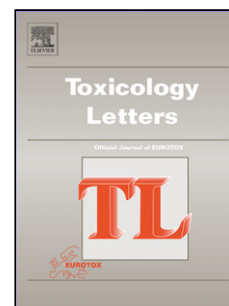


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Toxicology Letters

Identifying low-dose-chemical-induced respiratory allergic responses in mice

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Key words

chemical-induced respiratory allergy; BALB/c mouse; trimellitic anhydride; toluene diisocyanate; 2,4-dinitrochlorobenzene.

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ABSTRACT

The inhalation of many types of chemicals, including pesticides, perfumes, and other low-molecular-weight chemicals, is a leading cause of allergic respiratory diseases. We attempted to develop a new test protocol to detect environmental chemical-related respiratory hypersensitivity at low and weakly immunogenic doses. We used long-term dermal sensitization followed by a low-dose intratracheal challenge to evaluate sensitization by the well-known respiratory sensitizers trimellitic anhydride (TMA) and toluene diisocyanate (TDI) and the contact sensitizer 2,4-dinitrochlorobenzene (DNCB). After topically sensitizing BALB/c mice (9 times in 3 weeks) and challenging them intratracheally with TMA, TDI, or DNCB, we assayed differential cell counts and chemokine levels in bronchoalveolar lavage fluid (BALF); lymphocyte counts, surface antigen expression of B cells, and local cytokine production in lung-associated lymph nodes (LNs); and antigen-specific IgE levels in serum and BALF. TMA induced marked increases in antigen-specific IgE levels in both serum and BALF, proliferation of eosinophils and chemokines (MCP-1, eotaxin, and MIP-1 β) in BALF, and proliferation of Th2 cytokines (IL-4, IL-10, and IL-13) in restimulated LN cells. TDI induced marked increases in levels of cytokines (IL-4, IL-10, IL-13, and IFN- γ) produced by restimulated LN cells. In contrast, DNCB treatment yielded, at most, small,

nonsignificant increases in all parameters. Our protocol thus detected respiratory allergic responses to low-molecular-weight chemicals and may be useful for detecting environmental chemical-related respiratory allergy.

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INTRODUCTION

The inhalation of many types of chemicals, including pesticides, perfumes, and other low-molecular-weight chemicals, is a leading cause of allergic respiratory diseases (Brown and Jason, 2007; Jeebhay and Quirce, 2007; Kreutzer et al., 1999). Environmental and lifestyle factors and increasing use of such chemicals have often been suggested as contributors to such diseases (Ban and Hettich, 2005; Peden, 2000). These diseases are characterized by episodic obstruction, inflammation, and nonspecific hyperresponsiveness of the airway to a variety of stimuli, including histaminergic and cholinergic agents (Arts et al, 2003; Sheffer and Taggart, 1993; Subcommittee on ‘Occupational Allergy’ of the European Academy of Allergology and Clinical Immunology, 1992). 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, this may result in the production of specific IgE antibodies, airway inflammation characterized by activated CD4⁺ T cells, eosinophils, and mast cells, and increased levels of interleukin (IL)-4 and IL-5 (Ban et al., 2006; Maestrelli et al., 1997; Mapp et al., 1999; Wisnewski and Redlich, 2001).

Several animal models have been used to identify chemical respiratory allergies (Arts *et al.*, 2003; Ban *et al.*, 2006). However, although environmental chemical allergens tend to have weak, minimal immunogenicity, these methods have focused on the detection of strong allergic reactions. Therefore, protocols are needed for the detection and treatment of weakly immunogenic and low-dose respiratory allergies. In a previous study, we developed a new detection method of environmental-chemical-related hypersensitivity using typical T helper (Th) 1 (2,4-dinitrochlorobenzene, DNCB) and Th2 (trimellitic anhydride, TMA; and toluene diisocyanate, TDI) sensitizers by a long-term dermal sensitization protocol (Fukuyama *et al.*, 2008). The study showed that, unlike low-dose 2,4-dinitrochlorobenzene (DNCB) treatment, treatment with low-dose TMA or TDI induced a typical Th2 response such as increase in antigen-specific serum IgE levels and IL-4 production in local lymph nodes (LNs). In contrast, secretion of Th1 cytokines in LNs was induced by treatment with the same low dose of the Th1 type allergen DNCB, with the production of high levels of interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and IL-2; Th1 cytokine production was not induced by the same concentrations of TMA or TDI (Fukuyama *et al.*, 2008). These results demonstrated that our method is able to detect allergic reactions caused by chemicals occurring at weakly immunogenic and low doses in the environment.

However, this test method is not fully capable of assessment of respiratory sensitizers because it lacks an airway challenge phase. In this issue, we attempt to develop our detection method of environmental-chemical-related hypersensitivity moreover using another challenge route (respiratory tract).

The respiratory tract is routinely exposed to a variety of environmental allergens. The presentation of their derivative epitopes to T lymphocytes, a key event in the adaptive immune response, takes place in the lung-associated LNs (Ban et al., 2006; Banchereau and Steinman, 1998; Lambrecht et al., 2000). In previous mouse studies, treatment with TMA and TDI provoked a strong Th2 immune response associated with increases in total serum concentrations of IgE and IgE-positive B cells in the respiratory tract (Ban and Hettich, 2001; Matheson et al., 2005). However, little is known about the mechanism of the effect of very low doses of environmental chemicals.

In light of these previous results, the aim of this study was to improve our methods for detecting environmental-chemical-related respiratory hypersensitivity at low and weakly immunogenic doses. To that end, we used long-term dermal sensitization followed by intratracheal challenge.

