoh, Harada, & Kosaka 2009, 2010; Nishino, Fukuyama, Watanabe, Kurosawa, Ueda, & Kosaka 2014). Now, we are focusing on NC/Nga mice to ascertain which strain of mice is more sensitive for the detection of ambient chemical exposure-induced respiratory allergy. Although NC/Nga mice have already been used as mouse models of atopic dermatitis in previous reports (Matsuda, Watanabe, Geba, Sperl, Tsudzuki, Hiroi, Matsumoto, Ushio, Saito, Askenase, & Ra 1997), to our knowledge, studies on altered ambient chemical exposure-induced allergy have not yet been reported. The objective of this study was to investigate the mechanism underlying hyper-expression of respiratory allergic symptoms in TMA-induced NC/Nga mice in comparison with TMA-induced BALB/c mice.

2. Materials and methods

2.1. Animals and reagents

BALB/cAnN and NC/Nga mice (female, 7-week-old) were purchased from Charles River Laboratories (Atsugi, Kanagawa, Japan) and housed individually in cages under controlled lighting (lights on, 07:00–19:00), temperature ($22 \pm 3^\circ\text{C}$), humidity ($55\% \pm 15\%$), and ventilation (at least 10 complete fresh-air changes/h). Standard rodent chow (Certified Pellet Diet MF; Oriental Yeast Co., Tokyo) and filtered water were provided ad libitum. All aspects of the current study were conducted in accordance with the Animal Care and Use Program of the Institute of Environmental Toxicology, Japan (IET IACUC Approval No. 13244). Trimellitic anhydride (TMA, $\text{C}_9\text{H}_4\text{O}_5$, >97%), acetone, and olive oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). For dermal sensitization, 1.0% (w/v) TMA was dissolved in acetone/olive oil (4:1). For intratracheal challenge, TMA was pulverized into particles with 2- μm diameter (Single track jet mill, FS-4, SEISIN ENTERPRISE Co., Ltd., Tokyo, Japan). Phosphate buffered saline (PBS), RPMI 1640 medium, heat-inactivated fetal calf serum (FCS), and Dynabeads mouse T-Activator CD3/CD28 were ordered from Life Technologies Co., Ltd., (Tokyo, Japan). The BD OptEIA mouse IgE Set for enzyme-linked immunosorbent assays (ELISAs), streptavidin-horseradish peroxidase, mouse BD Fc Block, biotin-conjugated anti-mouse IgE (rat IgG1, clone R35–118), FITC-conjugated anti-mouse IgE (rat IgG1, clone R35–72), PE-conjugated anti-mouse CD62L (rat IgG2a, clone MEL-14), APC-conjugated anti-mouse CD3 (hamster IgG1, clone 145-2C11), PE-Cy7-conjugated anti-mouse CD4 (rat IgG2a, clone RM4–5), PerCP-conjugated anti-mouse CD45R/B220 (rat IgG2a, clone RA3-6B2), APC-Cy7-conjugated anti-mouse CD11c (hamster IgG1, clone HL3), PerCP-Cy5.5-conjugated anti-mouse Gr-1 (rat IgG2b, clone RB6-8C5), FITC-conjugated anti-mouse CD23 (Fc ϵ RII; rat IgG2a, clone B3B4), PE-Cy7-conjugated anti-mouse CD117 (c-Kit; rat IgG2b, clone 2B8), APC-conjugated anti-mouse CD49b (hamster IgG1, clone HM α 2), PE-conjugated anti-mouse CD49d (rat IgG2a, clone 9C10), and the BD CBA Mouse Flex Set (cytometric bead array, IL-4, IL-5, IL-6, IL-9, IL-13, IL-17A, IL-17F, INF- γ , monocyte chemoattractant protein [MCP]-1, and TNF α) were purchased from BD Pharmingen (Tokyo, Japan). The Inject Immunogen EDC Kit was obtained from Pierce Biotechnology, Inc. (Rockford, MD, USA). NucleoSpin RNA II, a PrimeScript RT Reagent Kit with gDNA Eraser, and primers were purchased from TaKaRa Bio (Tokyo, Japan). The Quantikine ELISA Mouse CCL11/Eotaxin-

1 kit was purchased from R & D Systems (Minneapolis, MN, USA). Histamine EIA kit was from Bertin Pharma (Paris, France).

2.2. TMA-induced airway inflammation model

The experimental protocol was based on that described by Nishino, Fukuyama, Tajima, Miyashita, Watanabe, Ueda, & Kosaka (2013) with minor modifications and is depicted in Fig. 1. Following a 6-d acclimatization period, healthy mice (now 8 weeks of age) were allocated to each group for dosing (with TMA sensitization and challenge) or were not treated (untreated group) ($n = 6\text{--}8$ mice per group). On days 1–3, 8–10, and 15–17, a 25- μL aliquot of test solution (1% TMA in 4:1 acetone/olive oil) or solvent (4:1 acetone/olive oil) alone was applied to the dorsum of both ears of each mouse for dermal sensitization. Two weeks after the last sensitization (day 31), 0.25 mg of test substance was intratracheally induced using Dry Powder Insufflator™ (Penn-Century, Inc., Wyndmoor, PA, USA) for respiratory challenge. Four hours after the respiratory challenge, the clinical symptoms (abnormal respiratory sound) of each mouse were observed carefully and evaluated by a semi-quantitative score (0 = no signs, 1–4 = severe signs).

2.3. Animal euthanasia and necropsy

One day after the challenge (day 32), all animals were euthanized by exsanguination from the abdominal aorta and posterior vena cava following pentobarbital sodium injection (75 mg/kg, i.p.). The lung was removed from each mouse and stored until histological analysis and cytokine assays. The red coloration of the lung was evaluated based on a semi-quantitative score as a clinical symptom. Blood samples were taken from the inferior vena cava, and serum samples were assayed for IgE levels. Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lungs three times with 1 mL PBS supplemented with 1% FCS. The supernatants from the first BALF fraction were stored and cytokine/chemokine levels were measured. The cell pellets of all three fractions were resuspended, pooled, and used for differential cell counts by fluorescence-activated cell sorter (FACS). Single-cell suspensions were prepared from the hilar lymph node (LN) removed from each mouse by passage through a sterile 70- μm nylon cell strainer in 1 mL RPMI 1640 supplemented with 5% FCS. Single-cell suspensions were used to analyze the memory helper T cell counts, IgE-positive B cell counts, and cytokine production.

