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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

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ABSTRACT

Our aim was to improve a method for detecting respiratory hypersensitivity by testing three confirmed respiratory allergens (trimellitic anhydride [TMA], phthalic anhydride [PA] and toluene diisocyanate [TDI]), an environmental chemical of uncertain allergenicity (2,4-D-butyl [DB]), a confirmed contact allergen (2,4-dinitrochlorobenzene [DNCB]) and a standard irritant (sodium dodecyl sulfate [SDS]). BALB/c mice were topically sensitized (nine times in 3 weeks) with these chemicals, then challenged via the trachea. One day post-challenge, samples were taken from the mice to assay for total immunoglobulin (IgE and IgG₁) levels in serum and bronchoalveolar lavage fluid (BALF); differential cell counts and cytokine/chemokine levels in BALF; lymphocyte counts and surface antigen expression on B-cells within lung-associated lymph nodes (LNs); ex situ cytokine production by cells from these LNs; and gene expression in BALF (CCR3) and LNs (CCR4, STAT6 and GATA-3). The three confirmed respiratory allergens and DB induced immune response characteristic of immediate-type respiratory reactions, as evidenced by increased total IgE and IgG₁ levels; influx of eosinophils, neutrophils, chemokines and cytokines in BALF; increased surface antigen expression on B-cells in LNs; increased Th2 cytokine production in LNs; and increased respiratory allergy-related gene expression in both BALF and LNs. In contrast, DNCB and SDS treatments yielded, at most, insignificant increases in all respiratory allergic parameters. Thus, the protocol was equally suitable for use with an environmental chemical of unknown allergenicity and for typical respiratory allergens. Since the protocol differentiated respiratory allergens from contact allergens and irritants, it may be useful for detecting respiratory allergy related to environmental chemicals.

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1. Introduction

Some pesticides, perfumes and other low-molecular-weight chemicals can cause allergic respiratory diseases (Brown and Jason, 2007). Development of these diseases requires sensitization, which is thought to be triggered by dermal or respiratory exposure to the

sensitizing chemical, binding of the chemical to self-proteins, and subsequent presentation to chemical-specific lymphocytes. In susceptible individuals, sensitization may result in the production of specific immunoglobulin E (IgE) antibodies; airway inflammation, characterized by activated CD4⁺ T cells, eosinophils and mast cells; and increased levels of the interleukins (ILs) IL-4 and IL-5 (Ban et al., 2006).

The implementation of the European Union (EU) regulatory framework for registration, evaluation and authorization of chemicals (Boverhof et al., 2008) and the drafting and impending implementation of various EU directives have highlighted the need to develop assays that can identify respiratory allergens and differentiate them from contact allergens and irritants (Boverhof et al., 2008; European Commission, 2006a,b). Also, within the Globally Harmonized System for the Classification and Labeling of Chemicals, classification as a respiratory allergen relies heavily on human evidence and on animal studies (e.g. the IgE test in mice and pulmonary responses in guinea pigs), despite a lack of consensus and

Abbreviations: ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; DB, 2,4-D-butyl; DNCB, 2,4-dinitrochlorobenzene; EU, European Union; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IL, interleukin; Ig, immunoglobulin; LLNA, local lymph node assay; LN, lymph node; mAb, monoclonal antibody; OD, optical density; PA, phthalic anhydride; PBS, phosphate-buffered saline; PE, phycoerythrin; SDS, sodium dodecyl sulfate; STAT, signal transduction and activator of transcription; TDI, toluene diisocyanate; TMA, trimellitic anhydride.

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guidance for these assays (Boverhof et al., 2008; United Nations Economic Commission for Europe, 2007).

Several animal models have been used to identify chemicals as respiratory allergens (Arts et al., 2003; Ban et al., 2006). However, such methods have focused on the detection of strong allergic reactions, whereas environmental chemical allergens tend to have weak immunogenicity. Therefore, new protocols are needed for the detection of environmental chemicals that may act as respiratory allergens.

We have previously developed a method for detecting respiratory allergic diseases related to environmental chemicals (Fukuyama et al., 2008a,b, 2009b). The method involved analysis of serum, bronchoalveolar lavage fluid (BALF) and lung-associated lymph nodes (LNs), and could be used to detect and classify allergic reactions caused by weakly immunogenic chemicals at low doses. However, it was not clear whether the method could be applied to environmental chemicals, or whether it could differentiate between an allergic and an irritant response – such differentiation is important in identifying the immunological hazard posed by a chemical (Homey et al., 1998; Ku et al., 2009; Vohr et al., 2000). In addition, although our method has been used to identify respiratory allergens through dermal and respiratory exposure, there have been concerns that certain non-sensitizing irritants may yield false positive results through non-specific inflammatory processes.

In this paper, we attempt to overcome these limitations by using our original method to examine respiratory allergic reaction profiles following exposure to several types of chemicals: confirmed chemical respiratory allergens (phthalic anhydride [PA], toluene diisocyanate [TDI] and trimellitic anhydride [TMA]); an environmental chemical of uncertain allergenicity (2,4-D-butyl [DB]); a confirmed chemical contact allergen (2,4-dinitrochlorobenzene [DNCB]); and a non-sensitizing irritant (sodium dodecyl sulfate [SDS]). Exposure to PA, TDI or TMA leads to an increased respiratory allergic reaction (Arts et al., 2008; Ban et al., 2006; De Vooght et al., 2009; Fukuyama et al., 2008a,b, 2009a,b; Plitnick et al., 2003; Vanoirbeek et al., 2009). DB was found in two studies to elicit a respiratory allergy (Cushman and Street, 1982; Fukuyama et al., 2009b), but in another was not associated with wheezing (Hoppin et al., 2002). Exposure to DNCB causes delayed-type hypersensitivity but not respiratory hypersensitivity reactions (Fukuyama et al., 2008a,b; Vanoirbeek et al., 2006). SDS is used in controlled clinical testing as a standard contact irritant (Basketter et al., 1996; Ehling et al., 2005a,b).