1. MATERIALS AND METHODS

2.1 Chemicals

For dermal sensitization, each test chemical was dissolved in 4:1 acetone:olive oil (AOO). For intratracheal challenge, each test chemical was dissolved in a sterile physiological saline solution. TMA and DNCB were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). TDI (95% 2,4 isomer), acetone, and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DNCB is a contact allergen, whereas the others are respiratory allergens. The concentrations used are presented in Table 1. These concentrations of TMA, TDI, and DNCB were selected to avoid systemic toxicity and/or excessive local sensitization (particularly in the preliminary test) while still permitting comparisons of the sensitizing potencies of the chemicals. In addition, we focused on the EC₃ values (the concentration of a chemical required to elicit a threshold positive response) in local lymph node assay (LLNA) and LLNA was used to select concentrations of TMA, TDI, and DNCB.

2.2 Animals

Female BALB/c mice (age, 7 weeks) purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) were housed individually under controlled lighting (lights on from 0700 to 1900 h), temperature (22 ± 2 °C), humidity ($55\% \pm$

15%), and ventilation (at least ten 100% 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 Test and control groups

The mice were divided into five groups for each chemical (TMA, TDI, and DNCB): Group $-/-$ (both sensitization and challenge with solvent only), Group $-/+$ (sensitized with solvent only and challenged with high-dose test solution), Group $+/-$ (sensitized with high-dose test solution and challenged with solvent only), Group $+/+^{\text{low}}$ (sensitized with test solution and challenged with low-dose test solution), and Group $+/+^{\text{high}}$ (sensitized with test solution and challenged with high-dose test solution).

2.4 Sensitization and challenge protocol

After a 1-wk acclimatization period, mice were allocated randomly to dose and control groups ($n = 8$ per group). 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 each ear of each mouse for

dermal sensitization. Two weeks after the last sensitization (day 31), mice were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg), and an challenge with a 50- μ L aliquot of test solution or solvent alone was injected into intratracheal using a 29-G needle. On the day after the challenge (day 32), all mice were anesthetized with pentobarbital sodium (75 mg/kg) and sacrificed, and blood samples, BALF, and LNs were taken.

2.5 Antigen-specific serum IgE

TMA-ovalbumin (OVA) conjugate was prepared by Inject Immunogen EDC Kit in accordance with the manufacturer's protocol (Pierce Biotechnology, Inc. Rockford, MD, US). TDI-MSA conjugate (mouse serum albumin, Sigma Aldrich Chemical Company Inc., Tokyo, Japan) and DNCB-MSA conjugate were prepared as described, by a modified version of the method of Ye et al. (2006). Briefly, TDI or DNCB was added to 1% HSA in PBS with constant stirring. Aliquots were taken 5, 10, 20, 30, and 40 min after the beginning of the reaction. Excess ammonium carbonate (2 M, Sigma Aldrich Chemical Company Inc., Tokyo, Japan) was added to each aliquot to terminate the reactions. All reactive samples were centrifuged at 3000 $\times$ g for 40 min to remove unreacted TDI and DNCB. The samples were then extensively dialyzed for 3

days against 0.1 mol/L ammonium carbonate and precipitated with an equal volume of 20% trichloroacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples were then redissolved in 1 mol/L sodium hydroxide (Sigma Aldrich) and dialyzed with deionized water for 24 h. The protein content of each conjugated sample was determined by the Lowry method (Lowry et al., 1951).

Specific serum IgE was measured by ELISA. Briefly, each flat-bottomed microplate well (Nalge Nunc International K.K., Tokyo, Japan) was coated with chemical–protein conjugates (100 μ l, 0.1 mg/ml) in coating buffer (BD Pharmingen, San Diego, CA, USA) and incubated overnight at 4 °C. The content of each well was removed and the plate was washed five times with wash buffer (BD Pharmingen) following each addition–incubation procedure. Non-specific binding was blocked by incubation with 200 μ l 10% heat-inactivated goat serum (Sigma Aldrich Chemical Company Inc., Tokyo, Japan) in PBS for 1 h at room temperature. Mouse serum was diluted in PBS (from 1:4 to 1:4096). The dilute serum (100 μ l) was added to each well and incubated for 2 h at room temperature. Biotin-conjugated rat anti-mouse IgE monoclonal antibody (BD Pharmingen, 2 μ g/ml, 100 μ l) was added to each well and incubated for 1 h at room temperature. Streptavidin–horseradish peroxidase (SAv-HRP) conjugate (BD Pharmingen, dilution 1:1000, 100 μ l) was added to each well and incubated for 30 min

at room temperature. The plate was developed with tetramethylbenzidine (TMB) (100 μ l/well) in the dark, at room temperature, for 30 min. Optical density (OD) was read at a wavelength of 450 nm with an ImmunoMini reader (NJ-2300, Nippon Intermed, Tokyo, Japan).

2.6 BALF analysis

BALF was collected by cannulating the trachea and lavaging the lung three times with 1 ml phosphate-buffered saline (PBS, Gibco, Tokyo, Japan) supplemented with 1% heat-inactivated fetal calf serum (FCS; Gibco, Tokyo, Japan) warmed to 37 °C. The first BALF fraction from each animal was then centrifuged at 1300 rpm for 5 min, and then the all the supernatants from all mice in a group were pooled. The supernatant was assayed for chemokine and cytokine levels (IL-6, MCP-1, eotaxin, and MIP-1 β). The cell pellets of the first fraction and another two fractions were pooled and centrifuged at 1300 rpm for 5 min. The supernatant was removed and the cell pellet was resuspended in 1 ml PBS supplemented with 5% FCS for cell counts and differentials.

The number of cells was determined with a Coulter counter Z2 (Beckman Coulter Co., Tokyo, Japan). For differential cell counts, half of the cells were used for Cytospins (Shandon Inc., Pittsburgh, PA) and the remaining cells were used for flow

cytometric analysis (de Haar et al., 2005). The Cytospin preparations were stained with Giemsa stain (Wako Pure Chemical Industries, Ltd. Osaka, Japan), and 200 cells in each sample were examined to differentiate the numbers of macrophages, eosinophils, and neutrophils present (according to standard leukocyte typing).