2.4. Histology of the lung

Lung tissues were harvested, fixed in 10% formalin, and embedded in paraffin. Sections (5- μm thick) were affixed to slides, stained with hematoxylin–eosin, and imaged using an AZ100 Multizoom microscope (Nikon, Corporation, Tokyo, Japan) using a $40\times$ objective. The hematoxylin–eosin sections were evaluated in a blinded manner with respect to cell influx by semi-quantitative examination (0 = no influx, 1–3 = high influx).

2.5. Gene expression in the lung

Total RNA was extracted and reverse transcribed from accessory lobes by using a NucleoSpin RNA II kit and a PrimeScript RT Reagent Kit, according to the manufacturer's protocol. cDNAs were amplified by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), using a Thermal Cycler Dice system (TaKaRa Bio). The data acquired for each sample were normalized to the expression level of *Actb* (β -actin; a housekeeping gene). PCR primers for *IL4*, *IL5*, *IL9*, *IL13*, *IL17a*, *IFN γ* , and *Actb* are listed in Table 1.

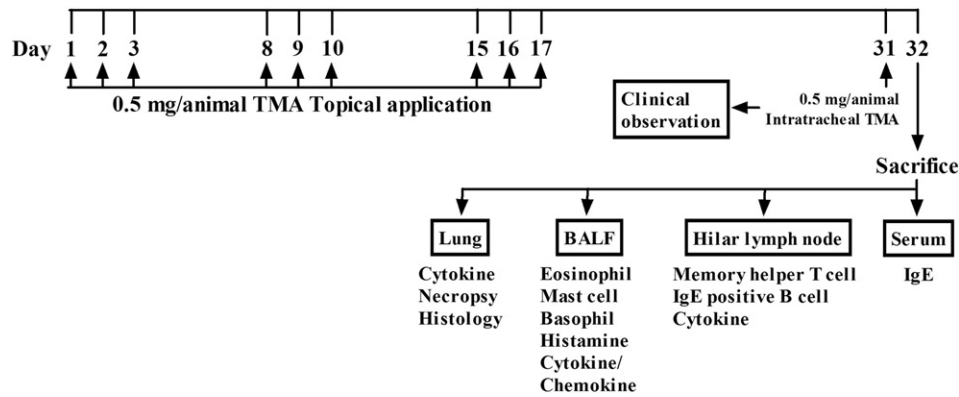


Fig. 1. Experimental protocol used in this study. See the [Materials and methods](#) for a detailed description. TMA, trimellitic anhydride; BALF, bronchoalveolar lavage fluid.

2.6. FACS analysis of BALF and LNs

To avoid nonspecific binding, 1×10^6 cells were first incubated with $1 \mu\text{g}$ Mouse BD Fc Block, followed by incubation with monoclonal antibodies. Cells were washed and analyzed on a FACSVerse flow cytometer (BD Pharmingen) by using the FACSuite program (BD Pharmingen). To analyze antigen expression, 5000 and 20,000 events were collected from BALF and LN samples, respectively. According to our results, total numbers of BALF cells in some untreated mice were less than 10,000 cells and there was no severe difference between result with 5000 events and that with 10,000 events in TMA-treated mice. Therefore, we used fewer 5000 events for BALF analysis.

2.7. Cytokine, chemokine, and histamine levels in BALF

The levels of the cytokines and chemokines (IL-4, IL-5, TNF α , and MCP-1) were measured using a cytometric bead array according to the manufacturer's protocol. The levels of eotaxin-1 and histamine were measured by ELISA, according to the manufacturer's protocol.

2.8. Cytokine determination of LNs

To stimulate T-cell receptor signaling, we cultured single cell suspensions recovered from LNs (5×10^5 cells/well) for 24 h with Dynabeads Mouse T-Activator CD3/CD28 ($12.5 \mu\text{g}$ /well) antibodies in 48-well plates at 37°C in an atmosphere containing 5% CO_2 . The levels of IL-4, IL-5, IL-6, IL-9, IL-13, IL-17A, IL-17F, and IFN γ in supernatants (cell culture medium) were measured using a cytometric bead array.

2.9. IgE measurements in serum

Total IgE levels in serum were measured by ELISA according to the manufacturer's protocol. TMA-specific IgE levels in serum were measured by an Imject Immunogen EDC Kit and ELISAs, as described previously (Fukuyama, Tajima, Ueda, Hayashi, Shutoh, Harada, & Kosaka 2009).

2.10. Statistical analysis

For the statistical evaluation of TMA-treated BALB/c mice vs TMA-treated NC/Nga mice, F test was first applied for each dataset. When group variances were homogeneous, Student's *t*-test was performed to detect any statistically significant difference between two groups. When group variances were heterogeneous, Aspin-Welch's test was used. The data were expressed as mean $\pm$ 1 S.D. and analyzed using Prism 4 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Abnormal clinical signs, lung appearance, and histological evaluations observed in the lung tissue of TMA-induced NC/Nga mice

Animals were carefully observed for their appearance, posture, behavior, and respiration, at 4 h after TMA challenge. Whereas there were no abnormal clinical signs in TMA-treated BALB/c mice, abnormal respiratory sounds were obviously noted in TMA-treated NC/Nga mice (Fig. 2 A). To examine whether TMA-challenge affected lung inflammation in the two mouse strains, we next monitored the lung appearance and histological features of airway inflammation in lung. Although

Table 1
Primer sets used in this study.

Gene symbol	Gene bank accession no.	Primers	Product (bp)
<i>IL4</i>	NM_021283.2	F: 5'-TCTCGAATGTACCAGGAGCCATATC R: 5'-AGCACCTTGGAAAGCCCTACAGA	183
<i>IL5</i>	NM_010558.1	F: 5'-TCAGCTGTGTCTGGGCCACT R: 5'-TTATGAGTAGGGACAGGAAGCCTCA	133
<i>IL9</i>	NM_008373.1	F: 5'-TGACCAGCTGCTTGTGTCTC R: 5'-TGTGGCATTGGTCAGCTGTAA	536
<i>IL13</i>	NM_008355.3	F: 5'-CAATTGCAATGCCATCTACAGGAC-3' R: 5'-CGAAACAGTTGCTTTGTAGCTGA-3'	150
<i>IL17a</i>	NM_010552.3	F: 5'-ACGCGCAAACATGAGTCCAG-3' R: 5'-AGGCTCAGCAGCAGCAACAG-3'	66
<i>Ifng</i>	NM_008337.3	F: 5'-CGGCACAGTCATTGAAAGCCTA-3' R: 5'-GTTGCTGATGGCCTGATTGTC-3'	199
<i>Actb</i>	NM_007393.3	F: 5'-CATCCGTAAAGACCTCTATGCCAAC-3' R: 5'-ATGGAGCCACCGATCCACA-3'	171

F: forward; R: reverse.

