

Endocrine disruptors that deplete glutathione levels in APC promote Th2 polarization in mice leading to the exacerbation of airway inflammation

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Endocrine-disrupting chemicals (EDC) are ubiquitous in environment and may have various undesirable effects on human health. In the present study, we have shown that some EDC [benzophenone, *p*-octylphenol, and tributyltin chloride (TBT)] promoted strong Th2 polarization *via* suppression and augmentation of Th1 and Th2 development, respectively, from naive CD4⁺ T cells primed with anti-CD3 and splenic antigen-presenting cells (APC). The effect was indicated to be indirect *via* suppression of IL-12 production and augmentation of IL-10 production of APC, which are critical for the Th1 and Th2 development, respectively. Such modulation of cytokine production by EDC was associated with reduction of intracellular glutathione levels in APC. IL-10 deprivation or the addition of *N*-acetylcysteine, which replenishes intracellular glutathione level during priming, cancelled the effect of EDC on the promotion of Th2 polarization. Oral administration of TBT, which most effectively promoted Th2 polarization *in vitro*, exacerbated airway inflammation in a murine model of allergic asthma with concomitant enhancement of Th2-type immunity. Collectively these results suggest that EDC such as benzophenone, *p*-octylphenol, and TBT promote Th2 polarization indirectly *via* the depletion of glutathione in APC and subsequent modulation of IL-10 and IL-12 production that might result in the exacerbation of allergic diseases.

Received 15/6/05

Revised 20/1/06

Accepted 21/2/06

[DOI 10.1002/eji.200535140]

Key words:

Allergy · Endocrine-disrupting chemical · Glutathione · Th1 · Th2

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Abbreviations: **BALF:** BAL fluid · **BP:** benzophenone · **DBP:** dibutylphthalate · **DCHP:** dicyclohexylphthalate · **EDC:** endocrine-disrupting chemical · **GSH:** glutathione · **NAC:** *N*-acetylcysteine · **NP:** nonylphenol · **OCS:** octachlorostyrene · **OP:** *p*-octylphenol · **TBT:** tributyltin chloride · **TDS:** T cell-depleted spleen cells

Introduction

Adaptive immune responses are initiated by the activation of naive CD4⁺ T cells that recognize specific antigen on antigen-presenting cells (APC) and subsequent differentiation into at least two distinct populations of T helper cells, Th1 and Th2 cells, which have different cytokine production profiles and discrete effector functions. Th1 cells secrete IFN- γ , IL-2 and

TNF- β , and stimulate cell-mediated immunity, whereas Th2 cells secrete IL-4, IL-5, IL-10 and IL-13, stimulate humoral immunity, and are prominent mediators of allergic responses [1].

The process of Th cell differentiation is tightly regulated in order to evoke appropriate immune responses, and any disturbance of this process results in the induction of undesired immune responses often leading to autoimmune, allergic, and infectious diseases [2]. Cytokines that are secreted by APC interacting with naive CD4⁺ T cells are of particular importance for this process [3]. IL-12, produced by DC and macrophages but not by B cells [4, 5], directs Th1 cell development, whereas IL-10 in concert with IL-6, which is produced by B cells as well as DC and macrophages, plays an indispensable role in the Th2 cell development [6, 7]. Accumulating evidence indicates that intracellular glutathione (GSH) levels in APC profoundly influence production of these cytokines and thereby direct type of immunity developing in the host. It has been shown that depletion of GSH in APC suppressed IL-12 production and augmented IL-10 production, which resulted in the predominance of Th2-type immunity [8–10].

Allergic diseases are caused by exaggerated Th2-type immune response toward common, usually innocuous environmental antigens. It has been suggested that enhanced oxidative status increases likelihood that the initial critical encounters between naive CD4⁺ T cells and allergens result in the dominance of Th2-type immunity [11]. Accumulating evidence clearly indicates that oxidative stress resulting from an oxidant/antioxidant imbalance, an excess of oxidants, or a depletion of antioxidants such as GSH, plays a critical role in the pathogenesis of allergic diseases [12].

