Toxicology xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Dysregulation of immune responses in an allergic mouse model following low-level toluene exposure

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ARTICLE INFO

Article history:
Received 16 March 2011
Received in revised form 18 April 2011
Accepted 5 May 2011
Available online xxx

Keywords:
Toluene
Inflammation
Transcription factor
Cytokine
Mice

ABSTRACT

To investigate the effect of low-level toluene inhalation on immune regulation in an allergic mouse model, C3H/HeN mice were exposed to 0, 5, 50, or 500 ppm of toluene for 6 h/day, 5 days/week for 3 or 6 weeks. For allergic mouse model, half of the mice in each group were immunized with ovalbumin (OVA). Allergic mice exposed to toluene for 3 weeks did not exhibit any changes in their plasma, lung or spleen samples. Although exposure to toluene alone for 6 weeks did not increase the number of inflammatory cells in bronchoalveolar lavage (BAL) fluid, coexposure to 50 ppm toluene and OVA increased the number of BAL cells. Histological changes and increased amounts of fibronectin were observed in the lungs of OVA-immunized, 50-ppm-toluene-exposed mice. Exposure to 500 ppm significantly increased the expressions of transcription factors STAT3, STAT4 and STAT5a mRNAs in spleen. In spleens from the allergic mouse model, the expressions of STAT3, STAT4, STAT5a, STAT6, GATA3 and Foxp3 mRNAs were significantly enhanced following exposure to 50 ppm toluene for 6 weeks, but the expression of T-bet mRNA was not increased. Regarding the Th1/Th2 balance, the expressions of IL-4 and IL-12 mRNAs were enhanced in the spleens of toluene-exposed mice. Total IgG1 antibody production in the plasma was significantly increased in the 50-ppm-toluene-exposed allergic mouse model. These results indicate that low-level toluene exposure might dysregulate the allergic responses to OVA in C3H/HeN mice.
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1. Introduction

Air pollution with volatile organic compounds (VOCs) is common in industrialized countries and leads to increased morbidity in susceptible individuals (World Health Organization [WHO] Regional Office for Europe, 2002; Venn et al., 2003). Epidemiological studies have indicated that domestic exposure to VOCs may increase the risk of childhood asthma and of allergic sensitization to food allergens (Rumchev et al., 2004; Lehmann et al., 2001). Toluene is a well-known VOC and a toxic indoor air pollutant that can occur at relatively high concentrations in homes and buildings, and chronic low-level exposure to this substance can impair immune functions. In assessments of the adverse effect of toluene, concentrations of 40 ppm (according to the European Union) and 88 ppm (according to WHO) have been reported to correspond to a "no observed adverse effect level" (Andersen et al., 1983; Foo et al., 1990). In Japan, the occupational exposure limit for toluene proposed by the Japan Society for Occupational Health is 50 ppm (Japan Society for Occupational Health, 1994), while the recently updated threshold limit value is 20 ppm in the United States (ACGIH, 2006). At present, evidence regarding the health implications of VOCs at concentrations commonly found inside houses remains insufficient.

Our previous studies using nose-only inhalation demonstrated that exposure to low levels of toluene aggravates airway inflammatory responses by modulating the number of inflammatory cells and by enhancing the plasma levels of nerve growth factor (NGF) in an allergic mouse model, compared with the effects in healthy normal models (Win-Shwe et al., 2007). Furthermore, the long-term exposure of male C3H/HeN mice to toluene increased the expression of NGF and TrkA mRNAs in the lungs of the immunized mice (Fujimaki et al., 2009a). We also investigated the role of T lymphocytes in NGF production following toluene exposure in immunized male C3H/HeN mice treated with anti-CD4 mAb (Fujimaki et al., 2009b). Treatment with anti-CD4 mAb completely abrogated the increased NGF content in the plasma, suggesting that $CD4^+$ T cells may be involved in the toluene-induced modulation of systemic NGF production. Our recent study indicated that exposure to low levels of toluene upregulated the expression of neurotrophins and their receptors in the adult mouse hippocam-

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doi:10.1016/j.tox.2011.05.002

Please cite this article in press as: Fujimaki, H., et al., Dysregulation of immune responses in an allergic mouse model following low-level toluene exposure. Toxicology (2011), doi:10.1016/j.tox.2011.05.002

pus in a strain-dependent manner and in accordance with allergic stress (Win-Shwe et al., 2010a). However, the biological mechanisms that occur via the neuro-immune axis and that regulate the response to toluene exposure are not fully understood.

As for the immunotoxicity of toluene, the exposure of male mice to low levels of toluene resulted in the induction of IL-2 production and the activation of the transcription factors NF-κB, the signal transducers and activators of transcription (STAT)5 and NF-AT in thymocytes (Liu et al., 2009). Occupational exposure to mixed organic solvents decreased the number of natural killer cells and T-lymphocyte subpopulations and increased the number of B lymphocytes from the peripheral blood (Tanigawa et al., 2001). However, the mechanism by which VOC might facilitate the development of allergic inflammation remains unclear, and the adverse effects of VOCs, which are major indoor pollutants, on the induction or augmentation of airway inflammation have not yet been fully characterized.

We hypothesized that exposure to low levels of toluene may aggravate allergic inflammatory responses through its effects on transcription factors. Therefore, the present study was designed to investigate whether whole body exposure to low levels of toluene enhances the immune response in lung and spleen using an allergic mouse model.