2. Materials and methods

2.1. Chemicals

For the dermal sensitization, each test chemical was dissolved in 4:1 acetone:olive oil (AOO). For the intratracheal challenge, each test chemical was dissolved in acetone and diluted in phosphate-buffered saline (PBS, Gibco, Tokyo, Japan). DNCB (98% pure) and TMA (97% pure) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Standard DB (98% pure), PA (<99.5% pure), SDS (<95% pure), TDI (2,4-isomer, 80% pure; 2,6-isomer, 20% pure), acetone and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The concentrations used are shown in Table 1. The test chemical concentrations were selected to avoid systemic toxicity or excessive local sensitization (particularly in the preliminary test, data not shown), while still permitting comparisons of sensitizing and challenge potencies. Also, in selecting the concentrations, we took into account the EC3 value (the concentration of a chemical required to elicit a threshold positive response) of each chemical, calculated from ³H-TdR incorporation data in a local lymph node assay (LLNA) (Basketter et al., 1996; Fukuyama et al., 2008a, 2009a). To confirm the allergenicity of the test chemicals, we adopted a sensitization dose that was less than the EC3 values, and a challenge dose that was less than 10 times that of the sensitization dose (see Table 1).

2.2. Animals

Female BALB/c mice (age, 7 weeks) were purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) and housed individually under controlled lighting (lights on from 0700 to 1900 hours), temperature (22 ± 3 °C), humidity

Table 1

Chemical concentrations used.

Chemical	Classification	EC3 values	Sensitization	Challenge
TMA	Respiratory allergen	0.2% ^a	0.2%	0.02%
PA	Respiratory allergen	0.3% ^b	0.3%	0.03%
TDI	Respiratory allergen	<0.03% ^a	0.03%	0.003%
DB	Environmental allergen	5.91% ^c	5%	0.5%
DNCB	Contact allergen	0.06% ^a	0.03%	0.003%
SDS	Irritant	5% ^b	3%	0.01%

TMA: trimellitic anhydride; PA: phthalic anhydride; TDI: toluene diisocyanate; DB: 2,4-D-butyl; DNCB: 2,4-dinitrochlorobenzene; SDS: sodium dodecyl sulfate.

^a Fukuyama et al. (2008a).

^b Basketter et al. (1996).

^c Fukuyama et al. (2009a).

(55 ± 15%) and ventilation (at least 10 fresh-air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available ad libitum.

This study was conducted in accordance with the *Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science* (Japanese Association for Laboratory Animal Science, 1987).

2.3. Experimental design

The mice were divided into three groups for each chemical; the groups were: –/– (sensitization and challenge with solvent only), +/– (sensitization with test solution and challenge with solvent only) and +/+ (sensitization and challenge with test solution). According to our preliminary study, each chemical –/+ treatment showed much the same patterns as –/– treatment in all parameters. Therefore, data of –/+ treatment are not shown in this issue.

After a 1-week acclimatization period, mice were allocated randomly to dose and control groups (*n* = 6 per group). Fig. 1 depicts the experimental protocol used. On days 1–3, 8–10 and 15–17, a 25-μL aliquot of the test solution or solvent alone was applied to the dorsum of both ears of each mouse for dermal sensitization. Two weeks after the last sensitization (day 31), mice were anesthetized by intraperitoneal (ip) injection of pentobarbital sodium (50 mg/kg), and were injected intratracheally through a 29-G needle with either a 50-μL aliquot of test solution or solvent alone. On the day after challenge (day 32), all mice were anesthetized with pentobarbital sodium (75 mg/kg) and sacrificed. Blood samples were taken from the inferior vena cava, and serum samples were assayed for IgE and IgG₁ levels. Each animal's lung-associated LNs were removed and pool by mouse in RPMI 1640 medium (Gibco, Tokyo, Japan). BALF was collected by cannulating the trachea and lavaging the lungs three times with 1 ml PBS, supplemented with 1% heat-inactivated fetal calf serum (FCS, Gibco) warmed to 37 °C. The first BALF fraction from each animal was centrifuged at 350 × *g* for 5 min, then supernatants from all mice in a group were pooled. The supernatant was assayed for levels of IgE, IgG₁, chemokine (MCP-1, MIP1-α, MIP1-β and EOTAXIN-1) and cytokine (IL-6 and TNF-α). The cell pellets of all three fractions were pooled and centrifuged at 350 × *g* for 5 min. The supernatant was removed, and the cell pellet was used for cell counts, differentials and RNA analysis.

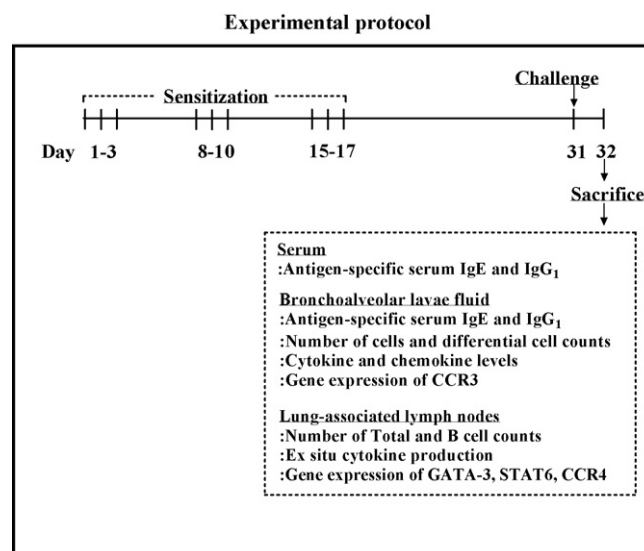


Fig. 1. Experimental protocol used in this study.

Table 2

Primer sets used.

Gene	GenBank Accession number	Primers	Product (bp)
<i>gata-3</i>	NM_008091	F: 5'-AGAGATTTCAGATCTGGGCAATGG-3' R: 5'-CAGGGACTGATTACAGAGCATGTA-3'	96
<i>stat6</i>	NM_009284	F: 5'-GTCCATCCACTCATTTACAGAGCCTA-3' R: 5'-ACATCTGAGCAAAGCATGGGTTC-3'	195
<i>ccr3</i>	NM_009914	F: 5'-GGCACTATGCAATAACCCATGAA-3' R: 5'-GGGTCTGTGTGCCAGAATAATGAA-3'	179
<i>ccr4</i>	NM_009916	F: 5'-TTGAAGCCTGGTTACAAGCGTAGAG-3' R: 5'-CCAAATGCACGGACATTTC-3'	107
<i>actβ</i>	NM_007393	F: 5'-CATCCGTAAGACCTCTATGCCAAC-3' R: 5'-ATGAGGCCACCGATCCACA-3'	171

2.4. Total IgE and IgG₁

The OptEIA™ Mouse IgE sets from BD Pharmingen (BD Biosciences, Tokyo, Japan) were used to measure total IgE in serum and BALF, in accordance with the manufacturer's protocol. Total IgG₁ in serum and BALF were assayed using plates coated with purified anti-mouse IgG₁ (which was also used as a standard), and using biotinylated anti-mouse IgG₁ and avidin horseradish peroxidase conjugate (BD Biosciences, Tokyo, Japan). Optical density (OD) was read at a wavelength of 450 nm with a microplate reader (spectra MAX 190, Molecular Devices, Tokyo, Japan).