In the past few decades, there has been significant increase in incidence and severity of allergic diseases [13]. Environmental factors such as pollutants and food additives that do not have allergic potential but exert a pro-allergic adjuvant effect have been believed to play indisputable roles in the induction and/or exacerbation of allergic diseases [13]. For this reason, identification of responsible factors and underlying mechanisms involved should be a research priority in order to make allergic diseases preventable.

Endocrine-disrupting chemicals (EDC), so called because of their ability to interfere with hormone-signaling pathways *via* estrogenic or androgenic activities, are ubiquitous in the environment and have been attracted to great deal of attention not only as a causative of reproductive abnormality but also of neurological defects and certain types of cancer development [14]. However, effects of EDC on the immune responses, especially on immune deviation into Th1 or Th2, have been poorly investigated. Our previous study has indicated that one EDC, tributyltin chloride

(TBT), which exerts androgenic activity [15], promoted Th2 polarization *via* suppression of Th1 development and enhancement of Th2 development from naive CD4⁺ T cells primed with anti-CD3 plus splenic APC [16].

Here we extended this approach to other EDC with estrogenic activities. In addition, we also investigated the mechanisms underlying EDC-induced Th2 polarization. Our present results indicate that benzophenone (BP) and *p*-octylphenol (OP) as well as TBT promoted Th2 polarization regardless of their androgenic or estrogenic activities. The effect of EDC on the Th2 polarization was shown to be indirect through depletion of GSH in APC and subsequent suppression and augmentation of IL-12 and IL-10 production, respectively. Exposure of TBT, which most efficiently promoted Th2 polarization *in vitro*, exacerbated allergic airway inflammation with concomitant enhancement of Th2 type immunity.

Results

Effect of EDC on Th development

In the first experiment, we assessed whether, in addition to TBT, the various EDC, which have ability to interfere with hormone-signaling pathway in mammalian cells, might also interfere with Th development. Naive CD4⁺ T cells were primed with 1 μ g/mL of anti-CD3 and T cell-depleted spleen cells (TDS) in the presence of graded concentrations of BP, OP, TBT, nonylphenol (NP), octachlorostyrene (OCS), dibutylphthalate (DBP), and dicyclohexylphthalate (DCHP). Six days later, the phenotype of the Th cells generated was assayed by intracellular staining of IFN- γ and IL-4 after restimulation with PMA plus ionomycin.

As shown in Fig. 1, BP, OP, and TBT strongly augmented Th2 development and suppressed Th1 development. DCHP similarly affected Th development, though the effect was relatively weak as compared with that of BP, OP, or TBT. CD4⁺ T cells could not be recovered from cultures that received more than 10 μ M of DCHP. In contrast, OCS augmented Th1 development and suppressed Th2 development. NP strongly suppressed Th1 development, but did not affect Th2 development. DBP repeatedly suppressed both Th1 and Th2 development. Since EDC that simultaneously suppress Th1 development and augment Th2 development might synergistically promote Th2 polarization, we used BP, OP, and TBT in the following experiments.

Indirect effect of EDC on Th development

To determine whether the suppression and augmentation of Th1 and Th2 development, respectively, were