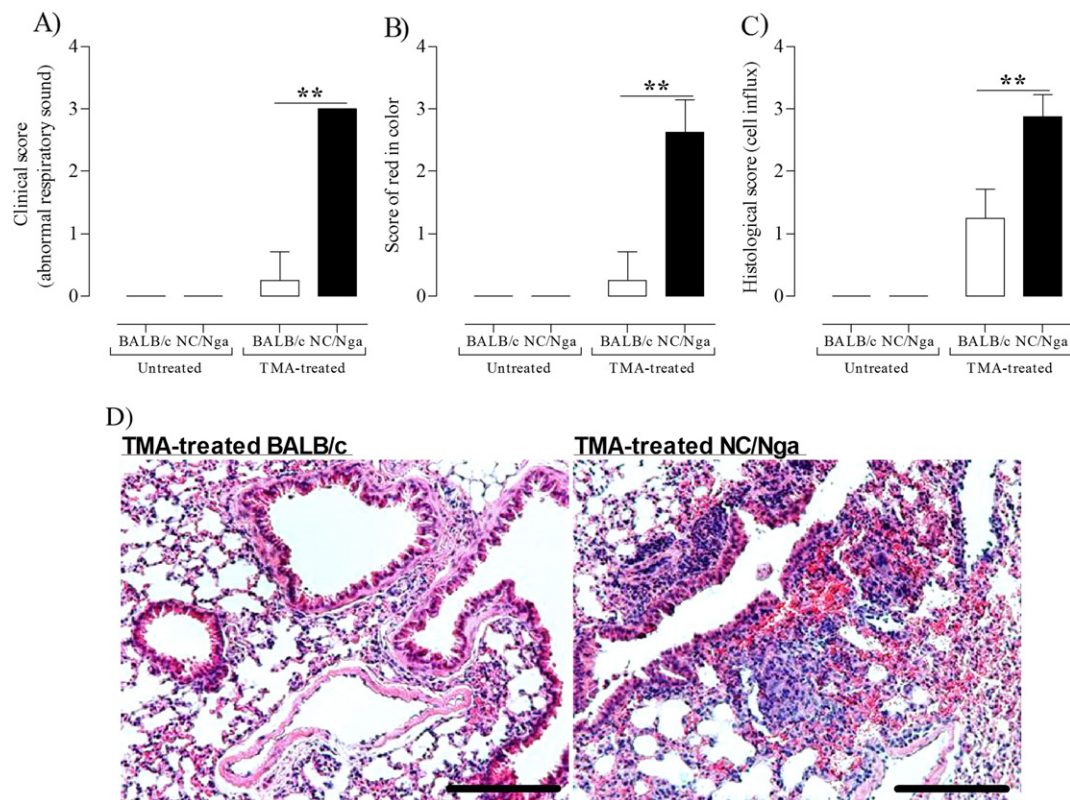


Fig. 2. Effects of respiratory TMA induction on abnormal clinical signs, lung appearance, and lung tissue. (A) Score of clinical observations. The grade was determined by semi-quantitative examination (0 = no change, 1–2 = severe changes). (B) Score of red coloration in the lungs. The grade was determined by semi-quantitative examination (0 = no change, 1–3 = severe changes). (C) Histological scores of hematoxylin–eosin-stained lung sections. Data were evaluated in a blinded manner for measurement of cell influx by semi-quantitative examination (0 = no influx; 1–3 cell layers = high influx). (D) Representative histological features of hematoxylin–eosin-stained sections in each group with $\times 40$ objective. All data represent the mean $\pm$ SD ($n = 6$ –8 per group). Values significantly different from those of another group are indicated by asterisks (** $P < 0.01$).

only slight features of lung abnormalities were noted in TMA-treated BALB/c mice, moderate or severe features of lung abnormalities were observed in TMA-treated NC/Nga mice (Fig. 2 B and C). In addition, hyperplasia of the alveolar wall, alveolar edema, and infiltration of inflammatory cells in the lung were significantly induced by TMA challenge in NC/Nga mice (Fig. 2 D).

3.2. Comparison of cytokine profiles in lung tissue between BALB/c and NC/Nga mice in the TMA-treated respiratory allergy model

To examine how TMA challenge affected the Th cell response in the two mouse strains, we first monitored the cytokine gene expression profiles in the lung tissue. When we looked at the Th2 related cytokine secretions in the lung, we found that only *IL4* gene expression was significantly increased ($P < 0.05$) in BALB/c mice compared with that in NC/Nga mice (Fig. 3 A–C). Contrary to *IL4* gene expression, Th1, Th9, and Th17-related cytokine gene expressions were increased in NC/Nga mice compared with those in the BALB/c group (Fig. 3 D–F). Particularly, *IL9* and *IL17a* gene expressions, which are involved in Th9 and Th17 immunoreactions, were significantly increased in NC/Nga mice compared with those in BALB/c mice (all $P < 0.05$).

3.3. Effects of intratracheal TMA induction on the differential cell counts, histamine levels, eotaxin-1 levels, and cytokine productions in BALF

To confirm the allergic reactions in the respiratory system, we measured the ratio of immunocytes (Table 2) as well as levels of histamine, eotaxin-1, and related cytokines in the BALF (Fig. 4). Whereas there were no significant differences in the percentage of lymphocyte and eosinophil rates between TMA-treated NC/Nga mice and

TMA-treated BALB/c mice, the percentage of neutrophils, mast cells, and basophils in NC/Nga mice were significantly enhanced by TMA as compared with those of TMA-treated BALB/c mice (all $P < 0.01$, Table 2).

When we measured the secretion of related cytokines, histamine, and eotaxin-1 in the BALF, the levels of histamine, eotaxin-1, IL-5, TNF α , and MCP-1 were significantly increased in TMA-treated NC/Nga mice compared with those in TMA-treated BALB/c mice (Fig. 4 A, B, D–F). On the other hand, the level of IL-4 was significantly increased in TMA-treated BALB/c mice compared with that in TMA-treated NC/Nga mice (Fig. 4 C).

3.4. Prominent enhancement of the IgE-positive B cells in the hilar LN in TMA-induced BALB/c mice

To confirm the allergic reactions in local LN, we first measured the cell counts of memory helper T cells and IgE-positive B cells. We could find significant induction of IgE-positive B cells in TMA-treated BALB/c mice compared with that of TMA-treated NC/Nga mice ($P < 0.01$, Fig. 5 B). The counts of helper T cell were almost comparable in the TMA-treated NC/Nga mice and TMA-treated BALB/c mice (Fig. 5 A).