2.5. Bronchoalveolar lavage fluid analysis

Cell numbers were determined with a Coulter counter Z2 (Beckman Coulter Co., Tokyo, Japan). For differential cell counts, Cytospin (Shandon Inc., Pittsburgh, PA) preparations and flow cytometric analysis (de Haar et al., 2005) were used. The Cytospin preparations were stained with Giemsa stain (Wako Pure Chemical Industries, Ltd.), and 200 cells in each sample were examined to determine the number of eosinophils and neutrophils present (according to standard leukocyte typing).

2.6. Lung-associated LN analyses

For cell counts, flow cytometry, RNA analysis and cell culture, single-cell suspensions of LNs in 1 ml RPMI 1640 supplemented with 5% heat-inactivated FCS were prepared by passage through sterile 70-μm nylon cell strainers (Becton Dickinson [BD] Falcon, Tokyo, Japan). The number of LN cells was determined with a Coulter counter Z2.

To stimulate T-cell receptor signaling, we cultured single-cell suspensions of LN (1×10^6 cells/well) with anti-CD3 (2 μg/ml) and/or anti-CD28 (2 μg/ml) (BD Pharmingen) T-cell antibodies for 24 or 96 h in 24-well plates (Multidish, Nalge Nunc International K.K., Tokyo, Japan) at 37 °C in a 5% carbon dioxide atmosphere. Interferon (IFN-γ) in the supernatants was quantified after culture for 24 h in the presence of anti-CD3 antibody. Interleukins in the supernatants were quantified as follows: IL-13 after culture for 96 h in the presence of anti-CD3, IL-4 after culture for 24 h in the presence of anti-CD3 and anti-CD28, and IL-5 after culture for 96 h in the presence of anti-CD3 and anti-CD28.

2.7. Antibodies and flow cytometry

The antibodies for flow cytometric analysis, purchased from BD Pharmingen, were fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgE (clone

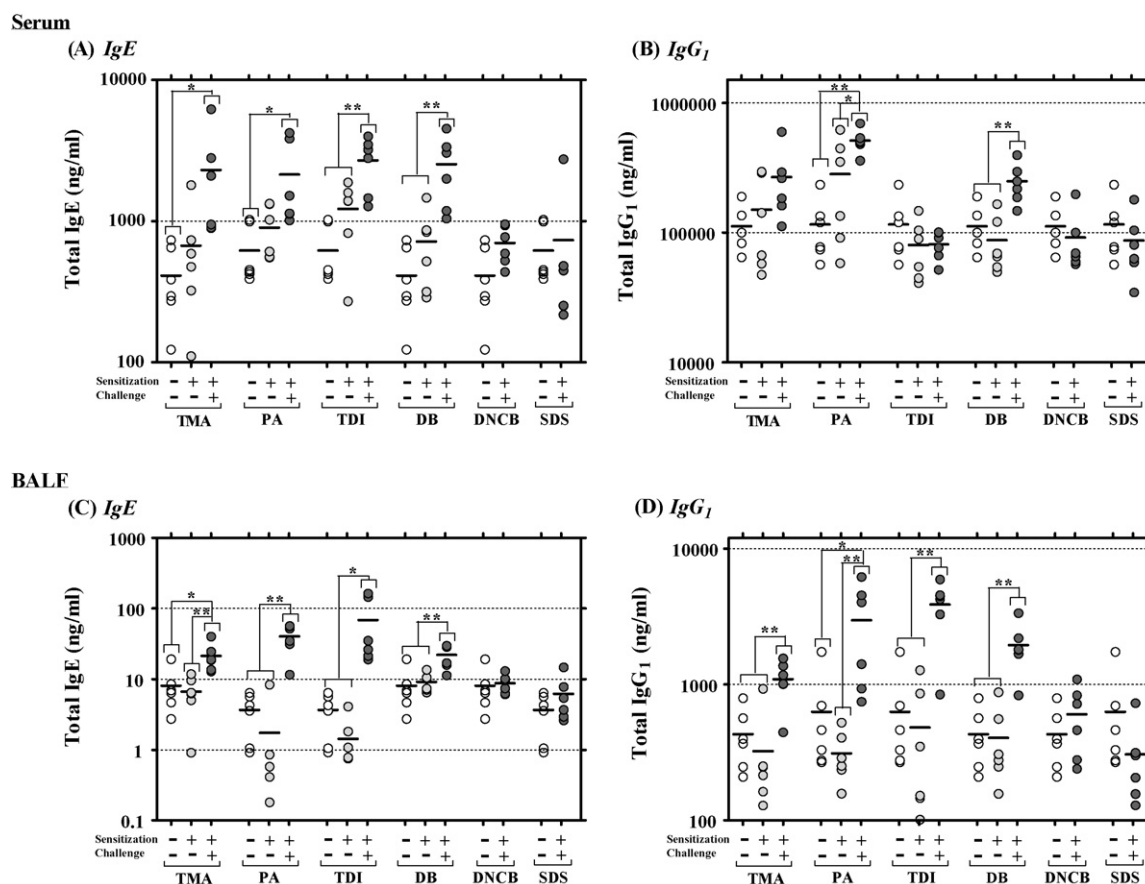


Fig. 2. Total IgE and IgG₁ levels in mouse serum (A and B) and BALF (C and D) isolated 1 day after challenge with test solution or solvent alone. Figure shows individual values of the IgE and IgG₁ levels (ng/ml), and mean values (shown as bars). Total IgE and IgG₁ levels were assayed by ELISA. Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

R35-72), PE-Cy5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2), FITC-conjugated rat anti-mouse Gr-1 (clone RB6-8C5) and phycoerythrin (PE)-conjugated hamster anti-mouse CD11c (HL3). To avoid non-specific binding, 1×10^6 cells were incubated with 20% normal goat serum (Sigma, St. Louis, MO, USA) for 10 min at 4°C, followed by incubation with FITC-, PE-, and PE-Cy5-conjugated monoclonal antibodies (mAbs) for 30 min at 4°C in the dark. The cells were washed twice with a buffer, resuspended at 1×10^6 cells per tube in 1 ml PBS, and then analyzed with a FACSCaliber flow cytometer (BD Pharmingen), using the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens.