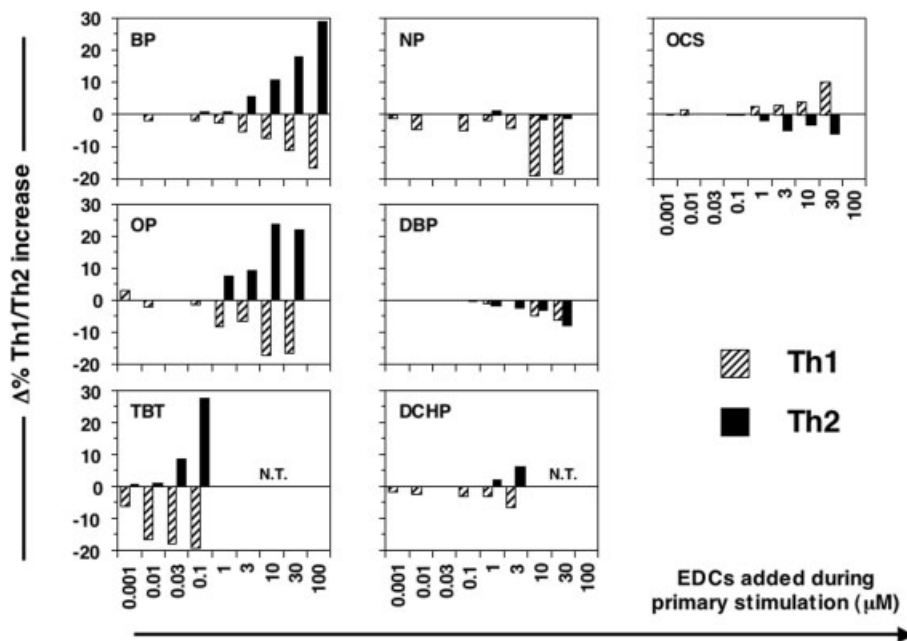


Figure 1. Effect of various EDC on Th1 and Th2 development. Naive CD4⁺ T cells were primed with 1 μ g/mL of anti-CD3 and TDS in the presence of graded concentrations of BP, OP, TBT, NP, OCS, DBP, and DCHP for 6 days. The phenotype of the Th cells generated was assayed by intracellular staining of IFN- γ and IL-4 after restimulation with PMA plus ionomycin. The numbers at histogram vertical axis were calculated by subtracting the % of Th1 or Th2 cells present in cultures in the absence of EDC from the % of Th1 or Th2 cells present in cultures in the presence of different EDC. The data shown are representative of three independent experiments with similar results.