3.5. Comparison of cytokine profiles in the hilar LN between BALB/c and NC/Nga mice in the TMA-treated respiratory allergy model

To examine how TMA challenge affected the helper T cell response in the two mouse strains, we next monitored the cytokine production profiles in the hilar LN (Fig. 6). Contrary to the IL-4 secretions in lung tissues, they were almost comparable between TMA-treated NC/Nga mice and TMA-treated BALB/c mice (Fig. 6 B). On the other hand, all Th1, Th9, and Th17-related cytokine gene expressions were increased in NC/Nga

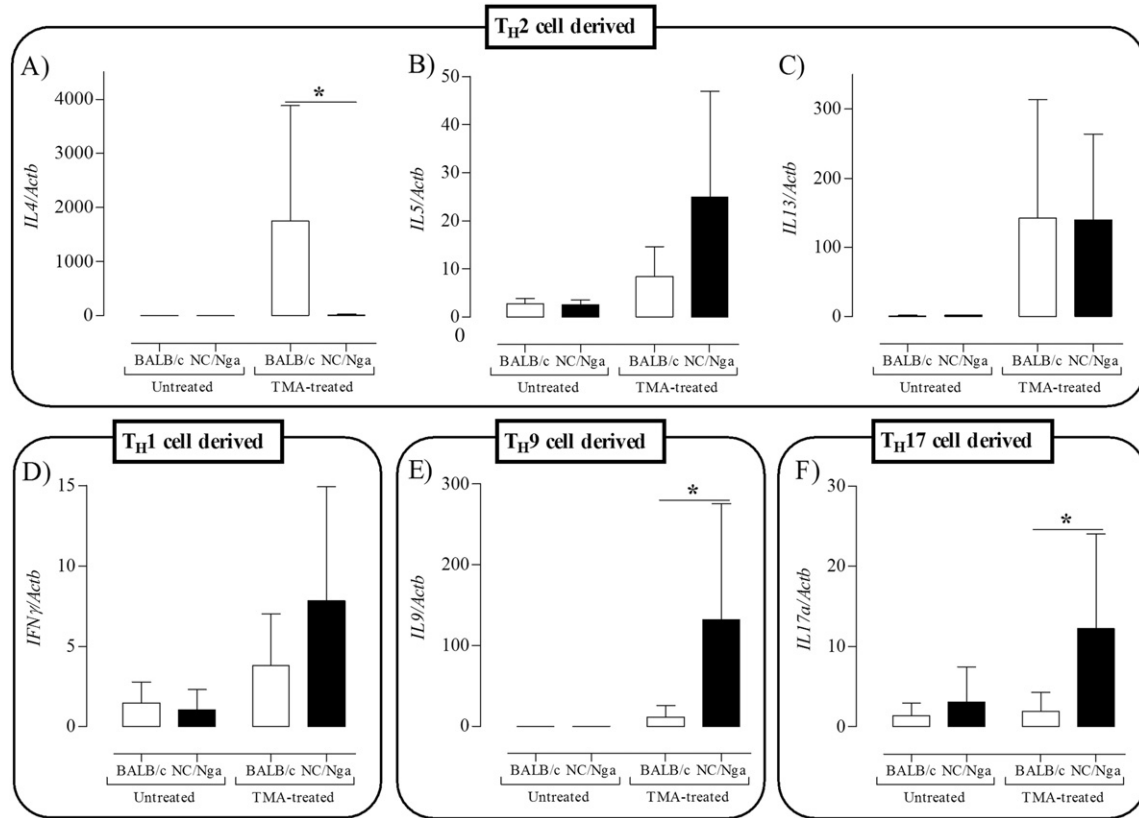


Fig. 3. Effect of cytokine gene expression profiles in lung tissues due to TMA exposure. Gene expressions of (A) *IL4*, (B) *IL5*, (C) *IL13*, (D) *IFNγ*, (E) *IL9*, and (F) *IL17a* were determined by qRT-PCR. All data represent the mean $\pm$ SD (n = 6–8 per group). All gene expression values are represented as the increase in expression compared with the value for the expression of *Actb*. Values significantly different from those of another are indicated by asterisks (*P < 0.05).

mice compared with those in the BALB/c group (all P < 0.01, Fig. 6 A, E–H). Corresponding to BALF analysis, the level of IL-5 was significantly increased in NC/Nga mice compared with that in BALB/c mice (P < 0.01, Fig. 6 C).

3.6. Prominent enhancement of the serum IgE levels in TMA-induced BALB/c mice

To confirm the enhancement of Th2 allergic reactions in lung and local LN, we examined the total and TMA-specific IgE levels in serum by ELISA (Fig. 7). In consequent, the TMA-treated BALB/c mice had significantly higher levels of both total and TMA-specific IgE when compared with NC/Nga mice treated with TMA (Fig. 7 A and B).

4. Discussion

The initial purpose of this project was to improve our original protocol for detecting the ambient chemical exposure-induced respiratory allergy by using a typical respiratory allergen TMA. To select the most sensitive mice strain for our protocol, we compared the allergic

potency induced by TMA in BALB/c and NC/Nga mice, which have been reported as highly sensitive strains to chemical respiratory allergens. In the process of this study, we incidentally noted abnormal clinical signs, redness of the lung, and inflammatory features in lung tissues of TMA-induced NC/Nga mice, whereas there were almost no signs in TMA-induced BALB/c mice. Therefore, the main objective of this manuscript reported here is to demonstrate the mechanisms of aggravated clinical symptoms and inflammatory features in NC/Nga mice focusing on the cytokine profile derived from Th cells.

BALB/c mice are known for their high response to Th2-mediated immunoreactions including IgE levels and are widely used to develop models of respiratory allergy (Lin, Lee, Jin, Yook, Quan, Ha, Moon, Kim, Kim, Lee, & Chang 2006). We previously examined the respiratory allergic responses in BALB/c mice by several Th2 type chemical respiratory allergens such as TMA, toluene diisocyanate, and phthalic anhydride (Fukuyama, Ueda, Hayashi, Tajima, Shuto, Saito, Harada, & Kosaka 2008; Fukuyama, Tajima, Ueda, Hayashi, Shutoh, Harada, & Kosaka 2010). Then, we successfully observed the significant increase in antigen-specific serum IgE levels, Th2 cytokine secretions, and proliferation of related immune cells in BALB/c mice. Thus, we thought that BALB/c mice are the best option to detect the potential of ambient chemical allergens so far. In fact, when we examined the Th2-mediated reactions such as IgE, IL-4, and IL-13 levels in this study, statistically significant changes were noted in TMA-treated BALB/c mice as mentioned in our previous reports.