2.7 Lung-associated-LN analyses

After completion of the BALF sampling, the lung-associated LNs were removed and all LNs from each mouse were pooled in RPMI 1640 medium (Gibco, Tokyo, Japan). For cell counts, flow cytometry, and cell culture, single-cell suspensions of LNs in 1 ml RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum were prepared by passage through sterile 70- μ m nylon cell strainers (Becton Dickinson [BD] Falcon, Tokyo, Japan). The number of LN cells was determined with a Coulter Z2 counter (Beckman Coulter Co.).

To stimulate T-cell-receptor signaling, we cultured single-cell suspensions of LN (1×10^6 cells/well) with T cell antibodies to anti-CD3 (2 μ g/ml) and anti-CD28 (2 μ g/ml) (Becton Dickinson [BD] Pharmingen, San Diego, CA, USA) for 48 h in 24-well plates (Multidish, Nalge Nunc International K.K., Tokyo, Japan) at 37°C in a 5% carbon dioxide atmosphere. Production of cytokines (IL-4, IL-10, IL-13, and IFN γ) was

analyzed in the supernatants prepared following culture for 48 h in the presence of anti-CD3 or anti-CD28 antibody.

2.8 Antibodies and flow cytometry

All antibodies for flow cytometric analysis—fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgE (clone 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)—were purchased from BD Pharmingen. To avoid nonspecific binding, 1×10^6 cells were incubated with 15% normal goat serum (Sigma, St. Louis, MO, USA) for 10 min at 4°C; this was followed by incubation with FITC-, PE-, and PE-Cy5-conjugated mAbs for 30 min at 4°C in the dark. The cells were washed twice with a wash buffer, resuspended at 1×10^6 cells per tube in 1 ml PBS, and then analyzed with a FACSCaliber flow cytometer (BD Pharmingen) by using the Cell Quest program (BD Pharmingen). For each sample, 10 000 events were collected and analyzed for the expression of antigens.

2.9 Cytokine and chemokine assays

Cytokines and chemokines (IL-4, IL-6, IL-10, IL-13, IFN γ , MCP-1, 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 in the supernatants of BALF was measured by ELISA (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's protocol.

2.10 Statistical analysis

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

3. Results

3.1 Antigen-specific IgE

We examined the antigen-specific IgE levels in the serum (Fig. 1A). The TMA +/+^{low} treatment group (sensitized with test solution and challenged with low-dose test solution; relative unit value: 144 ± 116) showed a significant increase ($P < 0.05$) in

specific serum IgE levels relative to the $-/-$ group (sensitized and challenged with solvent; relative unit value = 1) and the $-/+$ group (chemical challenge only; relative unit value = 1). The TMA $+/+^{\text{high}}$ treatment group (sensitized with test solution and challenged with high-dose test solution; 227 ± 142) showed a significant increase ($P < 0.01$) in specific serum IgE levels relative to the $-/-$, $-/+$, and $+/-$ (chemical sensitization only; 64 ± 48) groups. The TDI $+/+$ treatment group (both low and high: 17 ± 25 and 30 ± 42) showed an increasing trend, but the reaction was mild and no statistically significant differences compared with control groups were observed. There was no increase in antigen-specific serum IgE level in the DNCB treatment groups.

We then examined antigen-specific IgE levels in BALF (Fig. 1B). The TMA $+/+^{\text{high}}$ treatment group (25 ± 29) showed a significant increase in specific BALF IgE levels relative to other treatment groups ($-/-$, $-/+$, $+/-$, and $+/+^{\text{low}}$). In contrast, analysis of antigen-specific BALF IgE levels induced by TDI and DNCB challenges revealed no increase.

3.2 Bronchoalveolar lavage fluid analysis

We examined the the total numbers of macrophages, eosinophils, and neutrophils in BALF cells (Fig. 2A, B, C). To discriminate the different cell types in

BALF we used a combination of anti-Gr-1 and -CD11c antibodies. We also examined production of MCP-1, IL-6, EOTAXIN, and MIP-1 β in BALF response to each chemical (Fig. 2D, E, F).

Elevations in total and differential cell counts and production of MCP-1, IL-6, EOTAXIN, and MIP-1 β were not observed in mice sensitized and challenged with TDI or DNCB. In contrast, TMA $+/+^{\text{high}}$ and TMA $+/+^{\text{low}}$ groups showed greatly increased numbers of eosinophils and high levels of MCP-1, IL-6, EOTAXIN, and MIP-1 β . The number of eosinophils was significantly higher in the $+/+^{\text{high}}$ group than in the control groups ($-/-$, $-/+$, and $+/-$) ($P < 0.01$). In the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, the numbers of eosinophils were 4.52 ± 2.4 ($\times 10^4$ cells, mean $\pm$ s.d.) and 12.92 ± 10.6 , respectively—increases of approximately 400% and 1000%, respectively, over the control groups. Production of MCP-1 was significantly higher in the $+/+^{\text{high}}$ group than in the control groups ($-/-$, $-/+$, and $+/-$) ($P < 0.01$). In the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of MCP-1 was 16.70 ± 5.8 (pg/ml, mean $\pm$ s.d.) and 134.60 ± 157.8 , respectively—increases of approximately 300% and 2500%, respectively, over the production in the control groups. In the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of IL-6 was 4.23 ± 2.9 (pg/ml, mean $\pm$ s.d.) and 46.06 ± 73.0 , respectively— increases of approximately 400% and 5000%, respectively, over the production in the control groups.