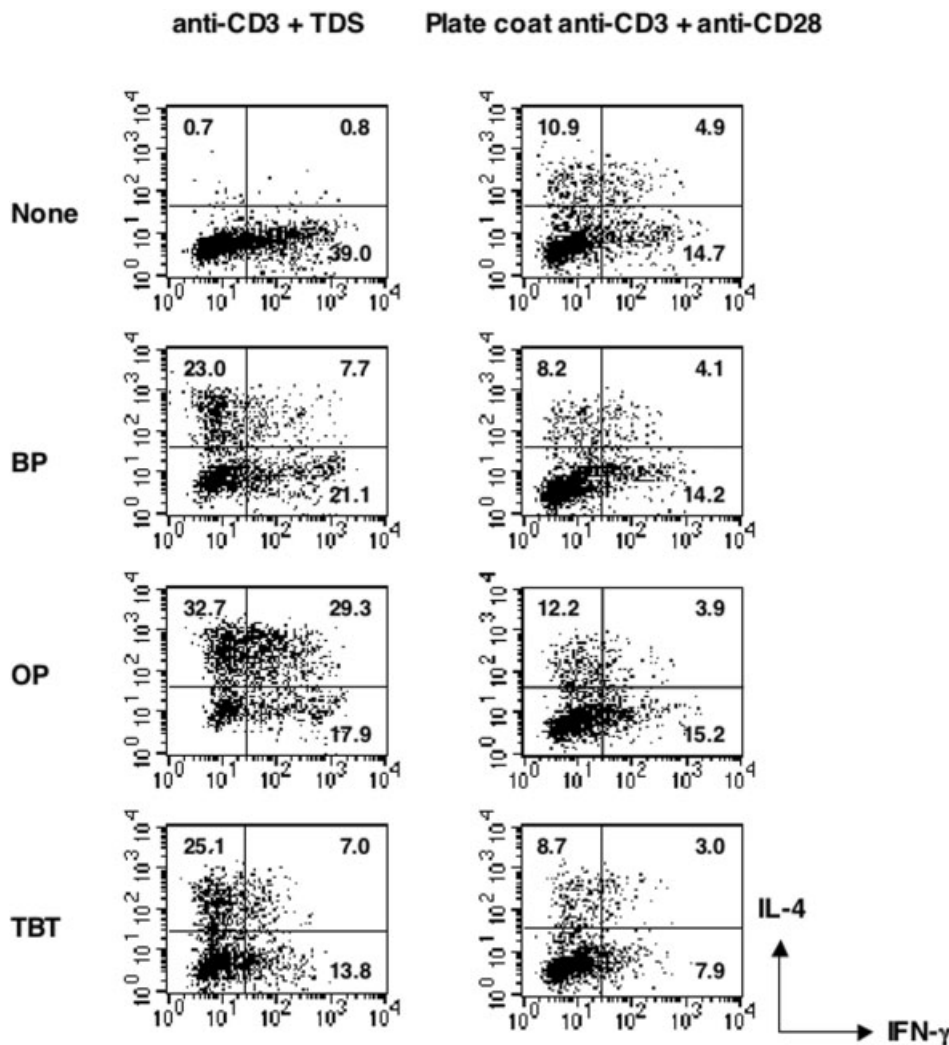


Figure 2. Requirement of APC for EDC to promote Th2 polarization. Flow cytometric analysis of intracellular IL-4 and IFN- γ staining of CD4⁺ T cells primed with plate-coated anti-CD3 plus anti-CD28 in the absence of TDS together with BP (30 μ M), OP (10 μ M), or TBT (0.1 μ M). The annotated numbers indicate the percentages of cells in each quadrant. The data shown are representative of three independent experiments with similar results.

direct effects of BP, OP, and TBT on naive CD4⁺ T cells, or indirectly caused through APC, Th cells generated from cultures driven by plate-coated anti-CD3 and anti-CD28 together with these EDC in the absence of TDS were assayed in the following experiment. When naive CD4⁺ T cells were primed in a well coated with anti-CD3 and anti-CD28 in the absence of TDS, the inclusion of EDC did not augment Th2 development (Fig. 2). TBT, but not BP and OP, slightly suppressed Th1 development even in the absence of TDS. Percentages of Th1 cells generated in the presence of TBT and those in the absence were $8.3 \pm 1.1\%$ and $16.2 \pm 2.5\%$, respectively, in three independent experiments ($p=0.02$).

Effect of EDC on the cytokine production of APC

In the above experiment, the effect of three EDC on Th development was indicated to be mainly indirect, possibly

working through APC. Therefore, we examined the effect of EDC on cytokine production of APC. TDS cultured with naive CD4⁺ T cells in the presence of anti-CD3 produced IL-12 p40 and IL-10. The addition of EDC to these cultures resulted in the decrease in IL-12 p40 and increase in IL-10 detected in the culture supernatant (Fig. 3A). Semiquantitative RT-PCR also revealed that IL-12 p40 and IL-10 mRNA expression in TDS were suppressed and augmented, respectively, by the addition of EDC, whereas IL-12 p35 mRNA and IL-6 mRNA expression remained unchanged (Fig. 3A). Essentially similar results were obtained when TDS were stimulated with LPS in the presence of EDC (Fig. 3B). Furthermore, we also confirmed that the EDC by themselves did not induce TDS to produce IL-12 or IL-10 without stimulations (data not shown). Taken together, these results indicated that EDC suppress IL-12 production and augment IL-10 production of TDS interacting with naive CD4⁺ T cells.