2.8. Cytokine and chemokine assays

Cytokines and chemokines (IL-4, IL-5, IL-6, IL-13, IFN- γ , TNF- α , MCP-1, MIP1- α and MIP1- β) in the supernatants of LN or BALF were assayed by BD Cytometric Bead Array (BD Pharmingen) in accordance with the manufacturer's protocol. EOTAXIN-1 in the supernatants of BALF was measured by ELISA (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's protocol.

2.9. RNA analysis

The cell pellet of BALF (1×10^4 cells/mouse) and single-cell suspensions of LN (1×10^6 cells/mouse) were pooled in RNeasy lysis buffer (Applied Biosystems, Tokyo, Japan) at -80°C . Messenger RNA was extracted and reverse transcribed into cDNA using μ MACS One-step cDNA Kit (Miltenyi Biotec KK, Tokyo, Japan) according to the manufacturer's protocol. PCR primers (see Table 2) for the genes encoding the following proteins were purchased from Takara Bio Inc., Shiga, Japan: chemotactic cytokine receptor 3 (CCR3), CCR4, signal transduction and activator of transcription 6 (STAT6), GATA binding protein 3 (GATA-3), and β actin (Act β). The corresponding cDNAs were amplified by quantitative real-time polymerase chain reaction (PCR) using the Thermal Cycler Dice system (Takara Bio Inc.). The levels of active gene expression were determined using a standard curve, and the data acquired for each sample were normalized to expression levels recorded for the housekeeping gene Act β .

2.10. Statistical analysis

The data were transformed logarithmically to equalize the variances, and the results were evaluated using analysis of variance (ANOVA). Where the ANOVA was significant, the differences between groups were assessed by the Tukey–Kramer test. A probability value (P) of <0.05 was considered to indicate significance.

3. Results

3.1. Total IgE and IgG₁

We examined total IgE and IgG₁ levels in serum and BALF (Fig. 2). Total IgE levels in serum (Fig. 2A) in the TDI and DB +/+ treatment groups showed a significant increase relative to the control groups (−/− and +/−). TMA and PA +/+ treatment groups showed a significant increase relative to the −/− group. Total IgE levels in BALF (Fig. 2C) in the confirmed respiratory allergen and DB +/+ treatment groups showed a significant increase relative to the control groups. Increasing trends were observed in the confirmed respiratory allergen and DB +/− treatment groups, but the reactions were mild. Total IgE levels were not elevated in serum or BALF in the DNCB- and SDS-treated groups. DNCB and SDS +/− treatment groups showed much the same patterns as −/− and +/+ treatment groups. Therefore, data of +/− treatment groups are not shown.

Total IgG₁ levels in serum (Fig. 2B) in the DB and PA +/+ treatment groups showed a significant increase relative to the control groups. Total IgG₁ levels in BALF (Fig. 2D) in the confirmed respiratory allergen and DB +/+ treatment groups showed a significant increase relative to the control groups. As with IgE, total IgG₁ levels were not elevated in serum or BALF in the DNCB- and SDS-treated groups (data of +/− treatment groups are not shown).

3.2. Bronchoalveolar lavage fluid analysis

We examined the total and differential cell counts of eosinophils and neutrophils (Fig. 3), chemokine and cytokine levels (Fig. 4), and expression of genes for CCR3 (Fig. 5) in BALF. The confirmed respiratory allergens and DB +/+ treatment groups showed increased numbers of eosinophils and neutrophils, and high levels of IL-6,

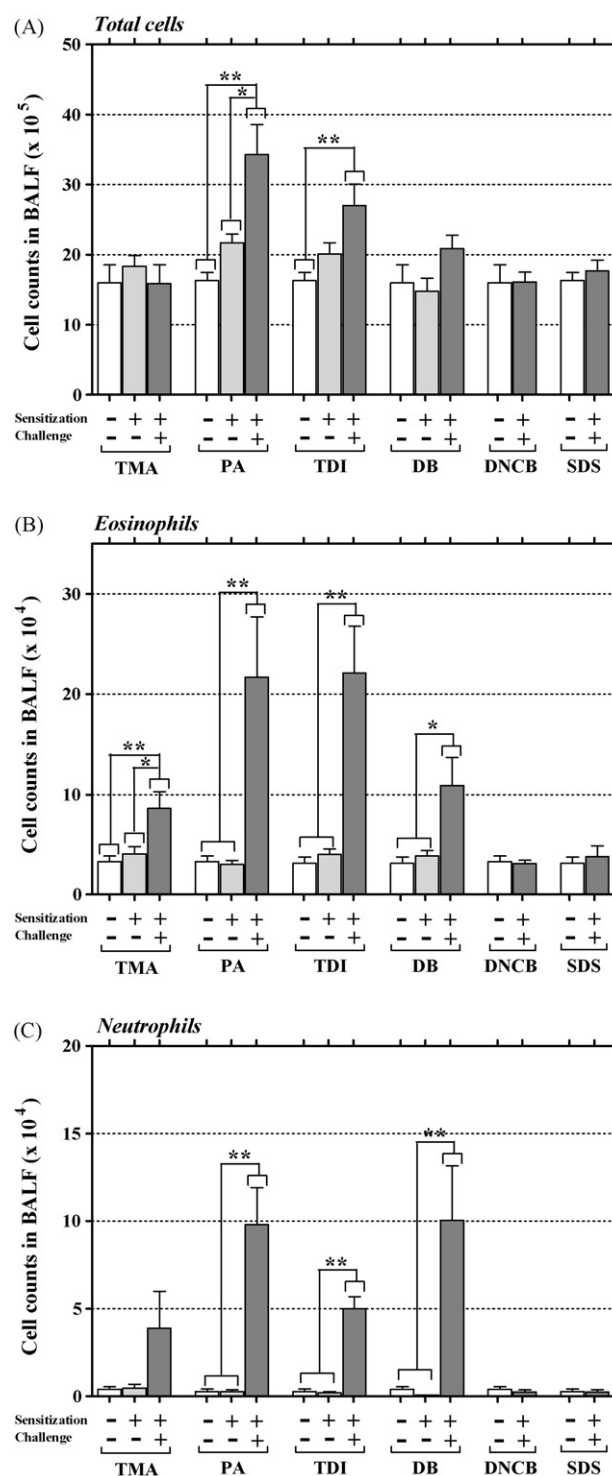


Fig. 3. Total cell (A), eosinophil (B) and neutrophil (C) counts in BALF of mice treated with test solution or solvent alone. Cell counts are expressed as mean (number of cells) $\pm$ S.D. BALF cells were differentiated by Gr-1 and CD11c staining and flow cytometry. The combination of these markers was used to discriminate the different cell types in BALF. Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

TNF- α , MCP-1, EOTAXIN-1, MIP1- α and MIP1- β . The number of eosinophils (Fig. 3B) was significantly higher in the confirmed respiratory allergen and DB +/+ treatment groups than in the control groups. The number of neutrophils (Fig. 3C) was significantly higher in the PA, TDI and DB +/+ treatment groups than in the control groups.