But the difference was not significant for this. Production of eotaxin was significantly higher in the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups than in the control groups ($-/-$, $-/+$, and $+/-$) ($P < 0.01$). In the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of eotaxin was 22.70 ± 12.0 (pg/ml, mean $\pm$ s.d.) and 34.08 ± 20.7 , respectively—increases of approximately 1000% and 1500%, respectively, over the control groups. Production of MIP-1 β was significantly higher in the $+/+^{\text{high}}$ groups than in the control groups ($-/-$, $-/+$, and $+/-$) ($P < 0.05$). In the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of MIP-1 β was 3.19 ± 3.9 (pg/ml, mean $\pm$ s.d.) and 9.43 ± 11.4 , respectively—increases of approximately 500% and 1000%, respectively, over the control groups.

In mice sensitized and challenged with TMA, TDI, or DNCB, the differential cell counts from the Cytospin preparations showed much the same patterns as those from the flow cytometric analysis (data are not shown).

3.3 Lung-associated-LN analysis

We examined the total numbers of lymphocytes and IgE-positive B cells ($B220^+ \text{IgE}^+$) in lung-associated LNs (Fig. 3A, B, C). In TMA-treated groups, the total number of lymphocytes was significantly higher in the $+/+^{\text{high}}$ group than in the $-/+$ group ($P < 0.05$). The numbers of IgE-positive B cells were significantly higher in the

+/+ groups (+/+^{low} and +/+^{high}) than in the control groups (-/-, -/+, and +/-) ($P < 0.01$). In the +/+^{low} and +/+^{high} groups, the populations of IgE-positive B cells were 23.84 ± 9.7 ($\times 10^5$ cells, mean $\pm$ s.d.) and 34.83 ± 19.5 , respectively—approximately 400% and 300% increases, respectively, over the control groups. In the TDI-treated groups, the total number of lymphocytes was significantly higher in the +/+ groups (+/+^{low} and +/+^{high}) than in the control groups (-/-, -/+, and +/-) ($P < 0.01$). The numbers of IgE-positive B cells were significantly higher in the +/+^{low} group than in the -/- and -/+ groups ($P < 0.05$). In the +/+^{low} and +/+^{high} groups, the populations of IgE-positive B cells were 16.52 ± 14.2 and 11.84 ± 9.4 , respectively—approximately 400% and 300% increases, respectively, over the control groups. However, in the DNCB-treated groups, no elevations of the total number of lymphocytes or the number of IgE-positive B cells were observed. In the +/+^{low} and +/+^{high} group, the respective populations of IgE-positive B cells were 5.82 ± 3.8 and 7.12 ± 2.0 .

We examined cytokine production (IL-4, IL-10, IL-13, and IFN- γ) in the lymph node supernatants of mice exposed to TMA, TDI, or DNCB (Fig. 3D, E, F). Marked increases in IL-4, IL-10, and IL-13 production were seen in the TMA and TDI treatment groups. Analysis of the IL-4 and IL-13 production induced by TMA treatment revealed significant differences between the +/+^{high} and control groups (-/-, -/+, and +/-) (IL-4,

$P < 0.05$ and IL-13, $P < 0.01$). Analysis of the IL-10 production induced by TMA treatment revealed significant differences between the $+/+$ ($+/+^{\text{low}}$ and $+/+^{\text{high}}$) and control groups ($-/-$, $-/+$, and $+/-$) ($+/+^{\text{low}}$; $P < 0.05$ and $+/+^{\text{high}}$; $P < 0.01$). Analysis of the IL-4, IL-10, and IL-13 production induced by TDI treatment revealed significant differences between the $+/+$ group ($+/+^{\text{low}}$ and $+/+^{\text{high}}$) and control groups ($-/-$, $-/+$, and $+/-$) ($P < 0.01$). However, there was no increase in IL-4, IL-10, or IL-13 production in the DNCB treatment group, and there was no significant difference between the $+/+$ ($+/+^{\text{low}}$ and $+/+^{\text{high}}$) and control groups ($-/-$, $-/+$, and $+/-$) in terms of the production of these cytokines in the DNCB-treated groups. When mice were treated with DNCB, production of IL-4 in the $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups was 4.92 ± 2.1 pg/ml and 4.32 ± 1.3 pg/ml, respectively (Fig. 4F). In the TMA $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of IL-4 was 8.65 ± 2.8 pg/ml and 10.97 ± 6.6 pg/ml, respectively—an approximately 200% increase over the DNCB treatment group. In the TDI $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of IL-4 was 19.76 ± 1.9 pg/ml and 17.33 ± 2.9 pg/ml, respectively—an approximately 400% increase over the DNCB treatment group. IL-10 production induced in the DNCB $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups was 9.89 ± 19.8 pg/ml and 2.97 ± 5.9 pg/ml, respectively. In the TMA $+/+^{\text{low}}$ and $+/+^{\text{high}}$ groups, production of IL-10 was 55.46 ± 36.8 pg/ml and 46.33 ± 21.0 pg/ml, respectively—an approximately 600%

increase over the DNCB treatment group. In the TDI $+/\text{+}^{\text{low}}$ and $+/\text{+}^{\text{high}}$ groups, production of IL-10 was 112.40 ± 39.1 pg/ml and 143.60 ± 66.6 pg/ml, respectively—an approximately 1500% increase over the DNCB treatment group.

In contrast, significant differences in IFN- γ production were revealed only in the TDI treatment groups and only between the $+/\text{+}$ ($+/\text{+}^{\text{low}}$ and $+/\text{+}^{\text{high}}$) and the control groups ($-/-$, $-/+$, and $+/-$) ($P < 0.01$). In the TDI $+/\text{+}^{\text{low}}$ and $+/\text{+}^{\text{high}}$ groups, production of IFN- γ was 722.60 ± 330.3 pg/ml and 881.80 ± 375.5 pg/ml, respectively—an approximately 2000% increase over the TMA and DNCB treatment groups.