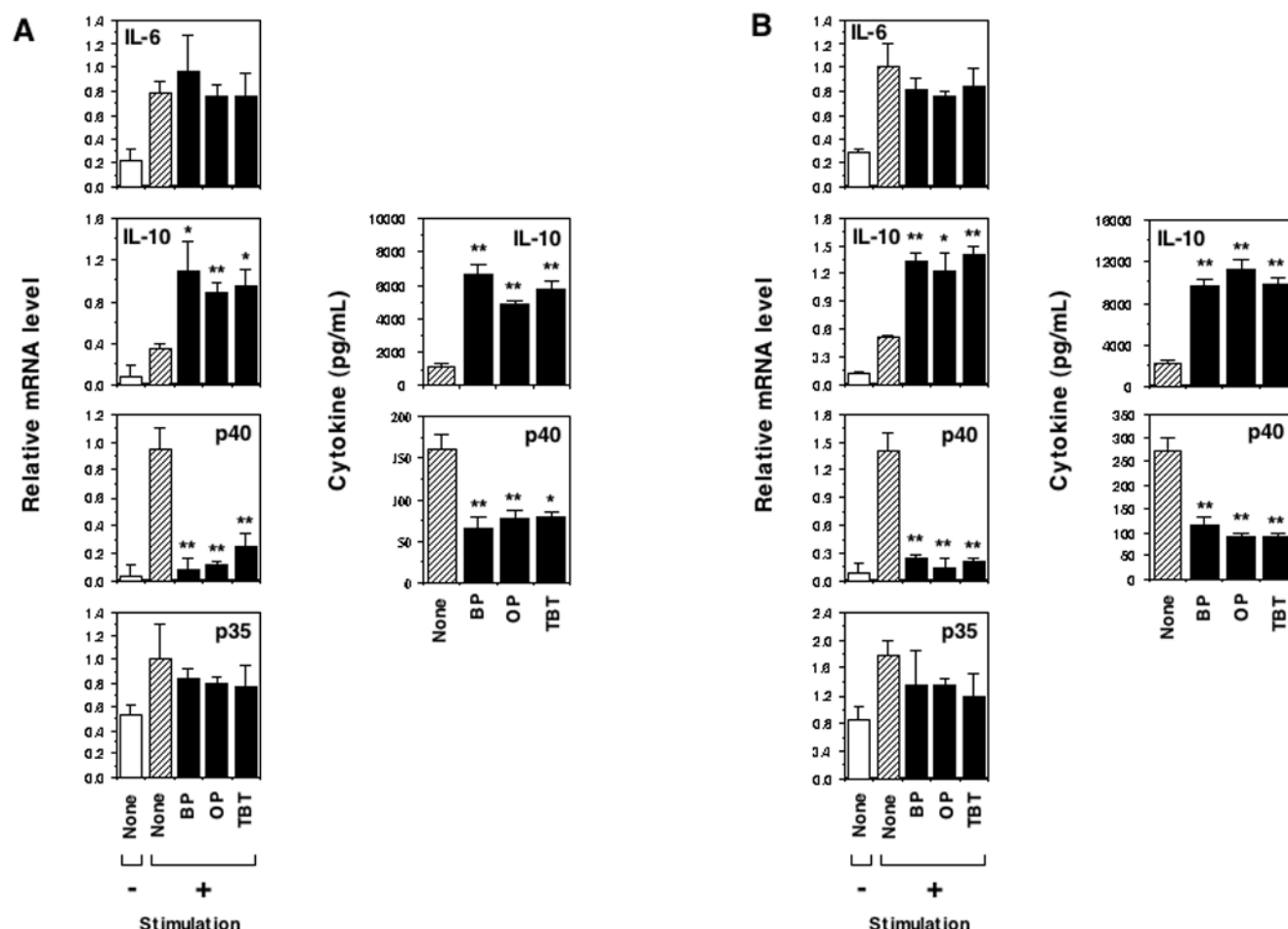


Figure 3. Suppression of IL-12 and augmentation of IL-10 production of APC interacting with naive CD4⁺ T cells by EDC. (A) TDS were cultured with naive CD4⁺ T cells and anti-CD3 together with or without BP (30 μ M), OP (10 μ M), or TBT (0.1 μ M). Culture supernatant and cells collected after 48 h and 24 h were assayed for cytokines by ELISA and cytokine mRNA by semiquantitative RT-PCR, respectively. (B) TDS were cultured with 20 μ g/mL of LPS together with or without BP (30 μ M), OP (10 μ M), or TBT (0.1 μ M). Culture supernatant and cells collected after 24 h and 12 h were assayed for cytokines by ELISA and cytokine mRNA by semiquantitative RT-PCR, respectively. The data shown are means $\pm$ SE of triplicate samples and representative of three independent experiments.

The role of IL-10 in the enhancement of Th2 development by EDC

In the next experiment, we tested whether the up-regulation of IL-10 production has relevance to the effect of EDC on the enhancement of Th2 development. Naive CD4⁺ T cells were primed with anti-CD3 and TDS in the presence of EDC at the doses that induced strong Th2 polarization and the effect of neutralization of IL-10 was examined. The addition of anti-IL-10 inhibited the effect of EDC on Th2 development (Fig. 4A). Furthermore, when TDS from IL-10-deficient mice were used for priming, enhancement of Th2 development by EDC was significantly diminished (Fig. 4B).