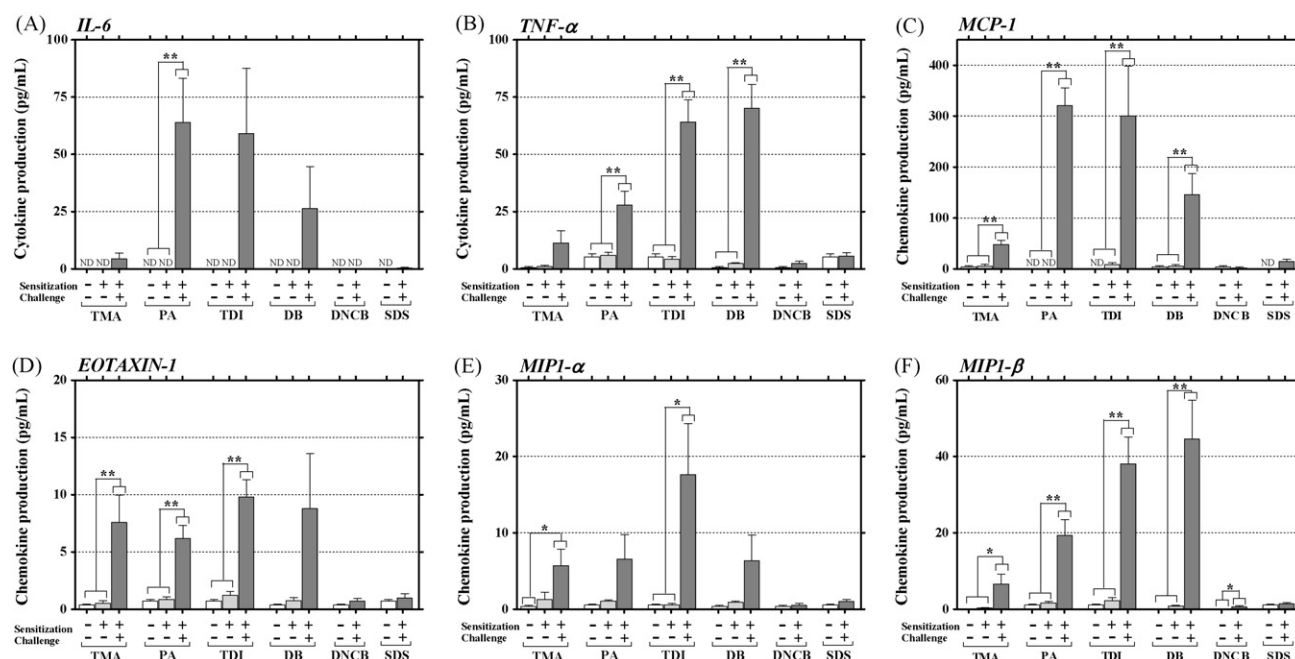


Fig. 4. Cytokine (A: IL-6; B: TNF- α) and chemokine (C: MCP-1; D: EOTAXIN-1; E: MIP1- α ; F: MIP1- β) levels in BALF of mice treated with test solution or solvent alone. Chemokine and cytokine levels are expressed as mean (pg/mouse) $\pm$ S.D. To determine the chemokine and cytokine levels, BALF was assayed by cytometric bead array. Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

Production of IL-6 (Fig. 4A) was significantly higher in the PA +/+ treatment group than in the control groups. Production of TNF- α (Fig. 4B) was significantly higher in the PA, TDI and DB +/+ treatment groups than in the control groups. Production of MCP-1 (Fig. 4C) was significantly higher in the confirmed respiratory allergen and DB +/+ treatment groups than in the control groups. Production of EOTAXIN-1 (Fig. 4D) was significantly higher in the confirmed respiratory allergen +/+ treatment groups than in the control groups. Production of MIP1- α (Fig. 4E) was significantly higher in the TMA and TDI +/+ treatment groups than in the control groups. Production of MIP1- β (Fig. 4F) was significantly higher in the confirmed respiratory allergens and DB +/+ treatment groups than in the control groups.

Total and differential cell counts and levels of IL-6, TNF- α , MCP-1, EOTAXIN-1 and MIP1- α did not increase in mice treated with DNCB or SDS. Significant increases in MIP1- β were observed in the DNCB +/+ treatment group, but MIP1- β increases were small compared with those in the respiratory allergen treated groups (Fig. 4F) (data of +/- treatment groups are not shown). The differential cell counts from the Cytospin preparations showed similar patterns to those of the flow cytometric analysis (data not shown).

Marked increases in CCR3 expression were seen in the confirmed respiratory allergen and DB +/+ treatment groups (Fig. 5). Significant differences were observed between the TMA, PA and DB +/+ treatment groups. CCR3 expression did not increase in any of the DNCB or SDS groups compared to the control groups (data of +/- treatment groups are not shown).

3.3. Lung-associated LN analysis

We examined the total numbers of lymphocytes, IgE-positive B-cells (B220⁺IgE⁺), and MHC Class II-positive B-cells (B220⁺MHC II⁺) in lung-associated LNs (Fig. 6). Total numbers of lymphocytes, IgE-positive B-cells, and MHC Class II-positive B-cells were significantly higher in the confirmed respiratory allergen and DB treatment +/+ groups than in the control groups; such increases were not seen in the DNCB- and SDS-treated groups (data of +/- treatment groups are not shown). With all chemicals studied, the total number of B-cells (i.e. B220⁺ cells) increased in tandem with the numbers of IgE⁺ and/or MHC Class II-positive B-cells (data not shown).

We examined cytokine production (IL-4, IL-5, IL-13 and IFN- γ) in the lung-associated LN supernatants (Fig. 7). Significant increases in IL-4, IL-5, IL-13 and IFN- γ production were seen in the confirmed respiratory allergen and DB treatment groups compared to the control groups; such increases were not seen in the DNCB- and SDS-treated groups (data of +/- treatment groups are not shown).