In the DNCB-treated groups, no elevation in cytokine production (IL-4, IL-10, IL-13, or IFN- γ) was observed.

DISCUSSION

Our primary objective was to upgrade our method of detecting environmental chemical-related respiratory hypersensitivity at weakly immunogenic and low doses. According to our results, low-level respiratory allergic responses were detected by our model by using long-term dermal sensitization followed by an intratracheal challenge. Indeed, TMA induced prominent allergic responses in parameters including IgE level (antigen-specific IgE level and IgE-positive B cell population), eosinophil proliferation,

local chemokine level (MCP-1, MIP-1 β , and eotaxin) and cytokine level (IL-4, IL-10, and IL-13). TDI induced significant increases in IgE-positive B cell populations and local cytokine production (IL-4, IL-10, IL-13, and IFN- γ). In contrast, DNCB sensitization groups yielded, at most, small, nonsignificant increases in all parameters.

Because we were particularly interested in low-level allergic reactions to the environment, we focused on the EC3 values (the concentration of a chemical required to elicit a threshold positive response) in local lymph node assay (LLNA). The EC3 values were calculated from ^3H -TdR incorporation data in the LLNA (Basketter et al., 1999). The EC3 value is an estimate of the amount of the test solution required to induce a stimulation index (SI, calculated by dividing the mean ^3H -TdR incorporation value for each treatment group by that of the solvent control group) of 3 (Basketter et al., 1999). In the standard LLNA, the criterion for a positive response is an SI of 3 or greater (Dearman et al., 1999; Basketter et al., 2002; Dearman et al., 1999; Gerberick et al., 2000). According to our previous LLNA results, the EC3 values of TMA, TDI, and DNCB in Balb/c mice were 0.20%, <0.03%, and 0.06%, respectively (Fukuyama et al., 2008). To confirm the validity of our model, we adopted a sensitization dose that was less than the EC3 values and a challenge dose that was 10 or 100 times lower than the sensitization dose (see Table 1).

To detect environmental chemical–related respiratory hypersensitivity at weakly immunogenic and low doses, we focused on several immune endpoints in this study: IgE antibody levels, eosinophil proliferation, and local chemokine and cytokine levels. Above all, the IgE antibody is a central marker of chemically induced respiratory allergy. Antigen-specific serum IgE levels and IgE-positive B cells from most draining LNs are especially useful for the detection of several types of allergies (Ban et al., 2006; Goutet et al., 2005; Kimber and Dearman, 2002; Manetz and Meade, 1999). Here, we analyzed the antigen-specific IgE levels (serum and BALF) by ELISA and the IgE-expressing B-cell population in lung-associated LNs by flow cytometry. According to our results, TMA induced significant increases in antigen-specific IgE levels (in both serum and BALF) and in IgE-expressing B-cell populations (in LNs) (see Figs. 1 and 3). In contrast, there was no increase in antigen-specific IgE levels and IgE-expressing B-cell populations in the DNCB treatment groups (see Fig. 1 and 3). Because TMA is a potential respiratory allergen (Ban et al., 2006; Vanoirbeek et al., 2003) and DNCB causes delayed-type hypersensitivity reactions but lacks the potential to cause respiratory hypersensitivity (Dearman and Kimber, 2000, Dearman et al., 1996, 2002, 2005), our TMA and DNCB results would be acceptable for characterizing chemical allergens. However, although TDI is recognized as a leading cause of respiratory allergy

(Ban et al., 2006; Chan-Yeung and Lam, 1986), our results revealed that TDI induced a significant increase only in the IgE-expressing B-cell population in the LNs. In terms of antigen-specific IgE levels, increasing trends were observed only in serum samples, but the reaction was mild and statistical significance was not shown.

Along with IgE levels, the airway responses to TDI presented here were unexpected in terms of our current understanding of airway allergic responses to an allergen challenge. In general, airway allergen challenge causes cellular inflammation in the airways, which is dominated by eosinophils, in both humans (Kroegel et al., 1994; Lee et al., 2005) and mice (Lee et al., 2005; Ohkawara et al., 1997). It has been suggested that eosinophils contribute to several of the clinical features of respiratory allergy, including tissue damage and airway hyperresponsiveness (Lotvall et al., 1998). Furthermore, eosinophils also have the capacity to release substantial amounts of allergy-related chemical mediators, such as MCP-1, eotaxin, and MIP-1 β (Janway et al., 2004). Therefore, we analyzed the eosinophil proliferation and chemokine levels (MCP-1, eotaxin, and MIP-1 β) in BALF by flow cytometry. In our study, eosinophil proliferation and the production of various chemokines in BALF cells from TMA-sensitized and challenged mice were enhanced, but TDI- and DNCB- sensitized and challenged mice showed no increase (see Figure 2).

The mechanism of chemical-induced respiratory allergy is dependent on Th2 type cytokines (Andersson et al., 2000). Th2 cells produce IL-4, IL-10, and IL-13 (Mosmann and Coffman, 1989; Yssel and Groux, 2000), and these responses are down-regulated by Th1 cytokines (IFN- γ) (Gavett et al., 1995; Lack et al., 1996). Previous reports have shown that LN cells draining the site of dermal application of TMA- or TDI- induced respiratory allergy in murine models preferentially secrete Th2 cytokines including IL-4, IL-10, and IL-13 (Dearman et al., 2003; Plitnick et al., 2002). In our study, TMA induced a significant increase in the levels of Th2 cytokines (IL-4, IL-10, IL-13) produced by restimulated ex vivo lymph node cells (see Fig. 3). This up-regulation was associated with a subsequent increase in IgE production in BALF. In contrast, there was no increase in local Th2 cytokine production in DNCB-treated groups, and this lack of reaction was associated with the lack of increase in IgE production in the BALF analysis (see Fig. 3). It is possible that the lack of increase of IFN- γ (Th1 cytokines) in the DNCB treatment groups is partly a result of the choice of challenge route and the doses of chemicals used to sensitize and challenge (Ban et al., 2006). However, in the TDI treatment group, despite the prominent local cytokine production in the LNs and BALF reactions showed almost no increase. The local cytokine increases we observed in the LNs could not be correlated with reactions in

serum and BALF analysis.