The effect of EDC on Th2 development is mediated by depletion of intracellular GSH

Since intracellular levels of GSH in APC modulate their cytokine production [8–10], we examined, in the next

series of experiments, whether the depletion of intracellular GSH levels in APC by EDC was involved in the promotion of Th2 polarization. GSH levels in TDS treated with EDC at the concentrations that induce Th2 polarization were significantly decreased except those in TDS treated with 0.01 μ M TBT (Fig. 5). Although statistically not significant, TDS treated with 0.01 μ M TBT repeatedly showed a tendency to decrease in GSH levels.

We then examined the effect of *N*-acetylcysteine (NAC), which replenishes the cellular content in GSH [17], on the IL-10 and IL-12 production of APC. The concentration of NAC (0.24 mM) used for the experiment was chosen according to the preliminary experiment that revealed no effect on the Th development by itself (data not shown). As shown in Table 1, the addition of NAC partially cancelled the suppression of IL-10 production of TDS by BP, OP and TBT. NAC recovered IL-12 production suppressed by BP and OP, but not by TBT. This effect of NAC on IL-10 and IL-12 production of APC

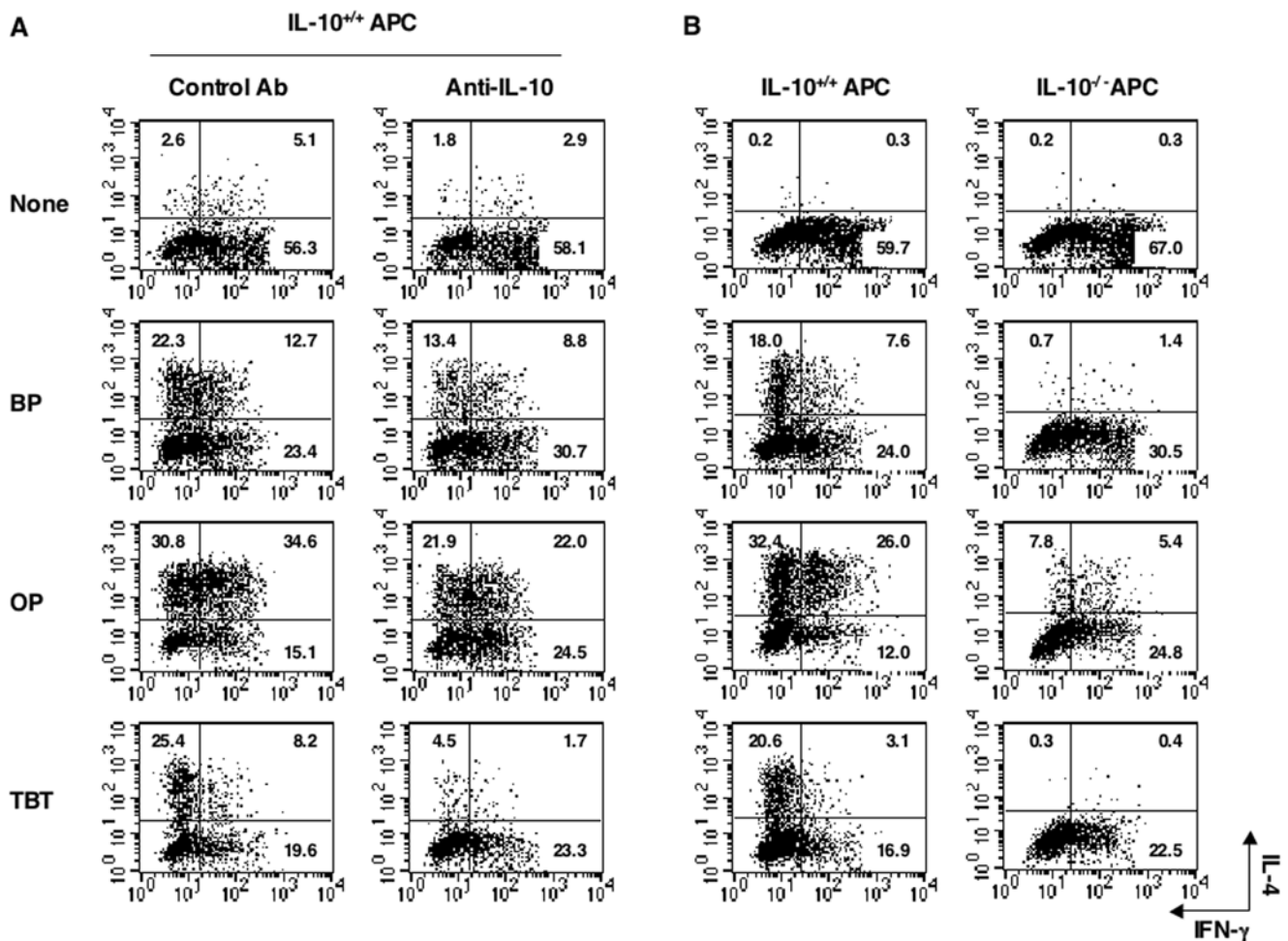


Figure 4. Role of IL-10 in the enhancement of Th2 development by EDC. Flow cytometric analysis of intracellular IL-4 and IFN- γ staining of CD4⁺ T cells primed with anti-CD3 plus TDS from IL-10^{+/+} mice in the presence of 5 μ g/mL anti-IL-10 or control mAb (A), or primed with anti-CD3 plus TDS from IL-10^{+/+} or IL-10^{-/-} mice (B). To these cultures, BP (30 μ M), OP (10 μ M), or TBT (0.1 μ M) were added. The data shown are representative of three independent experiments with similar results.

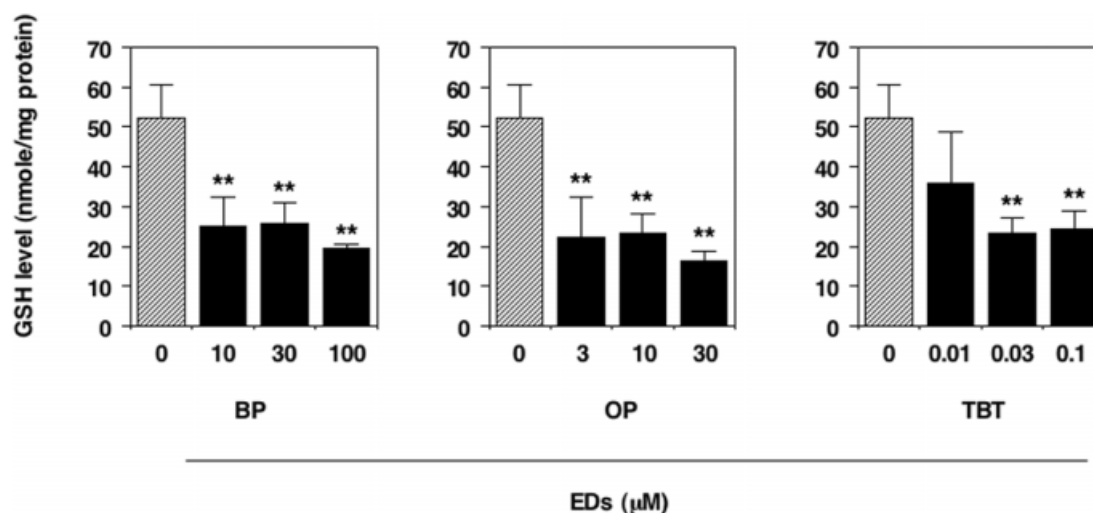


Figure 5. Effect of EDC on intracellular GSH levels in APC. TDS at 2×10^6 cells/mL were cultured in the presence of the indicated concentrations of BP, OP, or TBT for 18 h. Amounts of intracellular GSH were assayed as described in *Materials and methods*. The data shown are representative of three independent experiments with similar results; ** $p < 0.01$ vs. control.