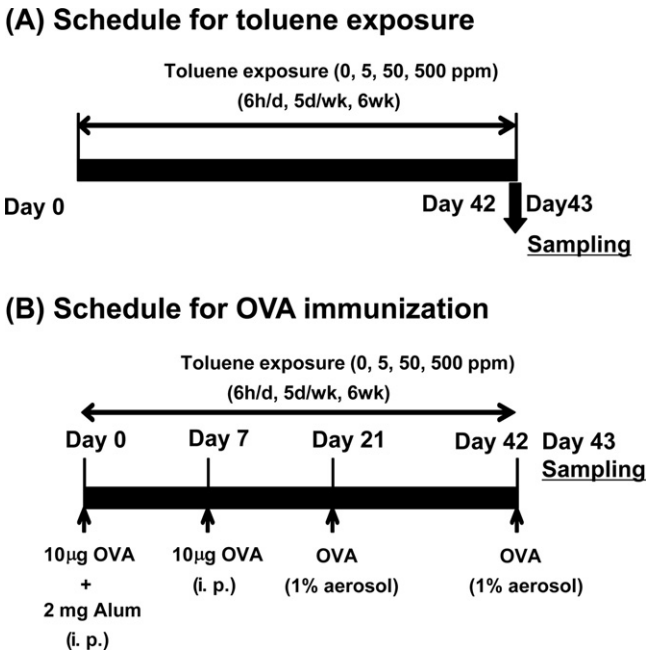

Fig. 1. Schedule for toluene exposure and OVA immunization.

2. Materials and methods

2.1. Animals

Seven-week-old male C3H/HeN mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and were used at an age of 8 weeks. Food and water were given *ad libitum*. The mice were housed in plastic cages under controlled environmental conditions (temperature, 23 ± 0.5 °C; humidity, 50 ± 5%; lights on between 07:00 and 19:00 h). This study was approved by the Ethics Committee for Experimental Animals of the University of Occupational and Environmental Health, Japan.

2.2. Experimental design

To investigate the effect of low-level toluene inhalation on immune responses to ovalbumin (OVA), C3H/HeN mice ($n = 6$) were exposed to either a filtered air control (0 ppm), 5, 50 or 500 ppm of toluene for 6 h (from 10:00 to 16:00 h) per day, 5 days per week for 3 or 6 weeks. One day following the final toluene inhalation, BAL fluid, lung, spleen and blood samples from six mice in each group were collected as described previously (Fujimaki et al., 2004). The allergic mouse model group was injected intraperitoneally with 10 μg of OVA plus 2 mg of alum on day 0 and with 10 μg of OVA alone on day 7; each of these mice was then challenged with nebulized OVA as a booster on days 21 and 42 (Fig. 1).

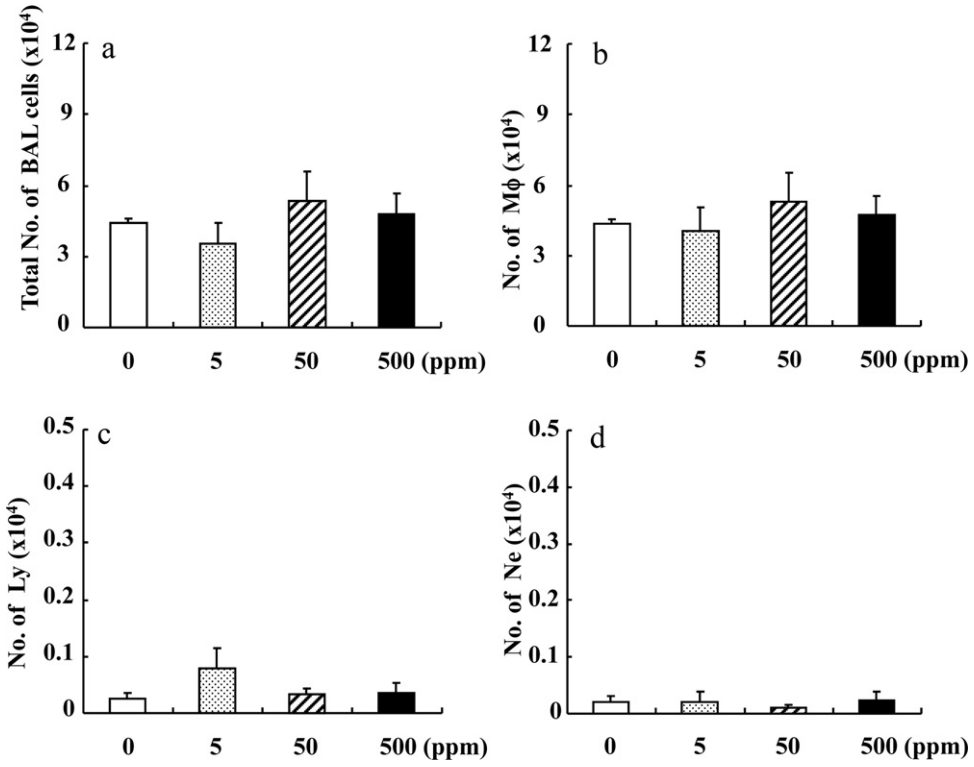

Fig. 2. Changes in the number of BAL fluid cells and inflammatory cells in OVA-immunized C3H/HeN mice exposed to toluene for 3 weeks. (a) Total BAL fluid cells, (b) number of macrophages, (c) number of lymphocytes, (d) number of neutrophils. Each bar represents the mean ± SE ($n = 6$).

