

Endocrine disruptors found in food contaminants enhance allergic sensitization through an oxidative stress that promotes the development of allergic airway inflammation



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ABSTRACT

In the past few decades, there has been a significant increase in incidence of allergic diseases. The hygiene hypothesis may provide some clues to explain this rising trend, but it may also be attributable to other environmental factors that exert a proallergic adjuvant effects. However, there is limited information on the risks of developing allergic asthma and related diseases through the ingestion of environmental chemicals found in food contaminants. In the present study, we have shown that oral administration of tributyltin, used as a model environmental chemical, induced oxidative-stress status in the bronchial lymph node, mesenteric lymph node and spleen, but not in the lung, where the initial step of allergic asthma pathogenesis takes place. Mice exposed to tributyltin exhibited heightened Th2 immunity to the allergen with more severe airway inflammation. Tributyltin also induced Treg cells apoptosis preferentially over non-Treg cells. All these effects of tributyltin exposure were canceled by the administration of glutathione monoethyl ester. Meanwhile, tributyltin did not affect airway inflammation of mice transferred with allergen-specific Th2 cells. Collectively, these results suggest that tributyltin exerts its pathological effect during the sensitization phase through oxidative stress that enhances the development of allergic diseases. The current study dissects the pathogenic role of oxidative stress induced by oral exposure to an environmental chemical during the sensitization phase of allergic airway inflammation and would be important for developing therapeutics for prevention of allergic diseases.

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Introduction

Allergic asthma is a chronic airway inflammatory disorder characterized by airway hyperresponsiveness (AHR) and reversible airway obstruction, eventually leading to airway remodeling and irreversible changes in lung function (Bochner and Busse, 2005). Currently the number of patients suffering from asthma is estimated to be 300 million worldwide and with the rising trend it is expected to increase to 400 million by 2025 (Pawankar et al., 2011, 2012). Although genetic factors determine the predisposition to asthma and related allergic diseases (Holloway et al., 2013), environmental factors are most likely responsible for the upsurge in prevalence of these diseases during the past few decades, since population genetic variability does not change so rapidly (Sohn and Kim, 2012). The hygiene hypothesis has tried to

explain this trend in terms of reduced infectious or microbial exposure in early life (Strachan, 1989, 2000), but recent evidence does not provide an equivocal support for this hypothesis (Brooks et al., 2012). Clearly, other environmental factors that do not have allergic potential themselves but exert proallergic adjuvant effects, may also contribute to this uprising trend. In this regard, it is noteworthy that the rise in asthma prevalence approximately parallels the rise in common use of several chemicals (Mannino et al., 2002). Air pollution is well known for exacerbating asthma (Kelly and Fussell, 2011), but there is limited information on the risks of developing allergic asthma through the ingestion of environmental chemicals found in food contaminants. In order to make allergic diseases preventable, identification of responsible factors and elucidating underlying mechanisms involved remain an important research priority.

Endocrine disrupting chemicals, so called because of their ability to interfere with hormone-signaling pathways via estrogenic or androgenic activities, are ubiquitous in the environment and have attracted to great deal of attention as a causative not only of reproductive abnormality but also of neurological defects and certain types of cancer development (McLachlan, 2001). In addition, it is increasingly appreciated that they may also contribute to the pathogenesis of allergic asthma and related diseases (Chalubinski and Kowalski,

Abbreviations: TBT, Tributyltin; GSH, Glutathione; GSH-MEE, Glutathione monoethyl ester; BLN, Bronchial lymph node.

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2006), but pathophysiological actions appear to vary among the chemicals and underlying mechanisms remain poorly investigated. It is noteworthy that the development of allergic asthma comprises two main phases occurring in discrete sites, i.e., the secondary lymphoid organ and the lung, which is why the pathophysiological action of environmental factors may also vary in each phase. Previously, our *in vitro* studies have shown that environmental chemicals that are known to have an endocrine disrupting activity, such as tributyltin (TBT), benzophenone, and octylphenol, promote differentiation of naïve T cells into Th2 cells and induce apoptosis in Th1 cells preferentially, both of which are mediated by oxidative stress (Kato et al., 2006; Tada-Oikawa et al., 2008). Mice exposed to TBT, found in food contaminants and the most potent oxidative stressor among them, at environmentally relevant concentrations had exacerbated allergic airway inflammation with concomitant enhancement of Th2-type immunity (Kato et al., 2004, 2006). Our results imply that environmental chemicals with an oxidative property function during the sensitization phase and make individuals vulnerable to allergic sensitization.

It is well appreciated that oxidative stress is involved in the pathogenesis of allergic asthma in the induction phase by exacerbating preexisting airway inflammation (Dozor, 2010; Koike et al., 2007; Riedl and Nel, 2008). Available data, including our own, do not clearly dissect the pathological roles that environmental chemicals play in the sensitization phase from those in the induction phase. In the present study, using TBT as a model endocrine disruptor, we have clearly dissected and elucidated the pathophysiological role of oxidative stress induced by oral exposure to TBT in the sensitization phase of allergic airway inflammation through enhancement of Th2 differentiation and induction of apoptosis in Treg cells.

Materials and methods

Mice

C57BL/6 mice, C57BL/6 (CD45.1) congenic mice and OT-II mice expressing TCR specific ovalbumin peptide (OVA_{323–339}) in the context of I^a_b were fed a standard diet, housed under specific pathogen free conditions, and used at 5–8 weeks of age. All animal experiments were conducted under protocols approved by the Animal Care and Use Committee of Mie University Life Science Research Center.

Medium and reagents

Culture medium used in the present experiments consisted of RPMI-1640 (Nissui, Tokyo, Japan) supplemented with 5×10^{-5} M 2-ME, streptomycin, and 10% FCS (JRH Biosciences, Lenexa, KS). Stock solution of tributyltin chloride (TBT; Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) was prepared in DMSO and diluted to working solutions in corn oil for *in vivo* experiments or in culture medium for *in vitro* experiments. The nominal concentrations of DMSO in any working solution used *in vivo* and *in vitro* experiments were 0.2% and 0.001–0.003%, respectively. Glutathione monoethyl ester (GSH-MEE) was purchased from Bachem AG.