We examined the expression of Th2 type allergy-related genes (GATA-3, STAT6 and CCR4) in lung-associated LN cells (Fig. 8). Marked increases in GATA-3, STAT6 and CCR4 expression were seen in the TMA and DB +/+ treatment groups; compared to the control

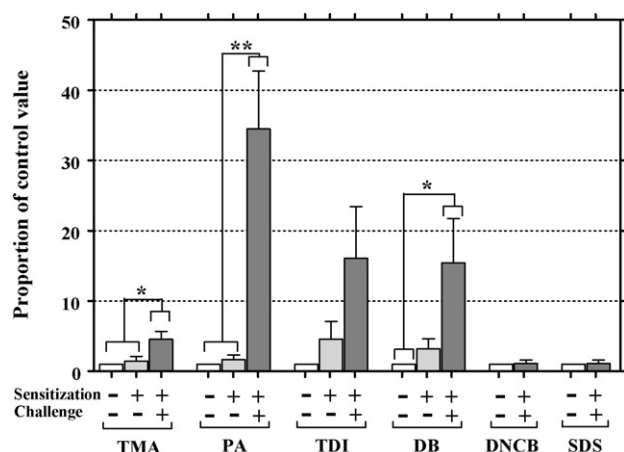


Fig. 5. Gene expression of CCR3 in BALF of mice treated with test solution or solvent alone. Values represent the increase in expression compared to the value for the -/- treatment group, as means $\pm$ S.D. Expression of genes was normalized to represent a proportion of the expression of Act β . Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

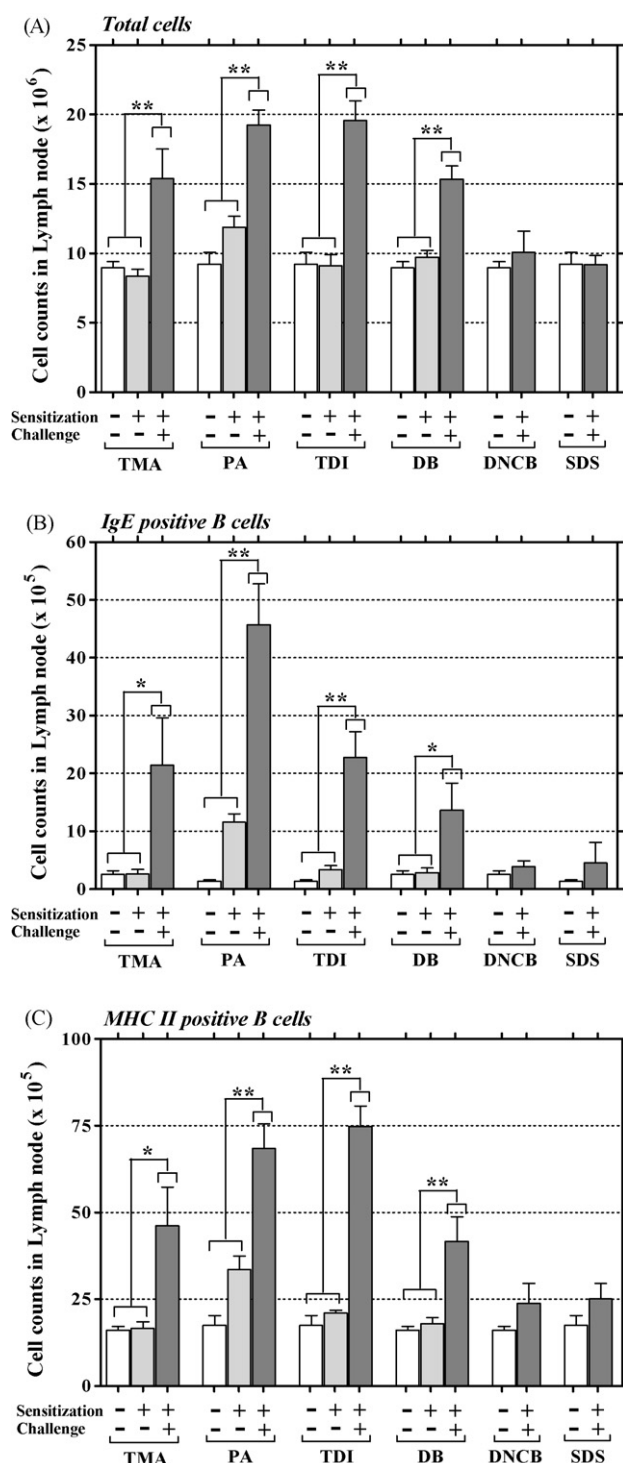


Fig. 6. Total cell (A), B220⁺IgE⁺ cell (B) and B220⁺ MHC Class II-positive cell (C) counts in lung-associated lymph nodes of mice treated with test solution or solvent alone. Numbers of lymphocytes are expressed as mean (number of cells) $\pm$ S.D. Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

group, the increase was significant for GATA-3 expression induced by TMA treatment ($P < 0.05$), for STAT6 expression induced by TMA and DB ($P < 0.05$), and for CCR4 expression induced by TMA and DB ($P < 0.05$ or $P < 0.01$). There was no increase in GATA-3, STAT6 and CCR4 expression in any of the PA, TDI, DNCB, or SDS groups (relative to controls) (data of DNCB and SDS +/- treatment groups are not shown).

4. Discussion

This study focused on several immune endpoints. We examined IgE and IgG₁ levels because these immunoglobulins are involved in respiratory allergies, and are thus useful in identifying chemicals as respiratory allergens (Ban et al., 2006; Fukuyama et al., 2008a,b; Goutet et al., 2005). We found that the confirmed respiratory allergens (TMA, PA and TDI) induced significant increases in total IgE and IgG₁ levels and in IgE-expressing B-cell populations. Similar results were found with the environmental chemical DB, indicating that the method can be used for identifying respiratory allergens of weak immunogenicity at low doses. In contrast, there was no increase in IgE and IgG₁ levels in response to DNCB and SDS. Taken together with our previous research and other reports (Ban et al., 2006; Dearman et al., 2005; Fukuyama et al., 2008a,b, 2009a,b), our findings suggest that our method would be useful for classifying chemical respiratory allergens.