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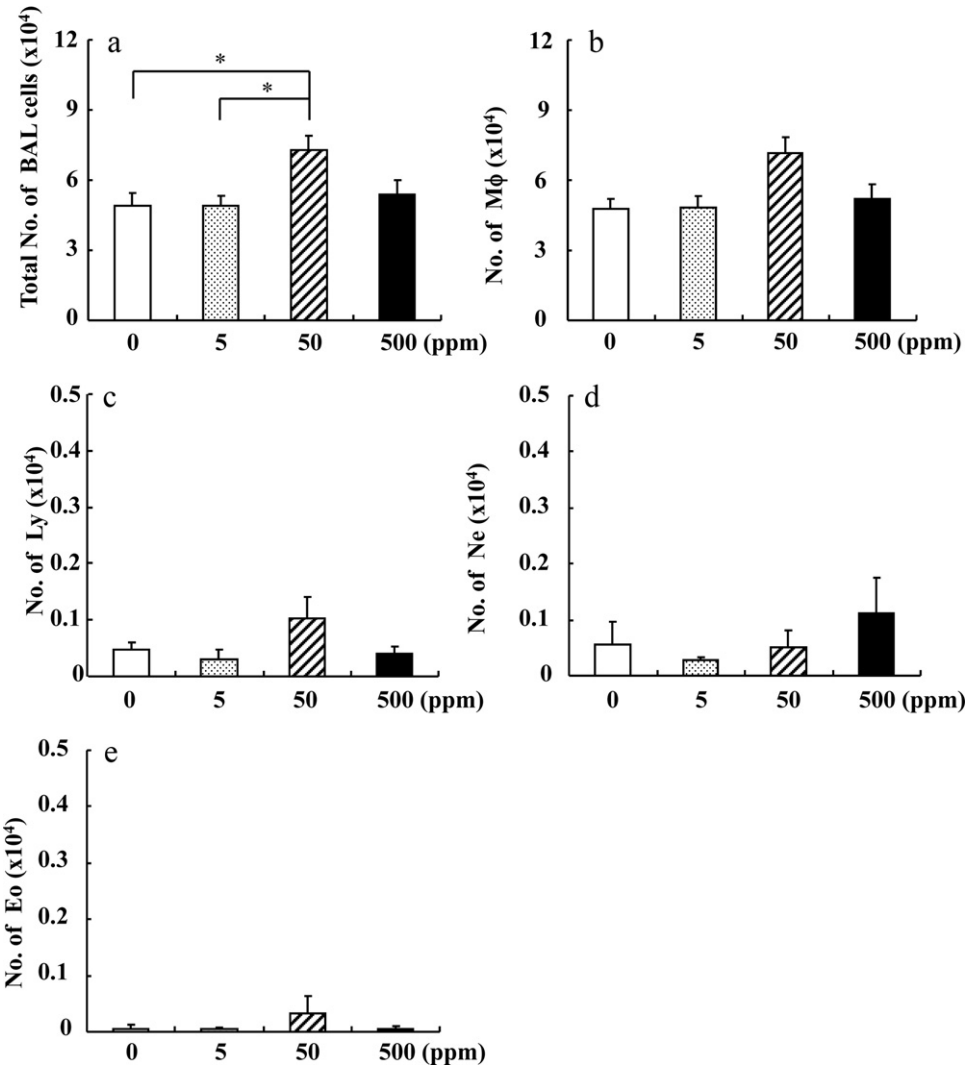

Fig. 3. Changes in the number of BAL fluid cells and inflammatory cells in OVA-immunized C3H/HeN mice exposed to toluene for 6 weeks. (a) Total BAL fluid cells, (b) number of macrophages, (c) number of lymphocytes, (d) number of neutrophils, (e) number of eosinophils. Each bar represents the mean ± SE ($n = 6$, $^*P < 0.05$).

2.3. Generation of toluene

Toluene vapor was generated using an organic solvent gas generator (Shibata Scientific Technology, Ltd.) and was diluted with clean, filtered air to achieve the desired gas concentrations. The vapor was then introduced into a stainless steel and glass chamber, as described previously (Hori et al., 1999). The average toluene levels (mean ± SD) in the 5-ppm, 50-ppm, and 500-ppm exposure chambers were 4.9 ± 0.4, 50.5 ± 2.0, and 505 ± 11.8 ppm, respectively.

2.4. Quantitative real-time PCR

One day following the final toluene inhalation, the mice were sacrificed under deep pentobarbital anesthesia and the spleens were collected from each group of mice, frozen quickly in liquid nitrogen, and then stored at −80 °C until the total RNA was extracted. The expression levels of STAT3, STAT4, STAT5a, STAT6, T-bet, Foxp3, GATA3, IL-4, IL-12, IL-10 and 18S mRNA in the lung were estimated using quantitative real-time PCR analysis with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Inc., USA), as described previously (Win-Shwe et al., 2007). Tissue 18S mRNA levels were examined as an internal control. The primer sequences were as follows: 18S forward, 5′-TACCACATCCAAGAAGGCAG-3′; and 18S reverse, 5′-TGCCCTCCAATGGATCCTC-3′. Some primers (STAT3, ID_20848; STAT4, ID_20849; STAT5a, ID_20850; STAT6, ID_20852; T-bet, ID_57765; Foxp3, ID_20371; GATA3, ID_14462; IL-4, ID_16189; IL-12, ID_16159; and IFN-γ, ID_15978) were purchased from Qiagen, Sample & Assay Technologies (Qiagen GmbH, Germany). Data were analyzed using the comparative threshold cycle method. The relative expression levels of each mRNA were normalized individually according to the 18S content in the respective samples and were expressed as mRNA signals per unit of 18S.

2.5. FACS analysis

We analyzed the immunophenotypes of the splenic cells derived from the toluene-exposed mice by fluorescence activated cell sorter (FACS) analysis using the following monoclonal antibodies purchased from PharMingen (San Diego, CA): phycoerythrin-labeled rat anti-CD4 IgG (clone GK 1.5), fluorescein isothiocyanate-labeled rat anti-CD8a IgG (clone53-6.7), phycoerythrin-labeled hamster anti-CD3 IgG (clone 145-2C11), fluorescein isothiocyanate-labeled rat anti-CD19 IgG (clone RA3-6B2), and the corresponding isotype-matched controls. The cell samples were analyzed on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, Mansfield, MA).