In recent years, several investigators have shown that dermal sensitization followed by airway challenge results in disparate immediate and late airway reactions that do not correspond to the elevated levels of IgE antibodies and cytokine production in the LNs draining the site of application (Ban et al., 2006; Dearman et al., 2002; Farraj et al., 2007; Fukuyama et al., 2008; Goutet et al., 2005; Manetz and Meade, 1999; Vandebriel et al., 2003). Farraj et al. (2007) demonstrated that Th2 cytokine production in LNs and serum IgE levels did not predict airway hypersensitivity to intranasal TDI exposure in mice. Kim et al. (1997) demonstrated that specific IgE antibodies to TDI were not associated with TDI-induced asthma. Fukuyama et al. (2008) showed elevations in the IgE-expressing B-cell population and Th2 cytokine levels following DNCB exposure without an increase in antigen-specific serum IgE levels after dermal DNCB exposure in mice. In addition, the potential of the contact allergen DNCB to induce a respiratory allergic reaction, as shown, for example, by an increase in the number of neutrophils in BALF and up-regulation of mRNAs for the chemokines in BALF cells, was examined in the highly Th2-polarized Brown Norway rat subjected to DNCB airway exposure (Kuper et al., 2008). These previous reports suggest that the responses to several types of allergens are dependent on many factors, including

differences in experimentation, e.g., sensitizing routes, doses of chemical used, application methods, and animal species. However, the important thing is not to overlook the minute signs and symptoms triggered when affected patients come into contact with a number of environmental chemical products at low doses. For that purpose, we focused on the detection of weak allergic reactions and attempted to develop a method for detecting environmental chemical allergens. We elicited positive reactions using typical allergens. However, whether the same events occur after sensitization by other chemicals that appear in many places in the environment remains uncertain. Experiments are currently under way in our laboratories.

In summary, our method detected low-level chemical-induced respiratory allergic responses, such as increased IgE levels, eosinophil proliferation, and increased local chemokine and cytokine levels. However, our results indicate that TDI may induce less intense respiratory allergic reactions than the current understanding of TDI responses suggests. As such, it should be recognized that the results of sensitization assays with several types of allergens might be greatly influenced by the sensitizing route, the dose of chemical, and the animal species used.