On the other hand, NC/Nga mice were developed as animal models for atopic dermatitis, characterized by intense scratching behavior and severe inflammation of the skin (Hashimoto, Arai, Takano, Tanaka, & Nakaike 2006). Along with the model for atopic dermatitis, Iwasaki et al. (2001a,b) previously attempted to develop NC/Nga mice as models of asthma with intranasal ovalbumin sensitization and it resulted in the increase of eosinophil counts compared with BALB/c mice subjected to

Table 2
Impact of TMA exposure on the ratio of differential cell counts in BALF.

	Untreated (%)		TMA-treated (%)	
	BALB/c	NC/Nga	BALB/c	NC/Nga
Lymphocytes	12.8 $\pm$ 7.0	30.0 $\pm$ 21.6	44.7 $\pm$ 12.1	38.3 $\pm$ 9.1
Eosinophils	0.8 $\pm$ 0.6	1.5 $\pm$ 2.1	14.3 $\pm$ 12.7	20.5 $\pm$ 12.3
Neutrophils	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	1.1 $\pm$ 1.3	13.8 $\pm$ 7.3 **
Mast cells	0.09 $\pm$ 0.05	2.54 $\pm$ 1.61	0.60 $\pm$ 0.33	2.73 $\pm$ 1.46 **
Basophils	0.57 $\pm$ 0.34	0.84 $\pm$ 0.23	0.15 $\pm$ 0.07	1.37 $\pm$ 0.86 **

All data are expressed as mean $\pm$ SD (%; n = 6–8 per group). Significantly different values are indicated by asterisks (**P < 0.01).

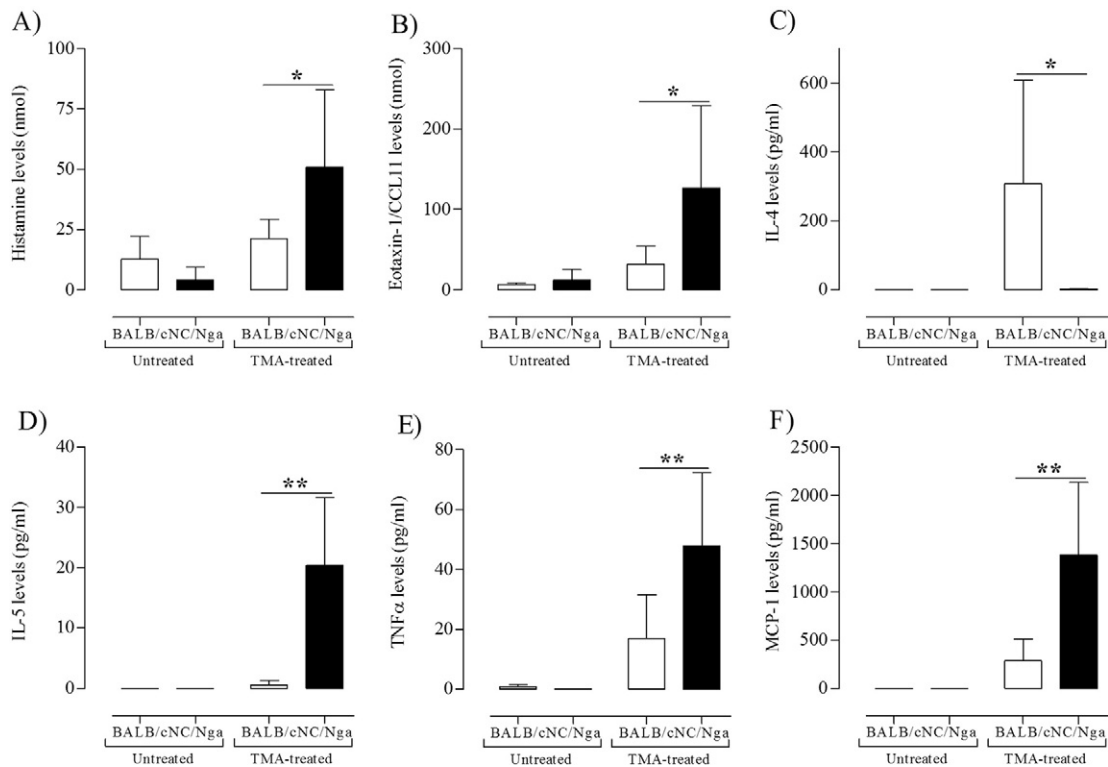


Fig. 4. Impact of TMA on histamine levels, eotaxin-1 levels and cytokine productions in BALF. The levels of (A) histamine and (B) eotaxin-1 were determined by ELISA. All data are expressed as mean $\pm$ SD (nmol; $n = 8$ per group). The levels of (C) IL-4, (D) IL-5, (E) TNF, and (F) MCP-1 were determined by cytometric bead array. All data are expressed as mean $\pm$ SD (pg/mL; $n = 6-8$ per group). Values significantly different from those of another are indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$).

the same treatment. Because eosinophil is one of the key triggers that induce respiratory allergic symptoms, we hypothesized that NC/Nga mice can develop more severe clinical symptoms induced by respiratory chemical allergens than BALB/c mice. Therefore, we first compared the sensitivity of BALB/c mice and NC/Nga mice by focusing on the clinical signs in this study.

According to our results, the clinical symptoms of NC/Nga mice were significantly more severe than those of BALB/c mice, and marked increases in the inflammatory reactions were also noted in TMA-treated NC/Nga mice as compared with BALB/c mice. It has already been reported by Hashimoto, Arai, Takano, Tanaka, & Nakaike (2006) that atopic dermatitis model in NC/Nga mice exhibited intense scratching behavior and severe inflammation of the skin compared with the responses in BALB/c, ICR, and C3H/HeN mice. However, there were no reports focusing on the asthmatic clinical signs and inflammatory features in

NC/Nga mice providing side-by-side comparison with other mice strains; our results are the first to be reported on this aspect.