2.6. Bio-Plex protein array system

Cytokine production in BAL fluid was measured in duplicate using the Bio-Plex protein array system (Bio-Rad), according to the instructions of the manufacturer. Briefly, 50 μL of a mixture of the 8 anti-cytokine-conjugated bead families was added to a 96-well filter plate prewetted with assay buffer. Fifty microliters of the lung samples or serially diluted cytokine standards were added to the filter plate containing the beads, followed by mixing at room temperature with mild agitation for 60 min. The cytokine-bound beads were washed three times with wash buffer using a filter manifold and incubated for 30 min at room temperature with 50 μL of biotin-conjugated detection antibodies. Bead-cytokine-antibody complexes were washed three times with wash buffer and incubated for 10 min at room temperature with phycoerythrin-conjugated streptavidin. The complexes were washed three times, resuspended in 125 μL of assay buffer, and the cytokine levels were measured in a Bio-Plex instrument using Bio-Plex manager software (Bio-Rad).

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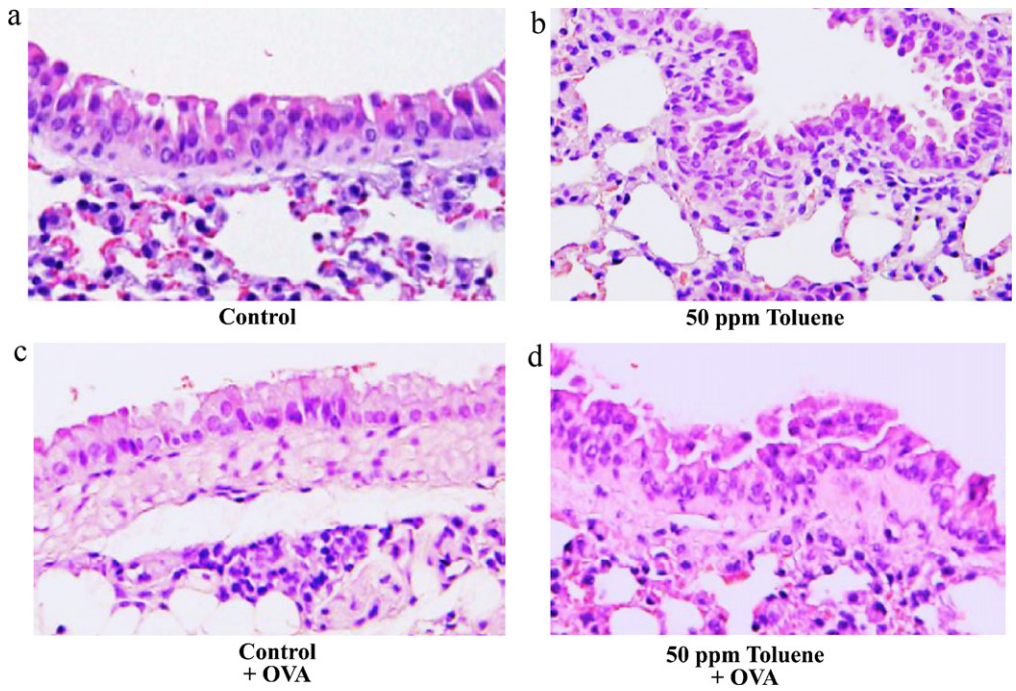

Fig. 4. Histological analysis of the lungs of C3H/HeN mice following the inhalation of 50 ppm of toluene for 6 weeks with or without OVA. (a) Control air, (b) 50 ppm of toluene, (c) control air + OVA, and (d) 50 ppm of toluene + OVA treatment groups (×400).

2.7. Measurement of total and anti-OVA IgE, IgG_1, and IgG_{2a} titers in plasma

Plasma total IgG_1 and IgG_{2a} antibodies were measured using enzyme-linked immunosorbent assay (ELISA), as previously described (Fujimaki et al., 2004). The ODs at 490 and 595 nm were determined using a microplate reader (Bio-Rad Laboratories). Total IgE antibodies in the plasma were measured using mouse ELISA kits (Seikagaku Co., Tokyo). The plasma titers of antigen-specific IgE, IgG_1 and IgG_{2a} antibodies were also measured using ELISA.

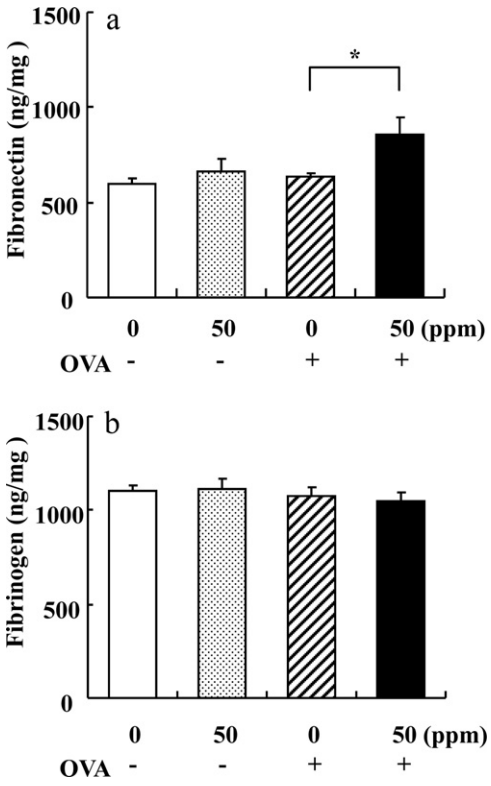

Fig. 5. Contents of fibronectin and fibrinogen in the lungs of C3H/HeN mice exposed to 50 ppm of toluene for 6 weeks with or without OVA immunization. (a) Fibronectin, and (b) fibrinogen. Each bar represents the mean ± SE ($n = 6$, $^*P < 0.05$).