In vitro Th differentiation

OVA-specific Th2-polarized cells along with Th cells differentiated in the presence of TBT were prepared from naïve CD4⁺ T cells of OT-II mice and their cytokine production profiles were assayed essentially as described previously (Saito et al., 2008; Torii et al., 2010). Briefly, CD4⁺CD62L⁺ T cells (1×10^5 cells/ml) were cultured with 35 Gy-irradiated C57BL/6 spleen cells (2×10^6 cells/ml) in the presence of 200 µg/ml OVA in a well of 24-well plate (Iwaki Glass Co., Japan). Cultures were also received IL-4 (10 ng/ml; Peprotech Inc.) plus anti-IL-12p40 (C17.8; 5 µg/ml; PharMingen) for Th2 polarization, vehicle

(DMSO), i.e. neutral condition, for Th0, or 0.1 µM TBT. The CD4⁺ T cells primed for 4 days were expanded into 4 wells with fresh medium containing 2.5 ng/ml IL-2 (rhIL-2, Ajinomoto Co., Japan) and cultured for another 2 days. On day 6, the CD4⁺ T cells were harvested over Ficoll and used for adoptive transfer experiments. The cytokine production profiles of these Th cell lines were assayed after stimulation with OVA in the presence of splenic APC followed by intracellular cytokine staining as described (Kato et al., 2006).

Mouse treatment

C57BL/6 mice were sensitized by i.p. injection with 10 µg of OVA and 1.125 mg of aluminum hydroxide (Imject Alum; Pierce) in 0.2 ml of saline on days 0 and 7. These mice were also treated with either corn oil (containing 0.2% DMSO as a control) or 1 µmol/kg TBT by oral gavage on days 0, 2, 4, 7, 9, and 11 (total 6 µmol/kg). GSH-MEE: Bachem AG solution was also given to these mice on days 0–4 and days 7–11 (2.5 or 25 mg/kg/day) via i.p. route. Where indicated, one day before the challenge with OVA, OVA-specific Th cell lines (1×10^6 cells) were adoptively transferred i.v. into naïve mice or mice treated with TBT in place of OVA sensitization. On days 14–16, the mice challenged with 10 µg OVA dissolved in 50 µl of saline intranasally under anesthesia with pentobarbital. On day 17, bronchoalveolar lavage fluid (BALF) along with spleen was collected. The supernatant and cells of BALF were subjected to cytokine ELISA and differential cell counts as described (Kato et al., 2004). Supernatant of splenocytes cultured at 4×10^6 cells/ml with 250 µg/ml OVA for 48 h were also subjected to cytokine ELISA as described (Torii et al., 2010).

In vivo assessment of T cell apoptosis

Total CD4⁺ T cells (1×10^7 cells) containing approximately 90% CD4⁺CD25[−] T cells and 10% CD4⁺CD25⁺ T cells from OT-II mice were i.p. transferred into C57BL/6 (CD45.1) congenic mice on day 1. These mice received i.p. injection with OVA/Alum on day 0 and intragastric administration with 1 µmol/kg TBT on days 0, 2, 4. A group of mice also received 25 mg/kg GSH-MEE via i.p. route from day 0 through day 4. On day 7, spleen cells from these mice were subjected to cell surface staining with Cychrome-conjugated anti-CD45.2 followed by intracellular staining with PE-conjugated anti-Foxp3 (FJK-16s; eBioscience) and FITC-conjugated anti-active caspase-3 (C92-605; BD Pharmingen), and expression of activated caspase-3 was analyzed after electronic gating of CD45.2⁺CD4⁺Foxp3[−] or CD45.2⁺CD4⁺Foxp3⁺ T cells on a FACScan.

Cytokine ELISA

IL-4, IL-5 and IFN-γ in BALF or culture supernatant were assayed by ELISA using paired mAbs specific for the corresponding cytokine as described previously (Torii et al., 2010). ELISA for IL-17 was conducted using commercial kit following manufacture's instruction (R&D). Lower detection limits of these assays were as follows: IL-4, 10 pg/ml; IL-5, 50 pg/ml; IL-17, 20 pg/ml; IFN-γ, 100 pg/ml.

Assay for serum tin concentration

Blood samples for serum were collected by cardiac puncture of lethally anesthetized mice. Sera were prepared by centrifugation of blood samples after incubation for 30 min at room temperature. The sample sera were diluted with 1% nitric acid and subjected to mass spectrometry analysis on ICP-MS (ICPM-8500, Shimadzu Co., Japan). The tin concentrations were calculated with reference to a standard curve obtained by using 0–100 pg/ml of tin.