We then tried to determine the key factor that makes a marked difference in clinical symptoms and inflammatory response between NC/Nga mice and BALB/c mice. When we looked at the cytokine secretions in the lung and hilar LN, significant increase in IL-9, IL-17A, and IL-17F levels was observed in TMA-treated NC/Nga mice compared with TMA-treated BALB/c mice, whereas Th2 immunoreactions such as IgE level and IL-4 production were significantly less than that in BALB/c mice. Therefore, we hypothesized that Th9 and Th17-related immunoreactions play a key role to aggravate the clinical signs or inflammatory symptoms due to ambient chemical exposure-induced respiratory allergy in NC/Nga mice. IL-9 is mainly released from Th9 cells, recently recognized as a new subset of helper T cells that generate IL-9 and IL-10, which were previously supposed to be Th2 type

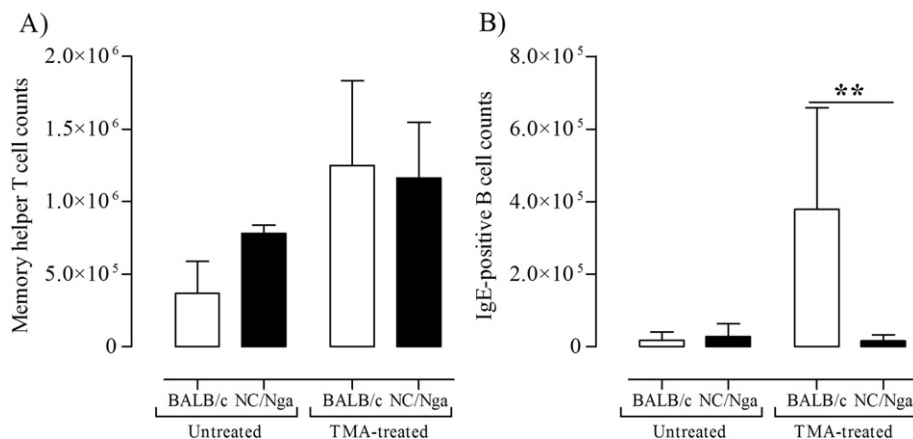


Fig. 5. Impact of intratracheal TMA induction on hilar lymph node activation. Cell counts of (A) memory helper T cell and (B) IgE-positive B cell were determined by FACS analysis. All data are expressed as mean $\pm$ SD ($n = 6-8$ per group). Values significantly different from those of another are indicated by asterisks (* $P < 0.05$).

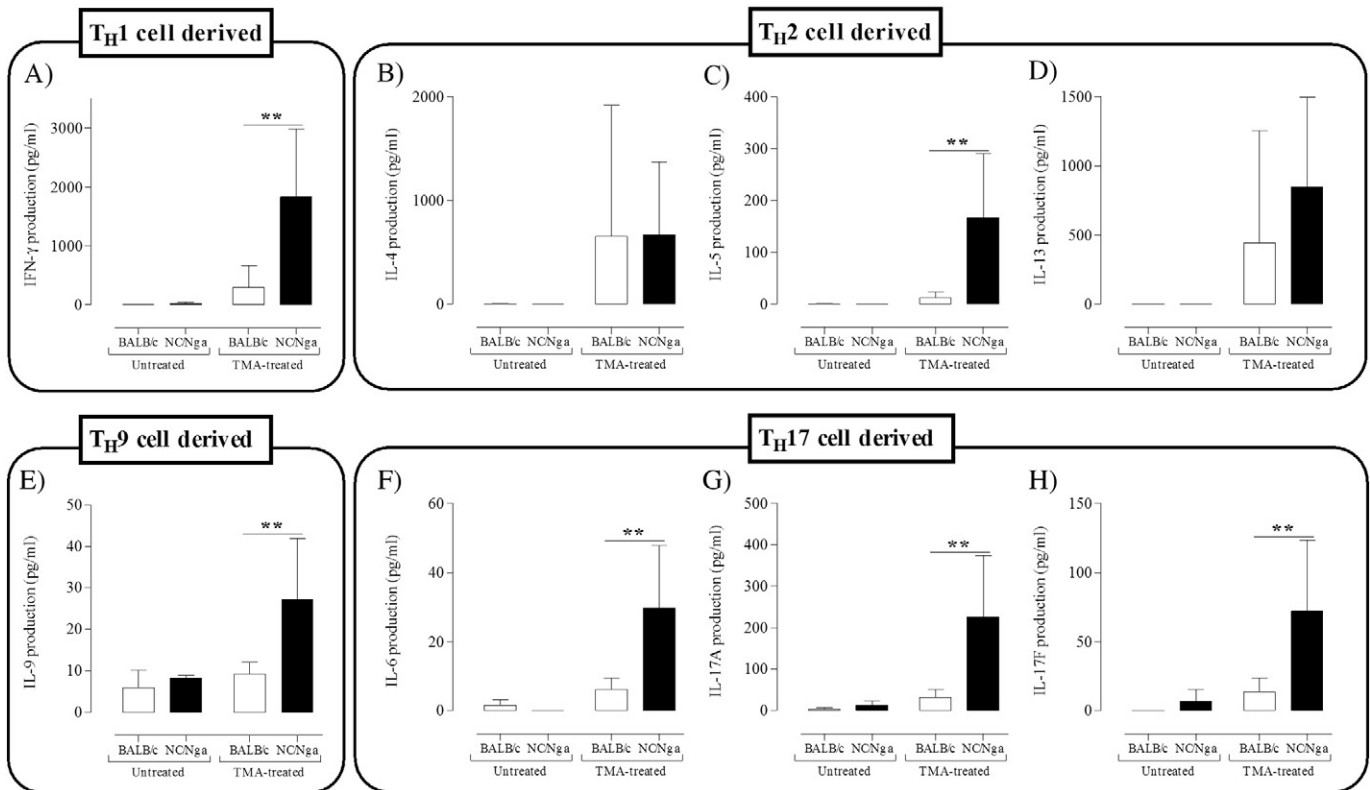


Fig. 6. Effect of cytokine production profiles in hilar lymph node due to TMA exposure. Production of (A) IFN- γ , (B) IL-4, (C) IL-5, (D) IL-13, (E) IL-9, (F) IL-6, (G) IL-17A, and (H) IL-17F was determined by cytometric bead array. All data represent the mean $\pm$ SD (pg/mL; n = 6–8 per group). Values significantly different from those of another are indicated by asterisks (**P < 0.01).

cytokines (Veldhoen, Uyttenhove, van Snick, Helmby, Westendorf, Buer, Martin, Wilhelm, & Stockinger 2008). Th9 cells differentiate from naive T cells as a result of stimulation by IL-4 and transforming growth factor β , and express the type B IL-17 receptor. IL-9 is enhanced during asthmatic inflammation, which induces mucus production by epithelial cells, and promotes the proliferation and differentiation of mast cells (Louahed, Toda, Jen, Hamid, Renaud, Levitt, & Nicolaides 2000 Matsuzawa, Sakashita, Kinoshita, Ito, Yamashita, & Koike 2003). Furthermore, IL-9 released from Th9 cells in models of allergic inflammation, play an important role in mast cell accumulation and activation (Sehra, Yao, Nguyen, Glosson-Byers, Akhtar, Zhou, & Kaplan 2015). According to our results, mast cells and their mediator histamine as well

as IL-5 levels in BALF were significantly induced by TMA in NC/Nga mice as compared with BALB/c mice. Mast cells are central to the pathogenesis of allergic diseases and release lipid mediators; cytokines, chemokines, and histamine. Mast cells rapidly synthesize and release lipid mediators via sneezing, coughing, bronchospasm, edema, and mucus secretion in the respiratory tract as a result of activation of mast cells by Fc ϵ RI due to polyvalent allergens (Stone, Prussin, & Metcalfe 2010). Therefore, it could be considered that the severe clinical symptoms observed in NC/Nga mice were derived from IL-9 to mast cell pathway.