2.8. Analysis of fibrinogen and fibronectin levels

Using a cell extraction buffer system (Invitrogen Corporation, CA), lung samples of whole cell lysates were prepared, the protein concentrations were determined, and the samples were stored at −80 °C. The concentrations of fibrinogen and fibronectin in the lungs were quantified using ELISA, in accordance with the manufacturer's directions (Immunology Consultants Lab., Inc., USA). Absorbance was measured using a microplate reader (BioRad, Model 550) at wavelengths of 450 and 630 nm.

2.9. Histological analysis

Lungs were removed from five mice in the control and toluene-exposed groups with or without OVA immunization after the animals had been deeply anesthetized with sodium pentobarbital; the lungs were then fixed with 10% formalin. The fixed lungs were dehydrated using a graded series of ethanol, cleared with xylene, and embedded in paraffin. Coronal paraffin sections were cut at a thickness of 4 μm using a cryostat and were mounted on 3-aminopropyltriethoxysilane-coated glass slides. Each section was stained with hematoxylin and eosin alone for histological examination.

2.10. Statistical analysis

All data were expressed as the mean ± standard error (S.E.). The statistical analysis was performed using the StatMate II statistical analysis system for Microsoft Excel, Version 5.0 (Nankodo, Inc., Tokyo, Japan). Dose–response data were analyzed using a one-way analysis of variance with a Bonferroni/Dunn post hoc analysis. Individual comparisons between groups were confirmed using the Student *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in the number of BAL cells and cytokine production

To investigate the effect of low-level toluene exposure on airway inflammation, we examined the number of BAL cells from C3H/HeN mice immediately after exposure to toluene for 3 or 6 weeks. As shown in Fig. 2, the exposure of immunized mice to toluene for 3 weeks resulted in no significant increase in BAL cells. In OVA-immunized C3H/HeN mice exposed to toluene for 6 weeks,

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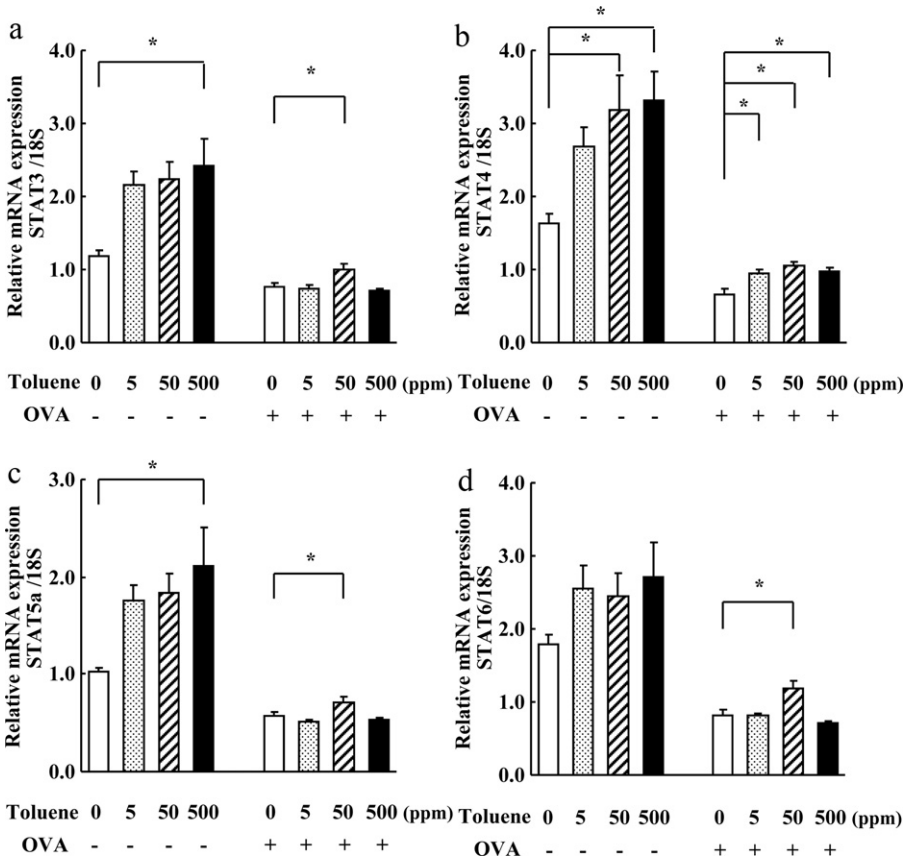

Fig. 6. Expression of STATs mRNAs in the spleens of non-immunized, and OVA-immunized mice following toluene inhalation. (a) STAT3, (b) STAT4, (c) STAT5a, (d) STAT6 mRNAs in C3H/HeN mice following exposure to toluene for 6 weeks. Each bar represents the mean ± SE ($n = 6$, $^*P < 0.05$).

the number of BAL cells from mice exposed to 50 ppm of toluene was significantly higher than that in control mice (Fig. 3a) and the number of macrophages, lymphocytes and eosinophils tended to be elevated, although the differences were not statistically significant. In non-immunized C3H/HeN mice, no significant increase in BAL cells from mice exposed to toluene for 3 or 6 weeks was observed (data not shown).

Regarding cytokine production, no significant increases in the production of IL-1ß, IL-2, GM-CSF, IFN-γ, or TNF-α were seen in BAL fluid from toluene-exposed, or toluene-exposed, OVA-immunized mice (data not shown).