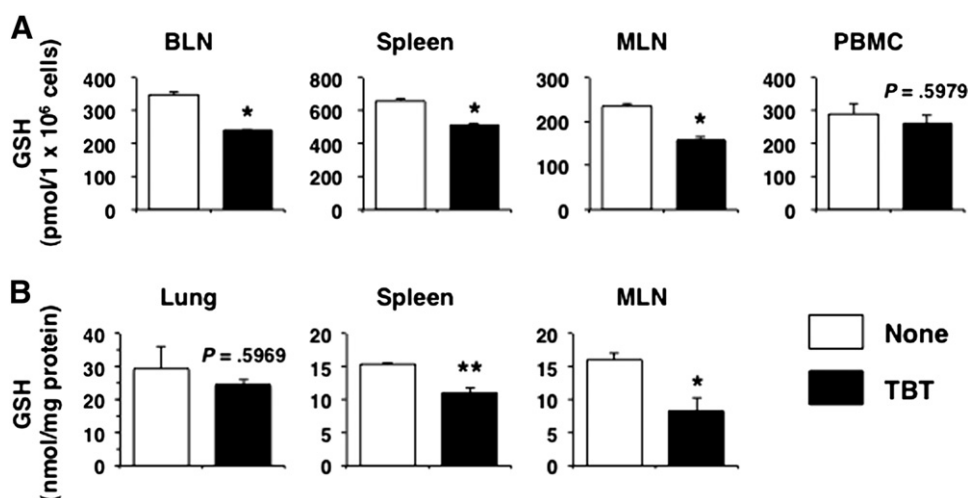


Fig. 1. Levels of GSH in mice exposed to TBT. C57BL/6 mice were treated with a multiple intragastric administration of 1 μ mol/kg/day TBT (on days 0, 2, 4, 7, 9, and 11). On day 12, lung, bronchial LN, spleen, MLN, and PBMC were collected and assayed for levels of GSH using commercial kit (A) and a system developed in-house (B) as described in the [Materials and methods](#). GSH levels of 1×10^6 cells of pooled tissues from 6 mice were measured in triplicate and the results are expressed as mean $\pm$ SEM of pmol GSH per 1×10^6 cells (A), and those individual tissues from 5 mice were measured independently in a single measurement and the results are expressed as mean $\pm$ SEM of nmol GSH per 1 mg protein (B). Statistical significance was determined by using two-tailed Student's t-test. * $p < 0.05$; ** $p < 0.01$.

Assay for intracellular GSH

Tissues and fractionated cells were collected from mice treated with GSH-MEE and/or TBT on day 12, which were subjected to the assay for concentrations of GSH using system developed in house as described (Hiraku et al., 2002; Kato et al., 2006). In some experiments, GSH concentrations were also measured by using a kit purchased from Promega (GSH/GSSG-Glo™ Assay).

Statistical analysis

Statistic analyses were performed with IBM SPSS Statistics Software Version 19 (IBM). Differences between two groups were compared using two-tailed Student's t-test and those among multiple groups were compared using Kruskal–Wallis with Bonferroni post hoc test. A p value of <0.05 was considered significant. All experiments were performed more than two times.

Results

TBT induces oxidative stress status in secondary lymphoid organs

Our previous in vitro studies have suggested that environmentally relevant concentrations of TBT augment Th2 immunity through an oxidative stress, which may lead enhanced airway inflammation. The first experiment, therefore, was conducted to determine whether TBT at doses that exacerbate airway inflammation induce oxidative stress in vivo. C57BL/6 mice were treated with a multiple intragastric administration of 1 μ mol/kg/day TBT (on days 0, 2, 4, 7, 9, and 11). On day 12, lung, BLN, spleen, MLN, and PBMC were collected and their levels of GSH were investigated using two different assay systems. As shown in [Fig. 1](#), TBT treatment increased oxidative stress status, as judged by reduced intracellular GSH levels, in BLN, spleen and MLN, but not in the lung and PBMC.

Anti-oxidant GSH-MEE that replenishes intracellular GSH cancels the effect of TBT on airway inflammation

Having confirmed that TBT increased oxidative stress status in secondary lymphoid organs where Th2 differentiation takes place, we next determined whether the increased oxidative stress status

induced by TBT has any relevance to the enhancement of Th2 development leading to the exacerbation of airway inflammation. GSH levels in the spleen and MLN were first measured in mice treated with TBT, as above, together with differential doses of GSH-MEE which is a membrane-permeable GSH precursor and replenishes intracellular GSH (Nishida et al., 1996). As shown in [Fig. 2](#), administration of GSH-MEE at 25 mg/kg/day, but not 2.5 mg/kg/day, resulted in significant increase in GSH levels both in spleen and MLN of

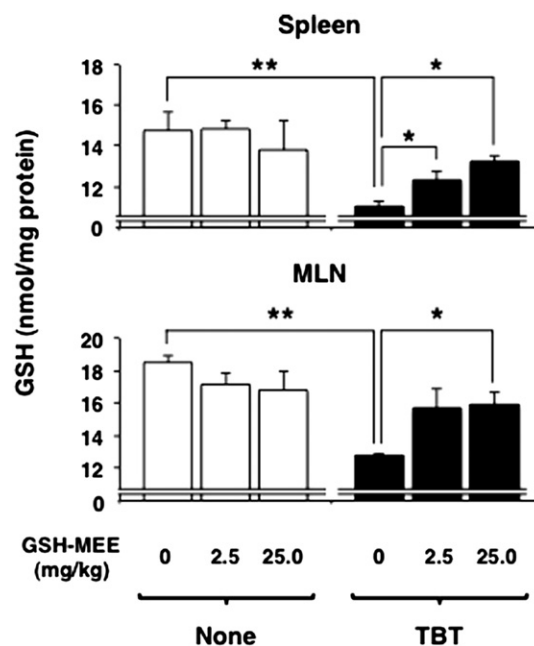


Fig. 2. Effect of GSH-MEE administration on oxidative stress status in mice exposed to TBT. C57BL/6 mice were treated with either corn oil (containing 0.2% DMSO as a control) or 1 μ mol/kg TBT by oral gavages on days 0, 2, 4, 7, 9, and 11 (total 6 μ mol/kg). GSH-MEE solution was also given to these mice on days 0–4 and days 7–11 (2.5 or 25 mg/kg/day). On day 12, spleen and MLN were collected and assayed for levels of GSH as described in the [Materials and methods](#). The results are expressed as mean $\pm$ SEM of indicated individual tissues from 4 to 5 mice. Statistical significance was determined by using Kruskal–Wallis with Bonferroni post hoc test. * $p < 0.05$; ** $p < 0.01$.

mice treated with TBT. Then, C57BL/6 mice were treated with TBT and/or GSH-MEE (25 mg/kg/day) during sensitization with OVA. Consistent with our previous results, TBT treatment exacerbated airway eosinophilia that was associated with increases in IL-5 levels detected in BALF (Fig. 3). However, simultaneous administration of GSH-MEE resulted in the reduction of eosinophilia and IL-5 levels in the lungs. Administration of GSH-MEE did not affect airway eosinophilia and IL-5 levels in BALF of mice without TBT treatment. When assayed for Th1, Th2, and Th17 signature cytokines produced by splenocytes from these mice upon restimulation with OVA in vitro, splenocytes from mice treated with TBT produced a higher amount of IL-4 as compared with those from mice without TBT treatment (Fig. 4). Administration of GSH-MEE to TBT-treated mice resulted in the reduction of IL-4 production and enhancement of IFN- γ production by splenocytes. Levels of IL-17 detected in BALF and produced by splenocytes of these mice were not affected either by the treatment with TBT and/or GSH-MEE.