Th17 cells, a new helper T-cell subset, which secrete IL-17A, IL-17F, IL-22, IL-23, and TNF α , also play an important role in the development

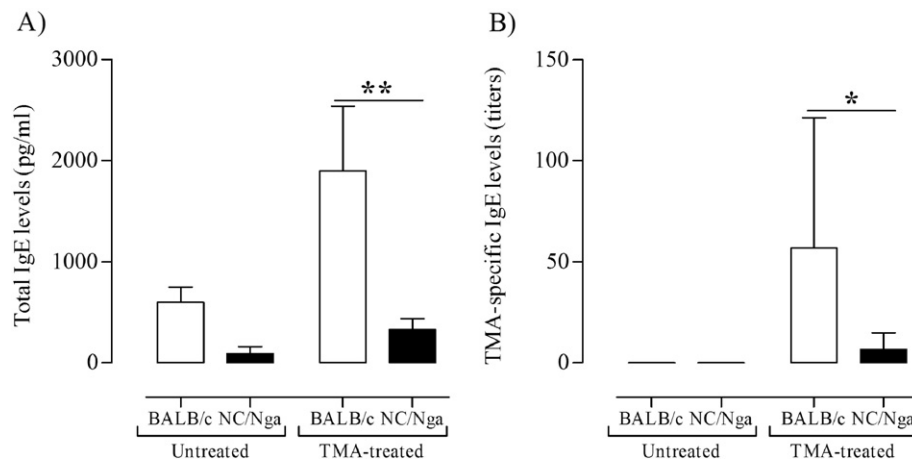


Fig. 7. Prominent enhancement of IgE levels in serum was observed in TMA-induced BALB/c mice compared with that in NC/Nga mice. (A) Total serum IgE levels (pg/mL) and (B) TMA-specific IgE titers in the serum were determined by ELISA. Data represent the mean $\pm$ SD (n = 8 per group). Values significantly different from that of the intact group are indicated by asterisks (*P < 0.05 and **P < 0.01).

of allergic airway inflammation (McGeachy & Cua 2009). In addition, the IL-17 family cytokines have been shown to induce neutrophilic airway inflammation (Hellings, Kasran, Liu, Vandekerckhove, Wuyts, Overbergh, Mathieu, & Ceuppens 2003; Oda, Canelos, Essayan, Plunkett, Myers, & Huang 2005). In this study, IL-17A and IL-17F levels in hilar LN were significantly increased in TMA induced NC/Nga mice compared with BALB/c mice, corresponding to marked infiltrations of neutrophils in the lungs of TMA induced NC/Nga mice. It has been reported that patients with symptomatic asthma have elevated levels of peripheral neutrophils that show signs of being activated. Both the numbers and activation levels of these neutrophils are lower in the absence of symptoms or after treatment and resolution of the allergic process (Monteseirin 2009, Wakushin, Hirose, Maezawa, Kagami, Suto, Watanabe, Saito, Hatano, Tokuhisa, Iwakura, Puccetti, & Iwamoto 2008). Therefore, IL-17A and IL-17F evoked neutrophil infiltration may be responsible for severe inflammatory response in lungs of NC/Nga mice.

Until now, Th2 type responses have been thought to play key roles in ambient chemical exposure-induced respiratory allergic diseases, as shown in our BALB/c mice model (Fukuyama, Ueda, Hayashi, Tajima, Shuto, Saito, Harada, & Kosaka 2008). However, we demonstrated in this study that Th9 and Th17 type cytokines were more responsible for aggravation of clinical symptoms and inflammatory response than were Th2 type immunoreactions, particularly in the acute phase of ambient chemical exposure-induced respiratory allergy. When developing a new detection method for ambient chemical exposure-induced respiratory allergy, time- and cost-effectiveness should always be considered. Therefore, we need to judge the short-term allergenicity of chemicals, which is classified as the acute phase. In this regard, we need to keep in mind that a wide variety of cytokine profiles including IL-9 and IL-17 should be taken into account based on our results reported in this study.

5. Conclusion

In this study, we incidentally discovered that allergic clinical symptoms and lung inflammatory responses to ambient chemical exposure-induced allergy in NC/Nga mice were significantly more severe than those in TMA-induced BALB/c mice. We then tried to examine the key trigger for these phenomena and found that cytokine secretions produced by Th9 and Th17 were significantly induced in the acute phase of ambient chemical exposure-induced respiratory allergy in NC/Nga mice. Taken together, we need to consider that a wide variety of cytokine profiles including IL-9 and IL-17 should be taken into account when developing a new detection method for ambient chemical exposure-induced respiratory allergy.

Conflict of interest statement

There are no conflicts of interest to report.

Acknowledgements

This work was supported by a research Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan (H-26-shokuhin-ippan-019). The authors wish to thank Dr. Y. Takizawa, Dr. H. Ueda, and Dr. Y. Shutoh at the Institute of Environmental Toxicology (Ibaraki, Japan) for their valuable suggestions and discussions.

References

Becker, S., Soukup, J. M., Gilmour, M. I., & Devlin, R. B. (1996). Stimulation of human and rat alveolar macrophages by urban air particulates: Effects on oxidant radical generation and cytokine production. *Toxicology and Applied Pharmacology*, 141, 637–648.

Carter, J. D., Ghio, A. J., Samet, J. M., & Devlin, R. B. (1997). Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicology and Applied Pharmacology*, 146, 180–188.

Dearman, R. J., Betts, C. J., Humphreys, N., Flanagan, B. F., Gilmour, N. J., Basketter, D. A., & Kimber, I. (2003a). Chemical allergy: Considerations for the practical application of cytokine profiling. *Toxicological Sciences*, 71, 137–145.

Dearman, R. J., Skinner, R. A., Humphreys, N. E., & Kimber, I. (2003b). Methods for the identification of chemical respiratory allergens in rodents: comparisons of cytokine profiling with induced changes in serum IgE. *Journal of Applied Toxicology*, 23, 199–207.