3.2. Histological analysis in the lung

Next, we examined histological changes in the lungs of toluene-exposed C3H/HeN mice. Compared with the lungs of the control, nonimmunized C3H/HeN mice, slight hyperplasia was observed in the bronchial epithelial cells after the inhalation of 50 ppm of toluene, whereas the accumulation of macrophages and neutrophils was not detectable in the lung sections of the mice (Fig. 4b). An increase in the hypertrophy and hyperplasia of the epithelial cells and mucus secretion in the lung was observed in the 50-ppm-toluene-exposed, OVA-immunized mice (Fig. 4d). However, no marked accumulation of inflammatory cells in the alveoli was observed. In oxidant-mediated lung injury, endogenous ligands such as fibronectin, fibrinogen and hyaluronan stimulate the TLR4 signaling pathway to induce an inflammatory response (Zhang et al., 2005; Jiang et al., 2006). We measured the contents of fibrinogen and fibronectin as useful markers for inflammation in the lungs from 50-ppm-toluene-exposed mice. As shown in Fig. 5, the levels of fibrinogen in lungs from control mice, 50-ppm-toluene-exposed mice, OVA-immunized mice, and 50-ppm-toluene-exposed plus OVA-immunized mice were 1106 ± 29, 1114 ± 57, 1078 ± 40, 1051 ± 40 ng/mg protein, respectively. The levels of fibronectin in lungs from control mice, 50-ppm-toluene-exposed mice, OVA-immunized mice, and 50-ppm-toluene-exposed plus OVA-immunized mice were 598 ± 27, 666 ± 65, 634 ± 23, 858 ± 93 ng/mg protein, respectively. Coexposure to OVA and 50 ppm of toluene significantly increased the levels of fibronectin in the lungs of mice, compared with OVA-immunized group ($P < 0.05$).

3.3. Expression of cytokine and transcription factor mRNAs in the spleen

We analyzed the immunophenotypes of the splenic cells derived from the toluene-exposed, or toluene-exposed and OVA-immunized mice. In non-immunized, or OVA-immunized mice, exposure to various concentrations of toluene for 6 weeks induced no significant changes in the percentage of CD3-, CD4-, CD8- or CD19-positive cells in the spleen (data not shown).

Next, we examined the expression of transcription factors and cytokine mRNAs related to the Th1/Th2 balance to clarify the factors related to sensitivity to low levels of toluene exposure. Activation of STATs is involved in differentiation of Th1 and Th2 type T cells. In non-immunized C3H/HeN mice, exposure to 500 ppm of toluene significantly increased the expressions of STAT3, STAT4, STAT5a mRNAs in spleen ($P < 0.05$, Fig. 6a–c). In OVA-immunized C3H/HeN mice, exposure to 50 ppm of toluene significantly increased the expressions of STAT3, STAT5a, STAT6 mRNAs. The expression of STAT4 mRNA was markedly increased by 5, 50, 500 ppm of toluene (Fig. 6b). In OVA-immunized C3H/HeN mice, exposure to 50 ppm of