TBT induces apoptosis in Treg cells preferentially over non-Treg cells

It is well established that intracellular GSH confers protection from oxidative-stress induced apoptosis (Franco and Cidlowski, 2009). We hypothesized that CD4⁺CD25⁺ regulatory T cells that play a critical

role in controlling development of Th2 immunity during sensitization (Saito et al., 2008) are sensitive to activation induced cell death through oxidative-stress mediated apoptosis. Therefore we examined the intracellular content of GSH in CD4⁺CD25⁺ T cells and compared it to the content in CD4⁺CD25⁻ T cells. As shown in Fig. 5A, the basal levels of GSH in Treg cells were significantly lower than those in non-Treg cells. Then CD45.1 mice were transferred with total CD4⁺ T cells from OT-II mice (CD45.2) and subsequently immunized with OVA and concurrently treated with TBT and/or GSH-MEE. Treg cell population, as defined by the expression of Foxp3 and CD45.2, contained more active caspase-3⁺ cells than Foxp3-CD45.2⁺ non-Treg cells (Fig. 5B). Moreover, TBT treatment significantly increased the frequency active caspase-3⁺ cells, whereas simultaneous treatment of GSH-MEE canceled this effect of TBT.

TBT does not affect eosinophilic airway inflammation induced by Th2 cells

Thus far, our results have indicated that the accelerated Th2 differentiation/immunity is one of the underlying mechanisms for the exacerbated airway inflammation in the lungs of mice exposed to TBT. To substantiate this, OVA-specific Th cells differentiated in the presence of TBT were examined to see whether they induced airway inflammation in unimmunized mice upon challenge with OVA. Naïve mice were

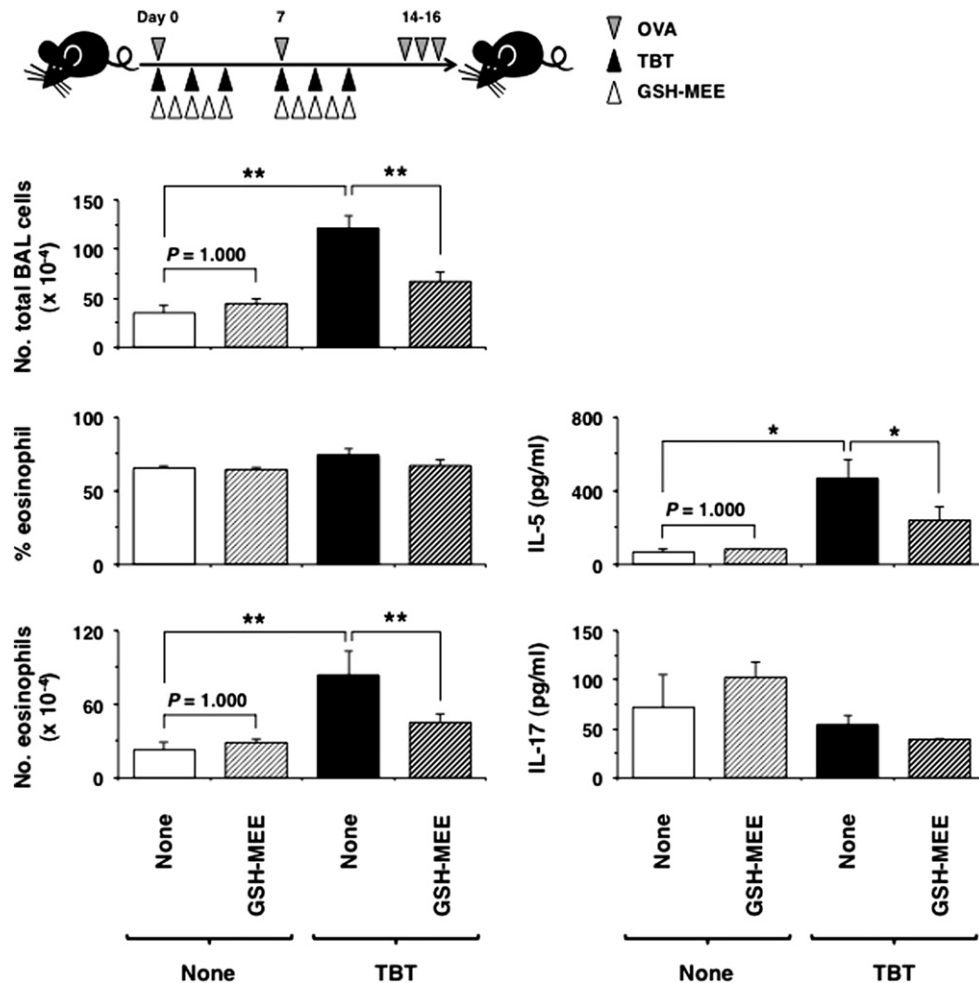


Fig. 3. Effect of GSH-MEE administration on airway inflammation of mice exposed to TBT. C57BL/6 mice were sensitized with OVA days 0 and 7, followed by an intranasal challenge with OVA on days 14–16. These mice were also treated with either corn oil (containing 0.2% DMSO as a control) or 1 μ mol/kg TBT by oral gavages on days 0, 2, 4, 7, 9, and 11 (total 6 μ mol/kg). GSH-MEE solution was also given to these mice on days 0–4 and days 7–11 (2.5 or 25 mg/kg/day). On day 17, BALF was collected, of which the supernatant and cells were subjected to cytokine ELISA and differential cell counts. The schematic representation of experimental procedure is shown on the upper side of the histograms. The results are expressed as mean $\pm$ SEM for 4–5 mice per group. Statistical significance was determined by using Kruskal Wallis with Bonferroni post hoc test. *p < 0.05; **p < 0.01.