Dearman, R. J., Basketter, D. A., & Kimber, I. (1992). Variable effects of chemical allergens on serum IgE concentration in mice. Preliminary evaluation of a novel approach to the identification of respiratory sensitizers. *Journal of Applied Toxicology*, 12, 317–323.

Fukuyama, T., Tajima, Y., Ueda, H., Hayashi, K., Shutoh, Y., Harada, T., & Kosaka, T. (2009). Allergic reaction induced by dermal and/or respiratory exposure to low-dose phenoxyacetic acid, organophosphorus, and carbamate pesticides. *Toxicology*, 261, 152–161.

Fukuyama, T., Tajima, Y., Ueda, H., Hayashi, K., Shutoh, Y., Harada, T., & Kosaka, T. (2010). A method for measuring mouse respiratory allergic reaction to low-dose chemical exposure to allergens: An environmental chemical of uncertain allergenicity, a typical contact allergen and a non-sensitizing irritant. *Toxicology Letters*, 195, 35–43.

Fukuyama, T., Ueda, H., Hayashi, K., Tajima, Y., Shuto, Y., Saito, T. R., ... Kosaka, T. (2008). Use of long-term dermal sensitization followed by intratracheal challenge method to identify low-dose chemical-induced respiratory allergic responses in mice. *Toxicology Letters*, 181, 163–170.

Hashimoto, Y., Arai, I., Takano, N., Tanaka, M., & Nakaike, S. (2006). Induction of scratching behaviour and dermatitis in various strains of mice cohabiting with NC/Nga mice with chronic dermatitis. *The British Journal of Dermatology*, 154, 28–33.

Hellings, W. P., Kasran, A., Liu, Z., Vandekerckhove, P., Wuyts, A., Overbergh, L., ... Ceuppens, L. J. (2003). Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *American Journal of Respiratory Cell and Molecular Biology*, 28, 42–50.

Iwasaki, T., Tanaka, A., Itakura, A., Yamashita, N., Ohta, K., Matsuda, H., & Onuma, M. (2001a). Atopic NC/Nga mice as a model for allergic asthma: severe allergic responses by single intranasal challenge with protein antigen. *The Journal of Veterinary Medical Science*, 63, 413–419.

Iwasaki, T., Tanaka, A., Matsuda, H., & Onuma, M. (2001b). Atopic NC/Nga mice as a model for allergic asthma: Cytokine profiles and eosinophil productivity of bone marrow. *The Journal of Veterinary Medical Science*, 63, 471–474.

Li, X. Y., Gilmour, P. S., Donaldson, K., & MacNee, W. (1997). In vivo and in vitro proinflammatory effects of particulate air pollution (PM10). *Environmental Health Perspectives*, 105, 1279–1283.

Lin, C. X., Lee, E., Jin, M. H., Yook, J., Quan, Z., Ha, K., ... Chang, H. W. (2006). Deoxyphodophyllotoxin (DPT) inhibits eosinophil recruitment into the airway and Th2 cytokine expression in an OVA-induced lung inflammation. *Planta Medica*, 72, 786–791.

Loftus, C., Yost, M., Sampson, P., Arias, G., Torres, E., Vasquez, V. B., ... Karr, C. (2015). Regional PM2.5 and asthma morbidity in an agricultural community: A panel study. *Environmental Research*, 136, 505–512.

Louahed, J., Toda, M., Jen, J., Hamid, Q., Renaud, J. C., Levitt, R. C., & Nicolaides, C. N. (2000). Interleukin-9 upregulates mucus expression in the airways. *American Journal of Respiratory Cell and Molecular Biology*, 22, 649–656.

Matsuda, H., Watanabe, N., Geba, G. P., Sperl, J., Tsudzuki, M., Hiroi, J., ... Ra, C. (1997). Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *International Immunology*, 9, 461–466.

Matsuzawa, S., Sakashita, K., Kinoshita, T., Ito, S., Yamashita, T., & Koike, K. (2003). IL-9 enhances the growth of human mast cell progenitors under stimulation with stem cell factor. *Journal of Immunology*, 170, 3461–3467.

McCormack, M. C., Belli, A. J., Kaji, D. A., Matsui, E. C., Brigham, E. P., Peng, R. D., ... Hansel, N. N. (2015). Obesity as a susceptibility factor to indoor particulate matter health effects in COPD. *The European Respiratory Journal*, 45, 1248–1257.

McGeachy, J. M., & Cua, J. D. (2009). Th17 cell differentiation: The long and winding road. *Immunity*, 28, 445–453.

Monteseirin, J. (2009). Neutrophils and asthma. *Journal of Investigational Allergy & Clinical Immunology*, 19, 340–354.

Nishino, R., Fukuyama, T., Tajima, Y., Miyashita, L., Watanabe, Y., Ueda, H., & Kosaka, T. (2013). Prior oral exposure to environmental immunosuppressive chemicals methoxychlor, parathion, or piperonyl butoxide aggravates allergic airway inflammation in NC/Nga mice. *Toxicology*, 309, 1–8.

Nishino, R., Fukuyama, T., Watanabe, Y., Kurosawa, Y., Ueda, H., & Kosaka, T. (2014). Effect of mouse strain in a model of chemical-induced respiratory allergy. *Experimental Animals*, 63, 435–445.

Oda, N., Canelos, B. P., Essayan, M. D., Plunkett, A. B., Myers, C. A., & Huang, S. (2005). Interleukin-17F induces pulmonary neutrophilia and amplifies antigen-induced allergic response. *American Journal of Respiratory and Critical Care Medicine*, 171, 12–18.

Sehra, S., Yao, W., Nguyen, E. T., Glosson-Byers, N. L., Akhtar, N., Zhou, B., & Kaplan, M. H. (2015). TH9 cells are required for tissue mast cell accumulation during allergic inflammation. *The Journal of Allergy and Clinical Immunology*, 136(433–440), e431.

Stone, K. D., Prussin, C., & Metcalfe, D. D. (2010). IgE, mast cells, basophils, and eosinophils. *The Journal of Allergy and Clinical Immunology*, 125, S73–S80.

Veldhoen, M., Uytendhoe, C., van Snick, J., Helmbly, H., Westendorp, A., Buer, J., ... Stockinger, B. (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature Immunology*, 9, 1341–1346.

Wakushin, H., Hirose, K., Maezawa, Y., Kagami, S., Suto, A., Watanabe, N., ... Iwamoto, I. (2008). IL-23 and IL-17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *American Journal of Respiratory and Critical Care Medicine*, 178, 1023–1032.