In general, an airway allergen challenge causes cellular inflammation that is dominated by eosinophils and neutrophils in the airways. Eosinophils and neutrophils may contribute to several of the clinical features of respiratory allergy, including tissue damage and airway hyperresponsiveness (Fukuyama et al., 2008b, 2009a; Lotvall et al., 1998). These cells have the capacity to release substantial amounts of allergy-related chemical mediators, such as IL-6, TNF- α , MCP-1, MIP-1 α , MIP-1 β and eotaxin (Janeway et al., 2004). The eotaxin receptor on eosinophils, CCR3, is an important chemokine receptor on eosinophils, because mice lacking functional CCR3 genes show impaired eosinophil recruitment to the lungs in a model of allergic airway inflammation (Humbles et al., 2002; Su et al., 2008). In mice treated with confirmed respiratory allergens and DB, we found an increase in eosinophil and neutrophil numbers, the levels of various chemokines and cytokines released (primarily) by cells likely to also be present in BALF, and CCR3 expression; however, no such increases were found in DNCB- or SDS-treated mice. Along with IgE and IgG₁ levels, the airway responses in our findings suggest that our method would be useful for classifying chemical respiratory allergens.

In the development of immune responses, activated local B lymphocytes can act as antigen-presenting cells for CD4⁺ and CD8⁺ T cells (Goutet et al., 2005). The CD4⁺ T cells that recognize MHC Class II molecules are of two functional types: Th1 and Th2 (Santana and Rosenstein, 2003). Th1 cells are specialized to activate macrophages that are promoted by, or have ingested, pathogens. Th2 cells are specialized for B-cell activation (Santana and Rosenstein, 2003). To evaluate the state of activation of Th1 and Th2 cells following chemicals sensitization and then challenge, we assessed the membrane expression of MHC Class II molecules important for antigen presentation and regulation of the immune response (Murray, 1998). In mice treated with confirmed respiratory allergens and DB, we found significant increases in MHC Class II-expressing B-cell populations; however, no such increases were found in DNCB- or SDS-treated mice.

Respiratory allergens that cause respiratory sensitization induce a Th2-type response as well as an increased serum IgE level (Anthoni et al., 2009). Th2 cells produce IL-4, IL-5 and IL-13 (Mosmann and Coffman, 1989), and these responses are down-regulated by Th1 cytokines (IFN- γ) (Gavett et al., 1995). In mice treated with confirmed respiratory allergens and DB, we found significant increases in the levels of Th1 and Th2 cytokines produced by re-stimulated ex vivo LN cells; again, there were no such increases in DNCB- or SDS-treated mice.

Recently, a number of noncytokine markers of Th2 responses have been described, including the transcription factor GATA-3, STAT6 and the chemokine receptor CCR4. GATA-3 has been described as 'master switch' in the development of Th2 cells, activating expression of key Th2 genes, including the prototypic

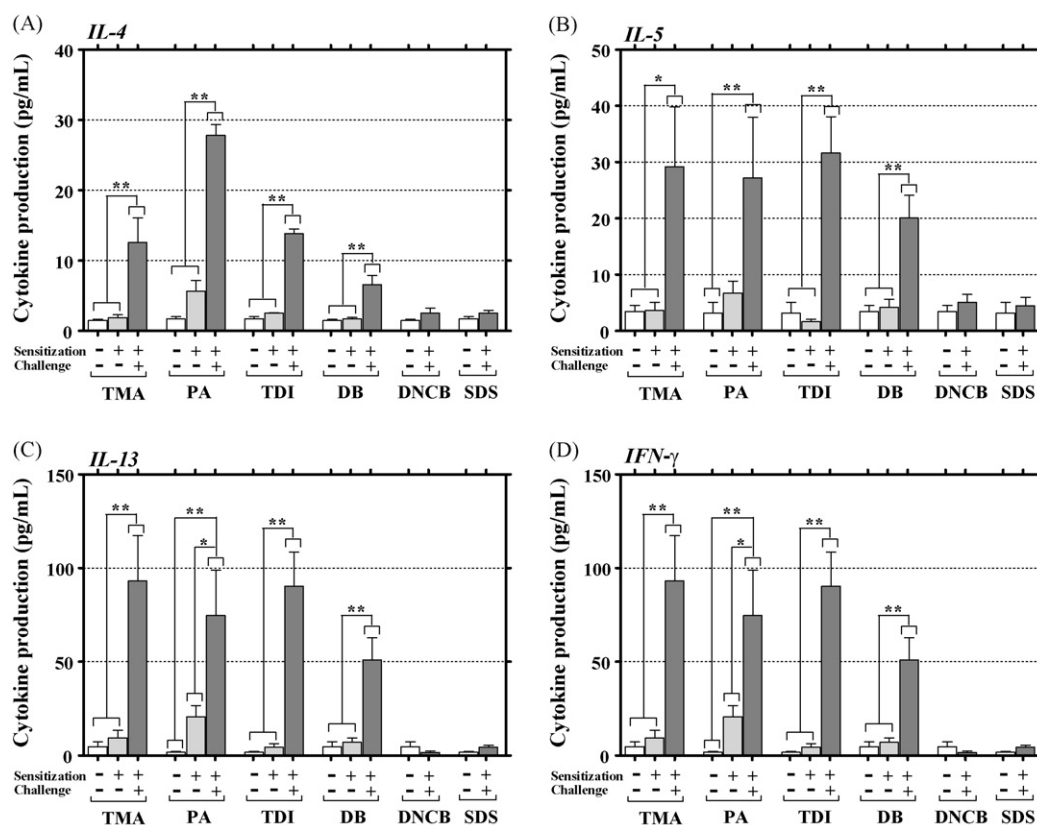


Fig. 7. Ex situ cytokine (A: IL-4; B: IL-5; C: IL-13; D: IFN- γ) production by lung-associated lymph node cells of mice treated with test solution or solvent alone. To determine the effects of cytokine production, lymph node cell suspensions were cultured with T-cell antibodies (CD3 and/or CD28) for 24 or 96 h, and the supernatant was assayed by cytometric bead array. Results are expressed as mean (pg/mouse) $\pm$ S.D. Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

cytokines IL-4, IL-5 and IL-13 (Ritz et al., 2004). Binding of IL-4 to IL-4R α receptor leads to rapid phosphorylation of STAT6, which in turn induces GATA-3 expression and subsequent production of the Th2 cytokines IL-4, IL-5 and IL-13 (Takeda et al., 1996). CCR4 is a chemokine receptor that is preferentially induced in Th2 cells during differentiation (Sato et al., 2009). Thus, to evaluate the state of activation of Th2 cells following chemicals treatment, we assessed the gene expression of GATA-3, STAT6 and CCR4 in the LN cells. We found that the TMA and DB treatment groups induced significant increases in the expressions of GATA-3, STAT6 and CCR4. However, although PA and TDI are recognized as Th2 responders, the reaction was mild and not statistically significant. Such a divergence in gene expression in the local lymph nodes probably has important functional consequences for the subsequent development of immune-inflammatory responses in the target organ. We have used these markers to more comprehensively assess the presence of Th2 responses in chemical-treated mice.