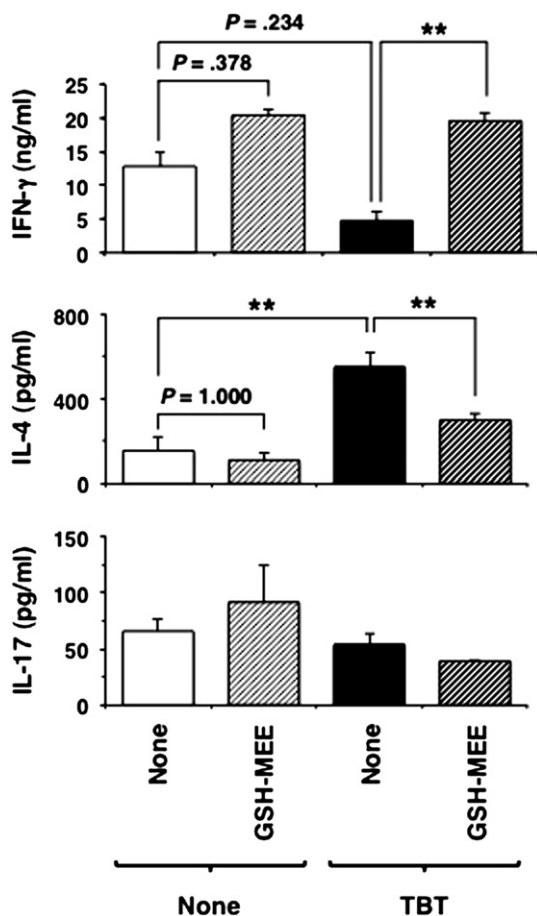


Fig. 4. TBT promotes development of Th2 immunity during sensitization phase through oxidative stress. Splenocytes from mice treated as described in legend for Fig. 3 were cultured in the presence of OVA. After 48 h, culture supernatants were collected and subjected to ELISA for IFN- γ , IL-4 and IL-17. The results are expressed as mean $\pm$ SEM of triplicate cultures. Statistical significance was determined by using Kruskal Wallis with Bonferroni post hoc test. * $p < 0.05$; ** $p < 0.01$.

transferred with CD4⁺ T cells from OT-II mice primed with OVA in vitro in the presence of TBT (TBT-Th) and challenged with OVA. CD4⁺ T cells primed under neutral (Th0) and Th2 condition (Th2) served as controls. Cytokine production profiles of these CD4⁺ T cell lines were examined after Ag-specific stimulation followed by intracellular cytokine staining with IFN- γ and IL-4, the results of which are shown in Fig. 6A. Because of the Th1 prone genetic background of C57BL/6 mice (Bix et al., 1998), Th cell lines developed under neutral conditions mostly produced IFN- γ but little IL-4. As shown in Fig. 6B, mice that received TBT-Th developed eosinophilic airway inflammation, typical of Th2-type allergic airway inflammation, which cannot be seen in mice transferred with the Th cell line differentiated in the absence of TBT (Th0). All Th cell lines induced significant airway inflammation, but only Th2 and TBT-Th cell lines induced eosinophilic airway inflammation, which is in sharp contrast to the airway inflammation without eosinophilia induced by Th0 cell line containing mostly Th1 cells as consistent with a previous report (Cohn et al., 1999). Next, we examined if TBT affects induction phase of Th2 immunity and/or acts directly on lung tissue cells to produce factor(s) involved in airway inflammation at later points in time. In this instance, TBT-treated but not OVA immunized mice were transferred with OVA-specific Th2 cell lines and then challenged with OVA intranasally. As shown in Fig. 6C, TBT pretreatment did not affect eosinophilic inflammation induced by transfer with Th2 cells.

Serum levels of tin in TBT-exposed mice that exhibited exacerbated airway inflammation

In a final experiment, we measured serum levels of TBT in mice treated with TBT at the dose that induces oxidative stress status in secondary lymphoid organ and exacerbates airway inflammation. TBT is mainly metabolized to DBT in the liver by P450 enzyme system (Ohhira et al., 2003; Ueno et al., 2003), but its ultimate metabolites include inorganic compounds via hydroxylation and dealkylation (Ueno et al., 1997). Therefore, blood was collected from the mice treated with TBT and serum levels of tin were measured by mass spectrometry. As shown in Fig. 7, serum tin levels in mice treated with TBT reached levels found in human blood (Whalen et al., 1999).

Discussion

In the present study, we demonstrate that exposure to environmentally relevant concentrations of TBT, a particularly notable environmental chemical, exacerbates allergic airway inflammation via enhancement of Th2 differentiation and preferential induction of apoptosis in Treg cells, in the process of which oxidative stress is critically involved. TBT exerts its pathological effect during the sensitization phase when the initial step of allergic asthma, i.e., differentiation of allergen specific Th2 cells, takes place in secondary lymphoid organ. On the other hand, TBT does not appear to play a pathological role in the induction phase when allergen specific Th2 immunity is already established.