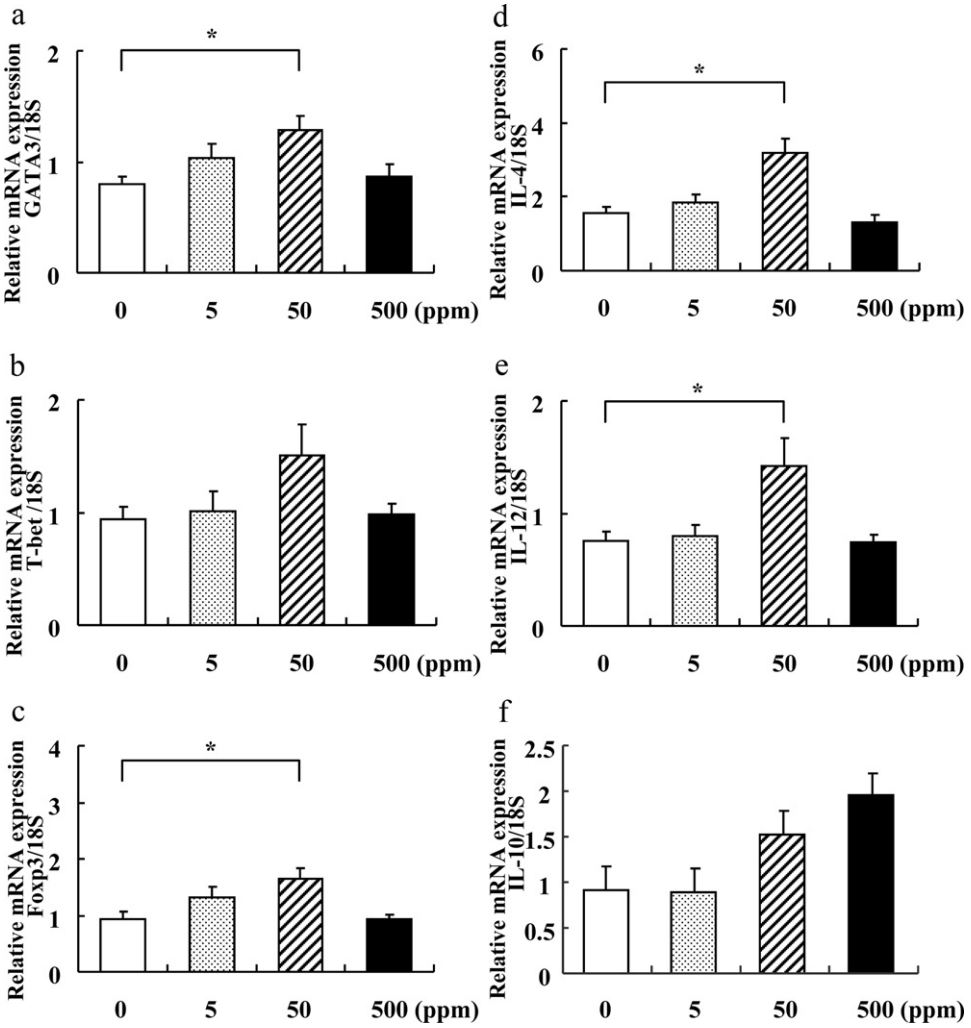

Fig. 7. Expression of transcription factor mRNAs in the spleen of OVA-immunized mice following toluene inhalation. (a) GATA3, (b) T-bet, (c) Foxp3, (d) IL-4, (e) IL-12, and (f) IL-10 mRNAs in C3H/HeN mice following exposure to toluene for 6 weeks. Each bar represents the mean ± SE ($n = 6$, $^*P < 0.05$).

toluene significantly increased the expressions of GATA3 and Foxp3 mRNAs ($P < 0.05$, Fig. 7b and c), but exposure to 500 ppm toluene did not. No change in GATA3, T-bet and Foxp3 mRNAs expression was observed in spleen from toluene-exposed mice without OVA immunization. Regarding Th1/Th2 balance, expression of IL-4 and IL-12 mRNA increased significantly in 50-ppm-toluene-exposed, OVA-immunized C3H/HeN mice (Fig. 7d and e). No significant increase was observed in IL-10 mRNA expression.

3.4. Antibody production in the plasma

Then we examined antigen-specific antibody production in the plasma of the toluene-exposed C3H/HeN mice, exposure to toluene induced no significant changes in the titers of antigen-specific antibody production in the plasma (Fig. 8). The total IgG_1 titer in the plasma of 50-ppm-toluene-exposed, immunized mice was significantly higher than that in the control group. No increases in the total IgE and IgG_{2a} titers in the plasma of the toluene-exposed, immunized C3H/HeN mice were observed. In non-immunized mice, there is no increase of anti-OVA IgE titers in plasma (data not shown).

4. Discussion

The major findings of the present study are that exposure to 50 ppm of toluene increases the total number of inflammatory cells in the lung and upregulates the expressions of STAT3, STAT4, STAT5, STAT6, GATA3, Foxp3, IL-4, and IL-12 as immune regulatory factors in the spleens of male C3H/HeN mice of allergic model. However, 500 ppm of toluene inhalation produced the increase in the mRNA expressions of STAT3, STAT4, and STAT5 mRNAs. Although the antigen-specific antibody responses did not differ significantly between toluene-exposed and control OVA-immunized C3H/HeN mice, the total IgG1 antibody production in the plasma was significantly increased in the 50-ppm-toluene-exposed allergic mice. Thus, exposure to a low level of toluene does not appear to have an adjuvant activity on IgE-mediated immune responses to OVA but instead has a dysregulatory role in a mouse model of allergic inflammation.

The accumulation of inflammatory cells and hypertrophy and hyperplasia of the airway epithelial cells were observed in the lungs of mice exposed to 50 ppm of toluene. However, no significant differences in the production of inflammatory cytokines were observed between toluene-exposed and control mice. Together with the increase in the fibronectin content as an endogenous TLR4 ligand, these findings indicate that coexposure to OVA sensitization and low-level-toluene inhalation induces the damage of the cells lining the airway but does not severely activate inflammatory responses in the lung, suggesting that OVA-sensitized mice may be sensitive to the adverse effects of low levels of toluene inhalation compared with non-sensitized mice.

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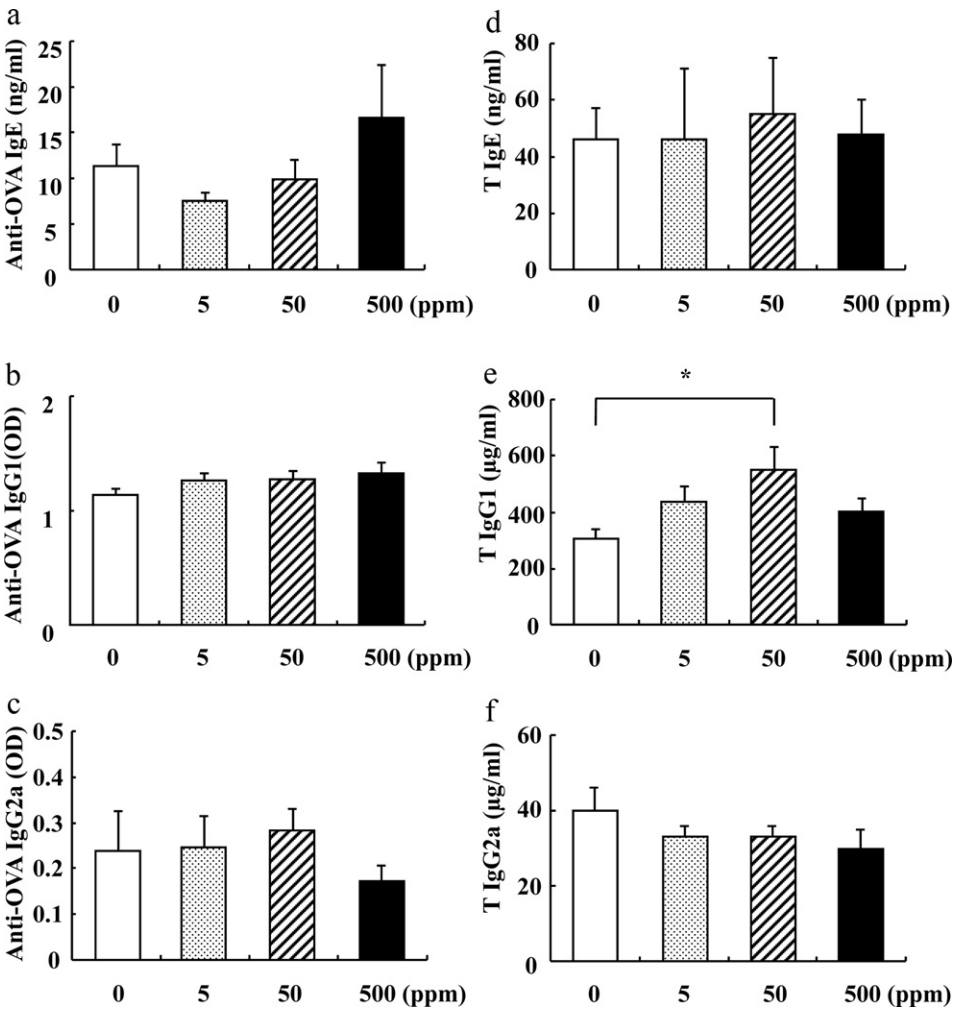