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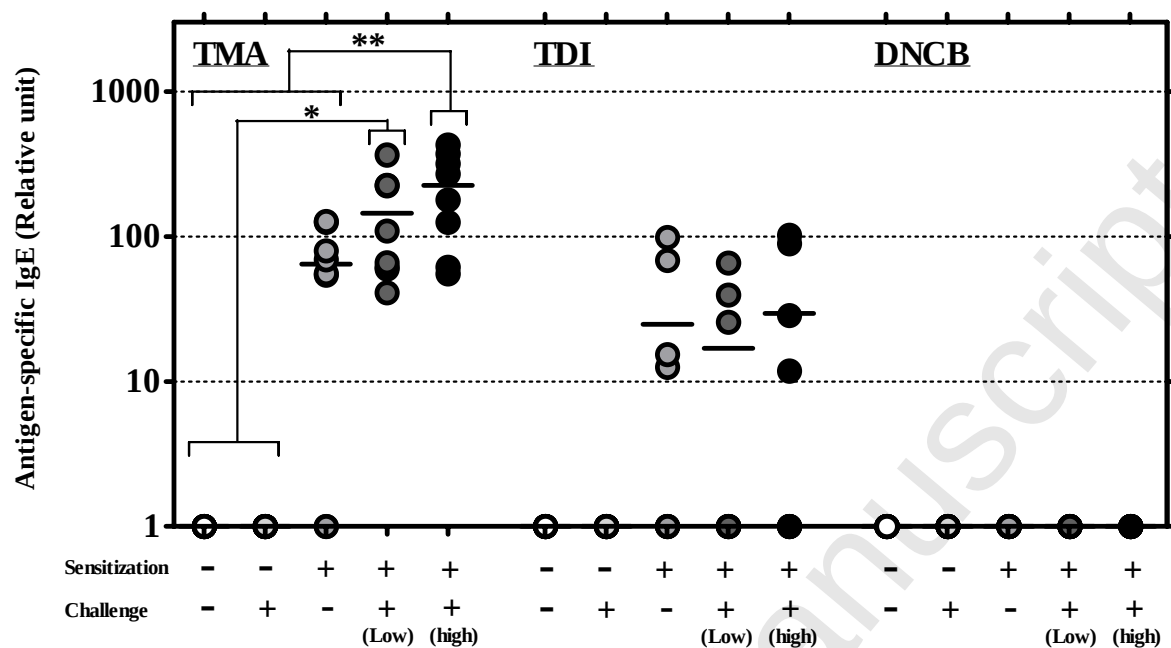
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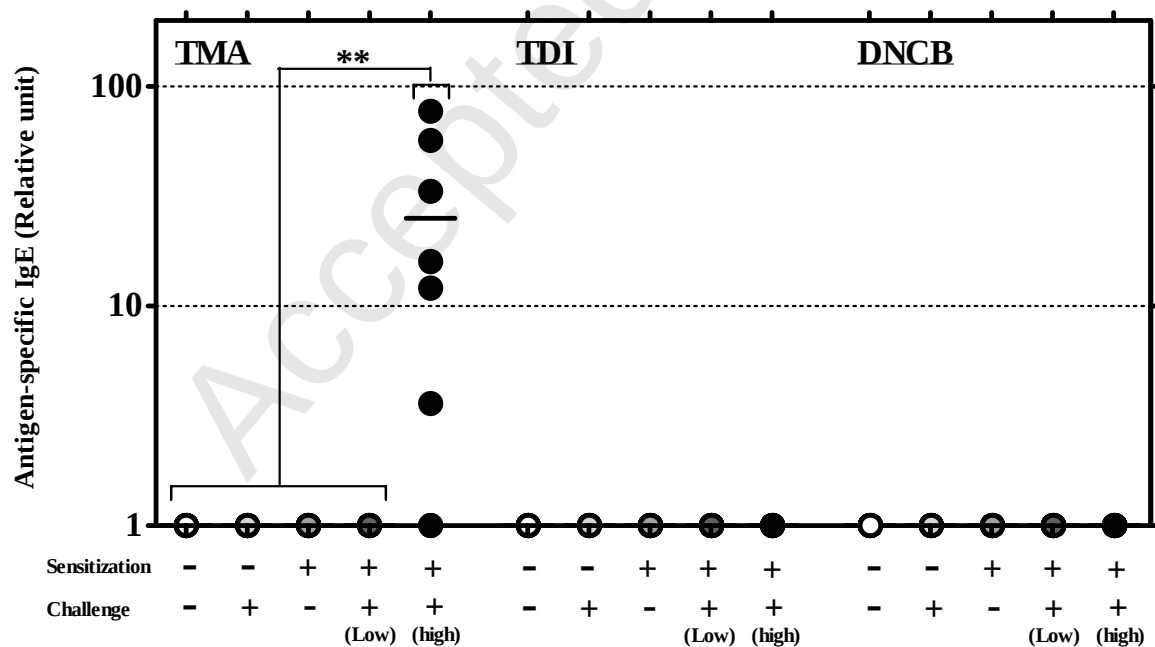
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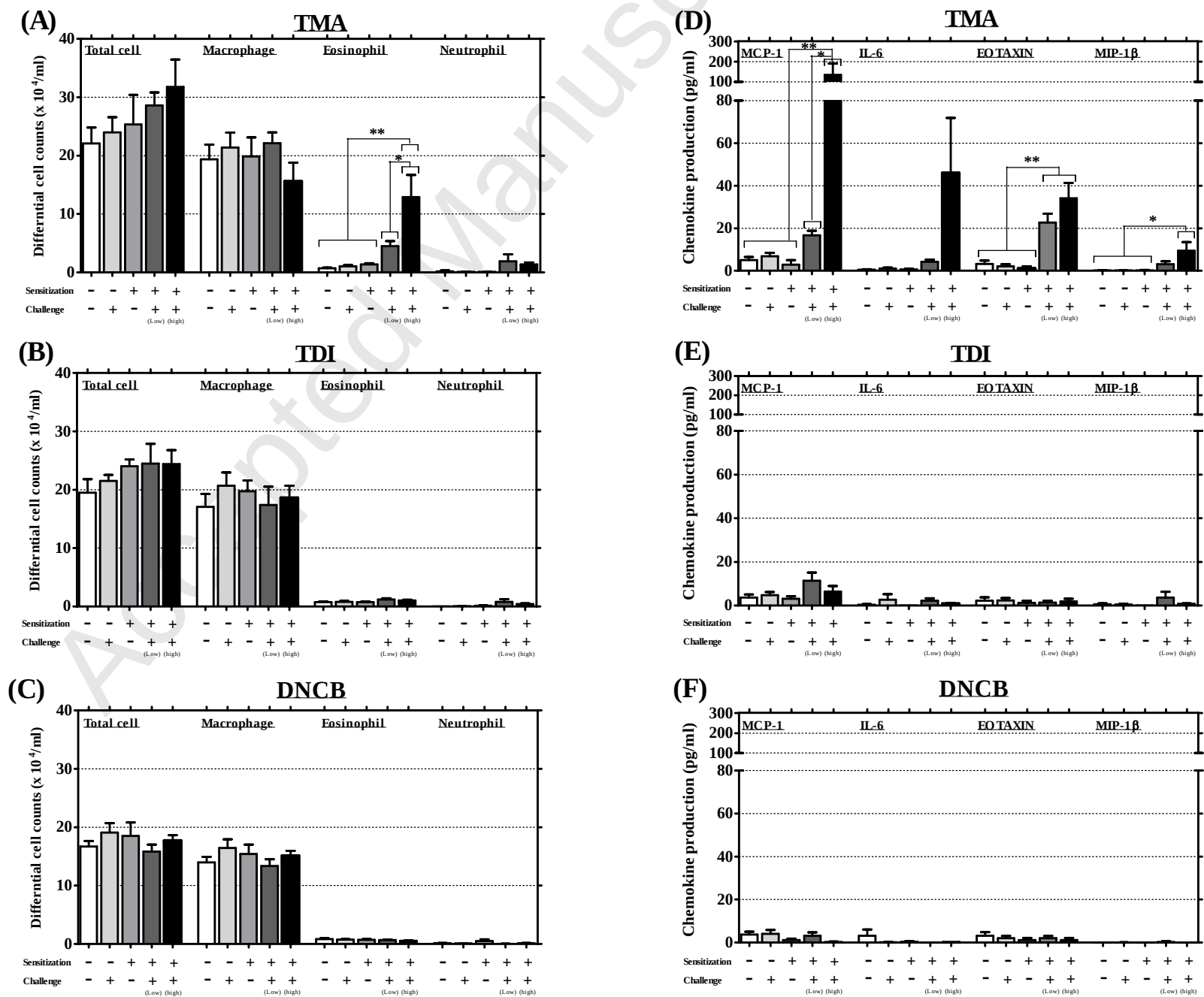
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(A)