Previously, we have shown that environmental chemicals such as benzophenone, octylphenol and TBT, all of which can be found in food contaminants, enhanced Th2 differentiation and induced apoptosis in Th1 cells in vitro (Kato et al., 2006; Tada-Oikawa et al., 2008). This effect on Th2 immunity was mediated indirectly through the depletion of GSH, i.e., oxidative stress, as a common underlying mechanism (Kato et al., 2006). We have further demonstrated that mice exposed to environmentally relevant doses of TBT developed enhanced Th2 immunity and airway inflammation (Kato et al., 2004, 2006). It has been increasingly appreciated that oxidative stress could be responsible for asthma exacerbation by exaggerating pre-existing airway inflammation and enhancing airway reactivity during the induction phase, and also providing an adjuvant effect to promote Th2 immunity (Bowler and Crapo, 2002; Cantin et al., 1987; Caramori and Papi, 2004). However, available data have been limited to in vitro models and a few studies have shown indirect evidence of oxidative stress in vivo (Dworski et al., 2001; Li et al., 2007; Riedl and Nel, 2008). Furthermore, very few studies have clearly dissected the pathological roles of oxidative stress induced by environmental chemicals played in sensitization phase from those in induction phase. Our data is the first, to our knowledge, to demonstrate that an oral exposure to environmental chemical, TBT, at environmentally relevant doses induces oxidative stress in secondary lymphoid organs including the BLN and enhances allergic sensitization, which contribute to the development of allergic asthma independently of exacerbating pre-existing Th2 response toward allergen. As is the case with in vitro (Kato et al., 2004, 2006), oral exposure to TBT affects antigen-presenting cells to produce a set of cytokines that promote Th2 differentiation in vivo, since APC from mice exposed to TBT induced Th2 differentiation of naïve CD4⁺ T cells in vitro (data not shown).

It is increasingly clear that Treg cells, the function of which is impaired in allergic and asthmatic diseases (Ryanna et al., 2009), control the development of allergic diseases in both sensitization and induction phases (Kearley et al., 2005; Saito et al., 2008). We have shown that Treg cells suppress the differentiation of allergen specific naïve T cells into Th2 cells in the allergen-draining lymph node and activation and/or recruitment of Th2 cells in the inflammatory site (Saito et al., 2008). In the present study, exposure to TBT induced apoptosis preferentially in Treg cells over non-Treg cells in vivo, which may contribute to the exaggeration of Th2 immunity toward allergens. Simultaneous administration of GSH-MEE canceled the effect of TBT exposure, indicating that

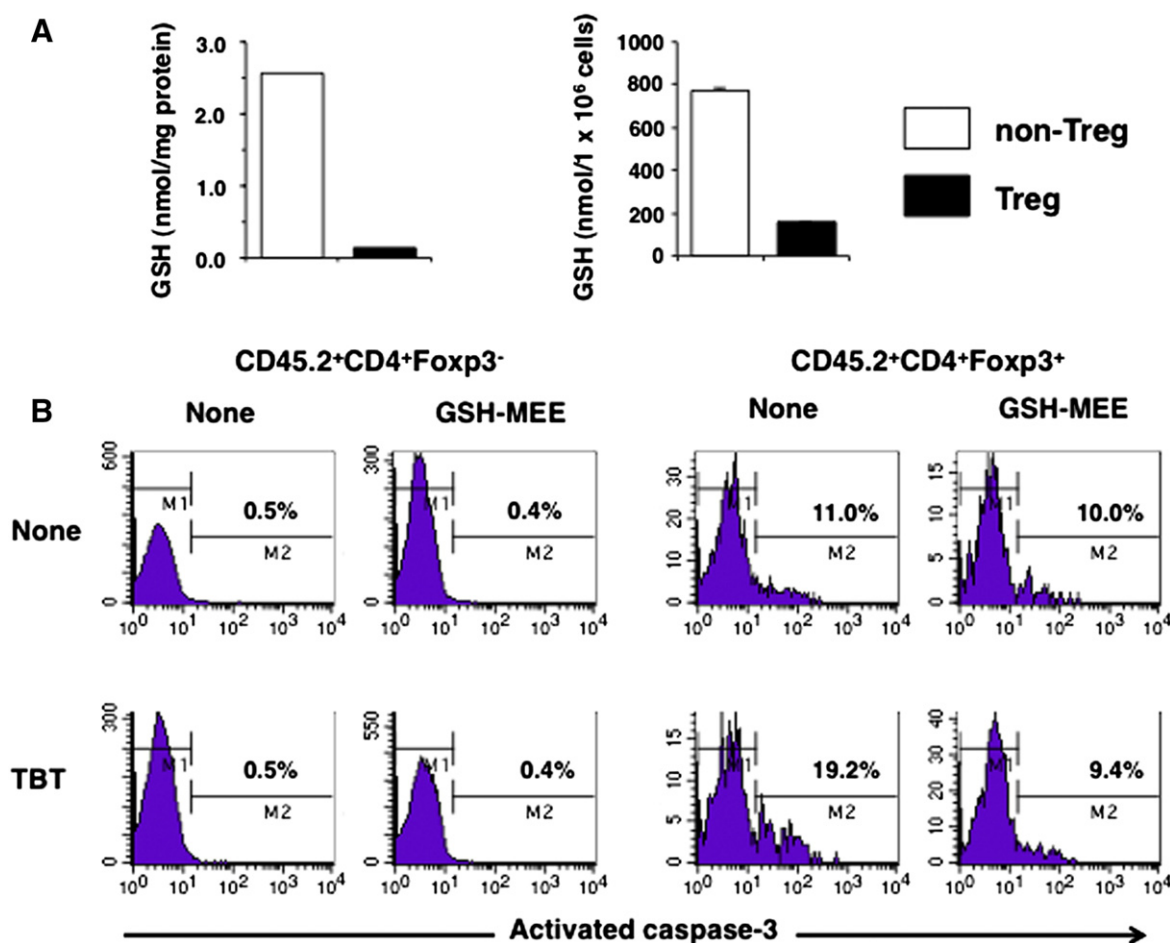


Fig. 5. TBT accelerates apoptotic cell death preferentially in Treg cells with low basal levels of GSH, which is canceled by GSH-MEE. **A**, the levels of intracellular GSH in Treg cells and non-Treg cells. Purified CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells from naïve mice were subjected to assay for levels of intracellular GSH. GSH levels in pooled T cell subpopulation from 3 mice were assayed by using an in-house method (left) and a commercial kit (right). **B**, flow cytometric analysis of activated caspase-3 staining in Treg cells and non-Treg cells. C57BL/6 (CD45.1) mice were transferred with total CD4⁺ T cells containing CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells from OT-II mice and received OVA immunization, along with exposure to TBT and/or GSH-MEE treatment as described in the [Materials and methods](#). Spleen cells from these mice were subjected to cell surface staining with anti-CD45.2 followed by intracellular staining with anti-Foxp3 and anti-active caspase-3. Expression of activated caspase-3 was analyzed after electronic gating of CD45.2⁺CD4⁺Foxp3⁻ or CD45.2⁺CD4⁺Foxp3⁺ T cells on a FACSscan. The results shown are representative of two (**A**) and three (**B**) independent experiments.