Fig. 8. Plasma levels of anti-OVA Igs and total Igs in immunized C3H/HeN mice exposed to toluene for 6 weeks. (a) Anti-OVA IgE antibody titers, (b) anti-OVA IgG_1 antibody titers, and (c) anti-OVA IgG_{2a} antibody titers, (d) total IgE antibody titers, (e) total IgG_1 antibody titers, and (f) total IgG_{2a} antibody titers ($n = 6$, $^*P < 0.05$).

Direct exposure of peripheral blood cells to VOCs in vitro suppressed Th1 and Th2 cytokine production (Wichmann et al., 2005). Our present study showed that the exposure of OVA-immunized mice to toluene markedly increased the expressions of Th2 type cytokine IL-4 and Th1 type cytokine IL-12 mRNAs in the spleen but not the expression of IL-10 mRNA. Moreover, expressions of STAT3, STAT4, and STAT5a mRNAs significantly increased in 500 ppm of toluene alone. STAT3 and STAT5a are thought to be responsible for lymphoid cell survival and growth (Battle and Frank, 2002; Nagy et al., 2006). IL-12 induces the tyrosine phosphorylation and DNA binding of STAT3 and STAT4 (Cho et al., 1996). Serine phosphorylation has been noted to increase transcriptional activation of STATs proteins (Cho et al., 1996). Therefore, the increase in the expression of these STATs mRNAs by 500 ppm toluene inhalation indicates the protection of lymphocytes against apoptosis induction. Coexposure to OVA and 50 ppm of toluene significantly increased the expression of STAT3, STAT4, STAT5a, and STAT6 mRNAs. STAT4 is important in promoting normal Th1 differentiation (Morinobu et al., 2002). On the other hand, STAT6 is necessary for the normal development of Th2 cells (Nelms et al., 1999). Taken together, these findings indicate that the exposure of immunized mice to toluene increased the expression of transcription factors in the spleen and total IgG1 antibody production in the plasma, but our study did not show a strong correlation between toluene exposure and exacerbation of allergic responses. Further studies are needed to elucidate the effect of low levels toluene exposure on tyrosine and serine phosphorylation in splenic lymphocytes.

Our findings indicate the presence of a critical effective dose of low-level toluene exposure on the manifestation of allergic inflammatory responses to OVA. However, instead of a dose-dependent pattern, we observed a reverse U-shaped curve for the inflammatory response. No mechanism has been proposed to explain the above stimulatory effect of low levels of toluene exposure, but one possibility is that toluene inhalation contributes to neural dysfunction related to sensory stimulation, and that neurotoxic reaction and homeostatic mechanisms compensate for toxicant-induced changes (Win-Shwe and Fujimaki, 2010b). Therefore, low-level toluene inhalation may induce the dysregulation of airway inflammation and splenic inflammatory response in a mouse model of allergic inflammation. Epidemiologically, domestic exposure to VOCs may increase the risk of childhood asthma and of allergic sensitization to food allergens (Rumchev et al., 2004; Lehmann et al., 2001). Our present study does not prove cause–effect relationship between allergic risk and toluene inhalation. The adverse outcome of developmental immunotoxicity may not appear until long after the early-life immune insult and may affect multiple health risks over a life course (Dietert and Zelikoff, 2009). The effect of toluene inhalation on early aging process in immune systems must be studied.

The characterization of homeostatic processes is particularly important at low, environmentally relevant doses, while the risk of toxic effects is typically only inferred from the effects observed at much higher doses. Recent data from the Pollutant Release and Transfer Register in Japan indicates that almost 180,000 t of

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toluene is released from factories and automobiles into the environment every year and that toluene is the chemical most commonly released into the air (Ministry of the Environment in Japan, 2007). Therefore, we examined reliable toluene concentrations between the occupational exposure level and the indoor air level and found the dysregulation of allergic inflammatory responses in an allergic mouse model.

In conclusion, low levels of toluene exposure in immunized mice might dysregulate immune responses to OVA via the activation of transcription factors. Our findings suggest that STATs transcription factors in spleen might be sensitive biomarkers in a mouse model following exposure to toluene at low levels.

Conflict of interest

None.

Acknowledgments

This research was partly supported by a grant from the Ministry of Education, Culture, Sports, Sciences and Technology, Japan (#21390198 to H.F.). We thank Dr. A. Furuyama for her useful discussion and Mrs. S. Murakami, and K. Ohnishi (National Institute for Environmental Studies) for their excellent technical assistance.

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Please cite this article in press as: Fujimaki, H., et al., Dysregulation of immune responses in an allergic mouse model following low-level toluene exposure. Toxicology (2011), doi:10.1016/j.tox.2011.05.002