Overall, these results show that our original method can be used to detect and classify compounds that are low-level respiratory allergens, and differentiate them from contact allergens and irritants. The confirmed respiratory allergens and DB were associated with eosinophil and neutrophil proliferation, increases in local chemokine and cytokine levels, and increased production of IgE and IgG₁ in serum and BALF. In contrast, treatment with a contact allergen or an irritant induced almost no Th2 responses in our protocol, and this lack of reaction was associated with a lack of increase in IgE and IgG₁ production in serum and BALF.

In recent years, several investigators have examined how to detect the chemical induced respiratory allergy. Arts et al. (2008) developed a respiratory LLNA which was chemical exposed by inhalation and cellular proliferation was determined in the res-

piratory tract draining LN. In analogy to the skin LLNA, they hypothesized that a chemical that induced an SI or 3 or more in the respiratory tract draining LN should be regarded a respiratory tract sensitizer. TMA, PA, TDI and DNCB were positive responses. Use of the respiratory LLNA has an advantage of exposure by inhalation is a realistic approach to test respiratory allergens. In addition, this test is more cost- and time-effective than our model. Despite these advantages, based on the local toxicity, the dose that can be applied is much lower than can be achieved by skin application. In addition, the contact allergens were as potent as the respiratory allergens, although the potency ranking differed from that in a skin LLNA. In contrast, our model can detect and classify weakly immunogenic and low-dose allergic reactions. Despite these advantages, the tests are time-consuming and costly. Therefore, our next step was to develop further our methods to detect environmental-chemical induced respiratory allergy (moreover) using shorter length-of-sensitization phases and exposure by inhalation in sensitization and challenge phase.

The animal species used may affect the results of assays for respiratory allergens. Limitations of mice are that their vasculature is the actual target of the anaphylactic response, their bronchial musculature is poorly developed and their small size can make it difficult to detect pulmonary reactions associated with the elicitation of respiratory allergy. In contrast, respiratory allergy models in other rodents (e.g. rats and guinea pigs) have focused extensively on pulmonary reactions during the elicitation stage of respiratory allergy (Briatico-Vangosa et al., 1994). Nevertheless, mouse models have been used to explore more mechanistic characterization of both the sensitization and challenge stage. Investigations into the cellular and molecular mechanisms of action in mice have been facilitated by their well-characterized immune system, the avail-

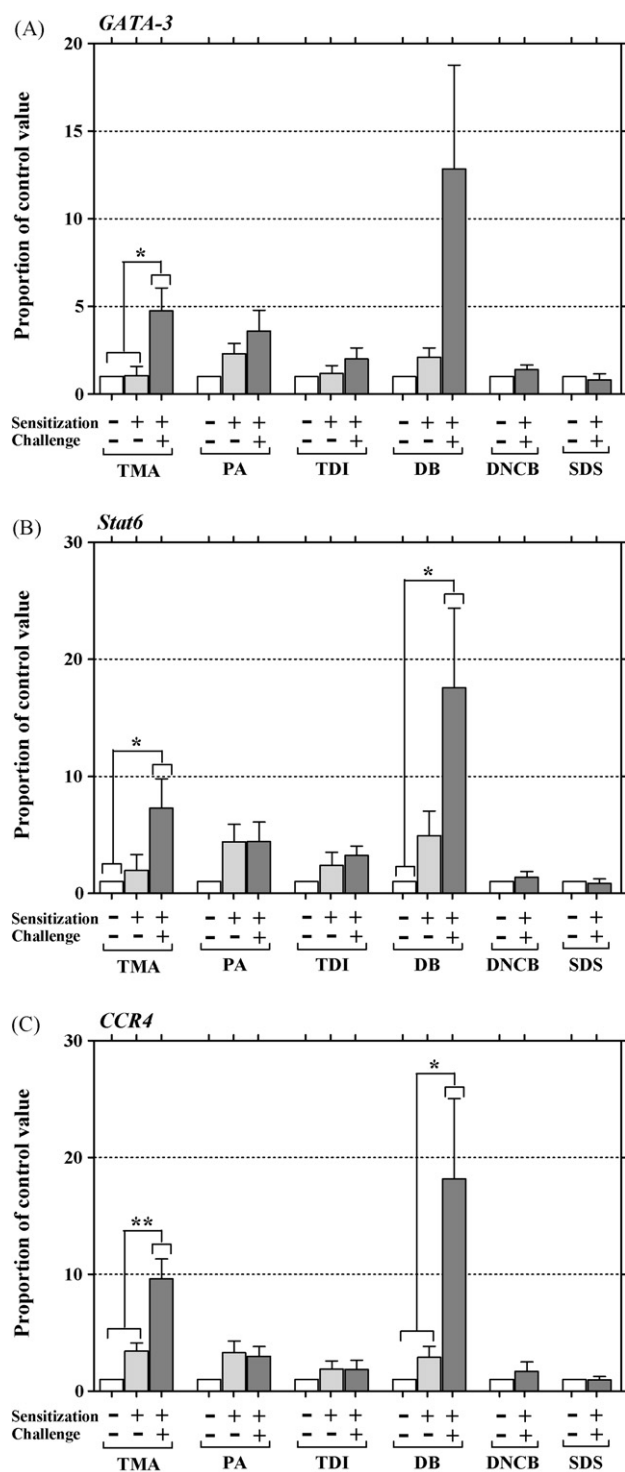


Fig. 8. Expression of genes (A: GATA-3; B: STAT6; C: CCR4) in lung-associated lymph node of mice treated with test solution or solvent alone. Values represent the increase in expression compared to the value for the $-/-$ treatment group, as means $\pm$ S.D. Expression of genes was normalized to represent proportion of the expression of Act β . Statistical significance is marked by asterisks: * for $P < 0.05$ and ** for $P < 0.01$ (Tukey's t -test). $n = 6$ per group.

ability of species-specific reagents and a fully sequenced genome. Mice also exhibit a number of similarities to humans in their response to respiratory allergens, including the development of IgE antibody and eosinophilic lung inflammation (Boverhof et al., 2008; Epstein, 2006). We believe that it is important not to overlook the minute signs triggered when affected patients come into contact

with any of a number of environmental chemical products at low doses, and important to classify false positive reactions induced by non-respiratory allergens. Thus, we feel that our mice model is useful and our studies continue to focus on the detection and classification of weak allergic reactions induced by environmental chemicals.

Conflict of interest statement

None

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