this is also mediated by oxidative stress. Oxidative stress has been shown to be critically involved in both the extrinsic and intrinsic apoptotic pathways (Circu and Aw, 2010). We have also shown that TBT induces H₂O₂ generation that drives apoptotic pathway where it involves a change in mitochondrial membrane potential and subsequent activation of caspase-3 (Tada-Oikawa et al., 2008). Similar to the case with Th1 cells, Treg cells exhibit reduced basal levels of intracellular GSH that can detoxify ROS, and this may confer vulnerability to oxidative stress-mediated apoptosis in Treg cells. In support of this notion, recent studies also show that Treg cells harbor low basal levels of GSH (Zhao et al., 2010) and cytotoxic effect of cyclophosphamide, a well-known reagent used in clinics to selectively ablate Treg cells at low-doses (Ghiringhelli et al., 2004; Lutsiak et al., 2005), is mediated by its ability to induce oxidative stress (Tripathi and Jena, 2009). Controversy exists as to the relative sensitivity to apoptosis in Treg cells over non-Treg cells (Yolcu et al., 2008), which may have originated from differences in activation stage of Treg cells and also experimental conditions used. Our experimental system may have an advantage over others in that Treg cells can obtain both pro- and anti-apoptotic signals from adjacent cells in vivo under physiological conditions.

In the present study, Th17 cells, which are named after their signature cytokine IL-17A (Zhu et al., 2010) and have emerged as an additional player in the regulation of allergic inflammation especially during sensitization phase (Cosmi et al., 2010; Nakae et al., 2002; Schnyder-

Candrian et al., 2006; Wang et al., 2010), seem not play a role in the exacerbation of airway inflammation by TBT.

Our study demonstrates that exposure to TBT during the induction phase does not exacerbate airway inflammation, where Th2 immunity is already established. In this regard, it is noteworthy that GSH levels in the lungs were not affected by TBT exposure in the present study. Since “the lungs are endowed with an armamentarium of a battery of endogenous agents called antioxidants” (Rahman et al., 2006), the oral exposure to TBT at environmentally relevant doses might not reach the levels that induce oxidative stress in the lungs and thus did not augment the activation of pre-existing Th2 cells. However, given that Treg cells are able to control the development of allergic diseases in the induction phase (Kearley et al., 2005; Saito et al., 2008), it was also possible that TBT also exacerbates airway inflammation during the induction phase through deprivation of Treg cells. We and others have shown that the defect in Treg cells during the sensitization phase has a more profound impact on the development allergic asthma (Baru et al., 2012; Saito et al., 2008). Therefore, it is tempting to speculate that exposure to environmental chemicals during sensitization phase plays a more important role that causes an increase in the prevalence of allergic diseases over recent decades, rather than simply increases symptoms in patients with allergic diseases.

TBT compounds have been the main active ingredients in biocides used to control a broad spectrum of organisms. Uses include wood