(B)





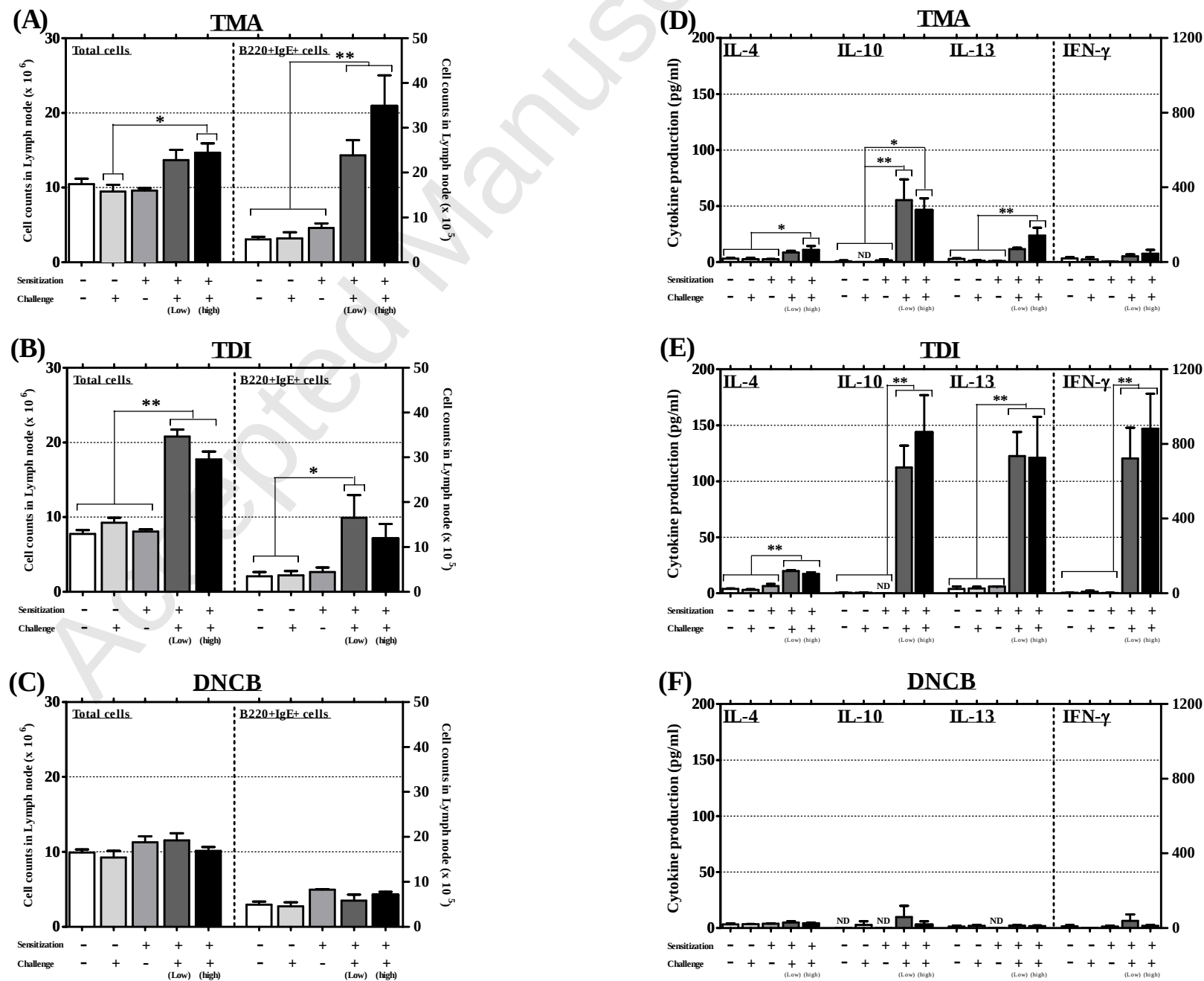


Figure Legends

FIG. 1. Antigen-specific IgE levels in mouse serum (A) and BALF (B) isolated 1 day after challenge with test solution. Results are expressed as mean (titer) $\pm$ S.D. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test).

FIG. 2. Chemokine levels (A: TMA; B: TDI; C: DNCB), and total and differential counts of cells (D: TMA; E: TDI; F: DNCB) in BALF of mice treated with test solution. Chemokine levels are expressed as means (pg/mouse) $\pm$ S.D. Total and differential counts are expressed as means (cells) $\pm$ S.D. To determine the chemokine levels, BALF were assayed by cytometric bead array. BALF cells were differentiated by using 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 $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test).

FIG. 3. Total numbers of lymphocytes and numbers of B220⁺ IgE⁺ cells (A: TMA; B: TDI; C: DNCB), and cytokine production (IL-4, IL-10, IL-13 and IFN- γ) (D: TMA; E: TDI; F: DNCB) in lung-associated lymph nodes of mice treated with test solution or solvent alone. Numbers of lymphocytes are expressed as mean (cells) $\pm$ S.D. Cytokine productions are expressed as mean (pg/mouse) $\pm$ S.D. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test). ND = none detected.

Table 1 Chemical concentrations used

Groups	Sensitization	Challenge
TMA	0.1%	Low: 0.001% High: 0.01%
TDI	0.01%	Low: 0.0001% High: 0.001%
DNCB	0.03%	Low: 0.0003% High: 0.003%

TMA: trimellitic anhydride

TDI: toluene diisocyanate

DNCB: 2,4-dinitrochlorobenzene