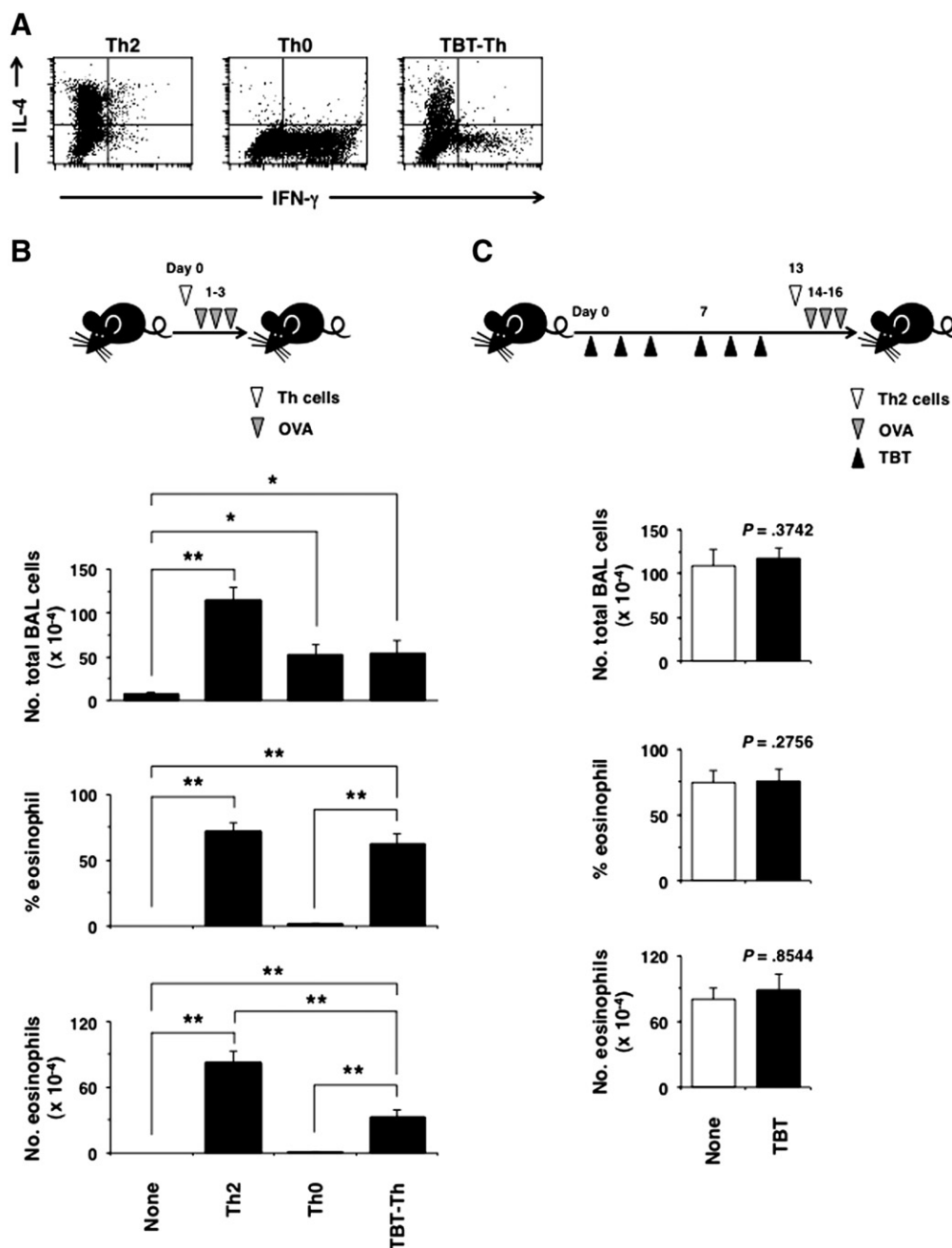


Fig. 6. TBT does not have an effect on already established Th2 immunity. A, the cytokine production profile of OVA-specific Th cell lines used for in vivo transfer experiments. Th cell lines were prepared and assayed for expression of intracellular IL-4 and IFN- γ as described in the [Materials and methods](#). B, Th cells developed in the presence of TBT induced airway eosinophilia. OVA-specific Th cells were generated in vitro under Th2- (Th2) and neutral- (Th0) condition, and in the presence of TBT (TBT-Th) were adoptively transferred into naïve mice in place of OVA sensitization and subsequently challenged with OVA for 4 consecutive days. The BALF was collected and subject to differential cell counts. The results are expressed mean $\pm$ SEM ($n = 5$ –6 mice/group). C, TBT did not exacerbate airway inflammation in mice already established Th2 immunity. One day before the challenge with OVA, OVA-specific Th2 cells were adoptively transferred into mice that had been treated with TBT as described in the [Materials and methods](#). After the challenge with OVA intranasally, BALF was collected, of which the supernatant and cells were subjected to differential cell counts. The results are expressed mean $\pm$ SEM ($n = 5$ –6 mice/group). The schematic representation of experimental procedures used in B and C are shown on the upper side of the histograms. Statistical significance was determined by using Kruskal Wallis with Bonferroni post hoc test (B) and two-tailed Student's t-test (C). * $p < 0.05$; ** $p < 0.01$.

preservation, marine antifouling paint for boats and ships, antifungal action in textile products including carpets and industrial water systems (Antizar-Ladislao, 2008). Although the use in marine antifouling paint has been banned since 2008 under the treaty of International Marine Organization, environmental contamination continues as a result of its chemical stability, non-marine uses, and application to ships prior and even after the ban (Gipperth, 2009). TBT accumulates and achieves bioconcentration easily in various marine organisms because of its

lipophilic property (Horiguchi, 2006; Lilley et al., 2012). Human exposure to TBT persists via consumption of contaminated drinking water and beverages, and in particular marine foods (Antizar-Ladislao, 2008). Although uncertainties in human–mouse toxicity extrapolation remains, the dose of TBT to be administered to mice, which induced increased airway inflammation in the present study, gave rise to serum levels of tin reaching levels detected in human serum as butyltins (Antizar-Ladislao, 2008). Together with the fact that the presence of a

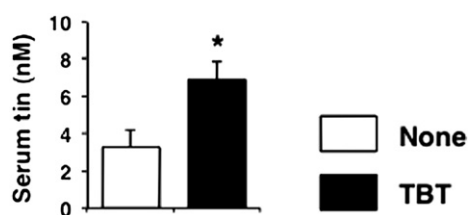


Fig. 7. Levels of tin in serum from mice treated with TBT. Sera from mice treated with TBT were assayed for tin as described in the **Materials and methods**. The results are expressed as means $\pm$ SEM between animals ($n = 5$). Statistical significance was determined by using two-tailed Student's t -test. * $p < 0.05$.

number of chemicals with oxidative stress activity (Kato et al., 2006) in real environmental matrices may act synergistically with TBT, it is plausible that TBT may have been contributing to the development of allergic diseases.

In summary, this study has shown for the first time that orally administered TBT, a recalcitrant environmental pollutant, induces exaggerated Th2 immunity and increased airway inflammation through a mechanism involving increased oxidative stress in secondary lymphoid organs. The current study dissects the pathogenic role of oxidative stress induced by an environmental chemical during the sensitization phase of allergic airway inflammation. It is noteworthy that simultaneous administration of antioxidant GSH-MEE canceled the effect of TBT. In previous human studies, it has been shown that therapeutic antioxidant supplementation has no clinical benefit in asthmatic patients who have already established Th2 immunity toward allergen (Fogarty et al., 2003, 2006; Shaheen et al., 2007). On the other hand, a recent observational study of adolescents showed the positive association of low dietary antioxidant intake and chronic bronchitic symptom (Burns et al., 2007). In the latter study, it is likely that allergic sensitization occurs in substantial number of eligible person during antioxidant intake. Together with our present study, it is suggestive that antioxidant intake may have a preventive, rather than therapeutic, effect on the development of allergic airway inflammation. Finally, our results do not formally exclude a role for environmental chemicals found in food contaminants in the pathogenesis of allergic diseases in the induction phase and future studies are needed to determine the relative contribution of this type of exposure to environmental chemicals in the pathogenesis of allergic airway inflammation. Nevertheless, persistent exposure to environmental chemicals, such as TBT, with oxidative stress may increase the likelihood of allergic sensitization in even individuals who are not pre-disposed to such sensitization and contribute to the increase in the prevalence of allergic airway inflammation and related diseases.

Conflict of interest statement

The authors declare no financial or commercial conflict of interest.

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