modulated by EDC was clearly associated with cancellation of enhancement of Th2 development and suppression of Th1 development by EDC (Table 2). The enhancement of Th2 development by all three EDC was significantly suppressed by the addition of NAC, whereas the suppression of Th1 development by BP and OP, but not by TBT, was recovered by NAC

Oral administration of TBT exacerbates airway inflammation

To determine whether the Th2 polarization induced by EDC has relevance to the exacerbation of allergic diseases, we examined the effect of EDC exposure on the exacerbation of airway inflammation in mice sensitized and challenged with OVA. Since TBT was

shown to be most effective in inducing Th2 polarization *in vitro* at the concentrations reported elsewhere in water, sediment, mussel tissue, and human blood [18, 19], we chose TBT in the following *in vivo* experiments.

Gastric inoculation with total 6 $\mu\text{mol/kg}$ TBT resulted in the exacerbation of airway eosinophilia that was associated with increases in IL-5 detected in BAL fluid (BALF) and IgE in serum (Fig. 6A). Furthermore, mice fed with laboratory chow containing 10 ppm of TBT for a month developed more severe airway eosinophilia with concomitant increase in IL-5 and IgE levels (Fig. 6B). Spleen cells from these mice restimulated with OVA *in vitro* produced less amount of IFN- γ (80.5 ± 10.5 pg/mL in TBT-treated mice vs. 255 ± 58 pg/mL in control, and 68.9 ± 15 pg/mL in TBT-fed mice vs. 156 ± 33 pg/mL in control; both

Table 1. Effect of NAC on the modulation of cytokine production by EDC^{a)}

	IL-10 (ng/mL)		IL-12 (pg/mL)	
	None	NAC	None	NAC
None	1.3 $\pm$ 0.1	1.3 $\pm$ 0.3	250 $\pm$ 14	255 $\pm$ 21
BP	13.9 $\pm$ 0.4	8.8 $\pm$ 0.3*	130 $\pm$ 14	272 $\pm$ 11*
OP	11.4 $\pm$ 0.7	5.0 $\pm$ 0.3*	145 $\pm$ 21	238 $\pm$ 25*
TBT	8.8 $\pm$ 0.4	4.1 $\pm$ 1.1*	125 $\pm$ 7	138 $\pm$ 11

^{a)} Naive CD4⁺ T cells were cultured with TDS and anti-CD3 in the presence of BP (30 μM), OP (10 μM), or TBT (0.1 μM). To these cultures, NAC (0.24 mM) was added. Culture supernatants were collected at 48 h and assayed for cytokines by ELISA. Data represent means $\pm$ SE of triplicate cultures and are representative of three independent experiments; * $p < 0.01$ compared to cytokines detected in cultures without NAC.

Table 2. Effect of NAC on the modulation of Th development by EDC^{a)}

	% Th1		% Th2	
	None	NAC	None	NAC
None	53.2 $\pm$ 6.4	51.4 $\pm$ 3.1	2.0 $\pm$ 1.0	1.6 $\pm$ 1.4
BP	22.5 $\pm$ 3.3	46.1 $\pm$ 5.2*	23.4 $\pm$ 4.6	9.9 $\pm$ 4.3*
OP	13.0 $\pm$ 4.1	25.8 $\pm$ 5.1*	33.5 $\pm$ 4.5	17.0 $\pm$ 2.7*
TBT	15.8 $\pm$ 2.8	12.4 $\pm$ 2.5	26.0 $\pm$ 4.2	13.8 $\pm$ 2.8*

^{a)} Naive CD4⁺ T cells were cultured with TDS and anti-CD3 in the presence of BP (30 μM), OP (10 μM), or TBT (0.1 μM). To these cultures, NAC (0.24 mM) was added. On day 6, the phenotype of the Th cells generated was assayed by intracellular staining of IFN- γ and IL-4 after restimulation with PMA plus ionomycin. Data represent means $\pm$ SE of four independent experiments; * $p < 0.01$ compared to Th1 or Th2 cells generated in cultures without NAC.

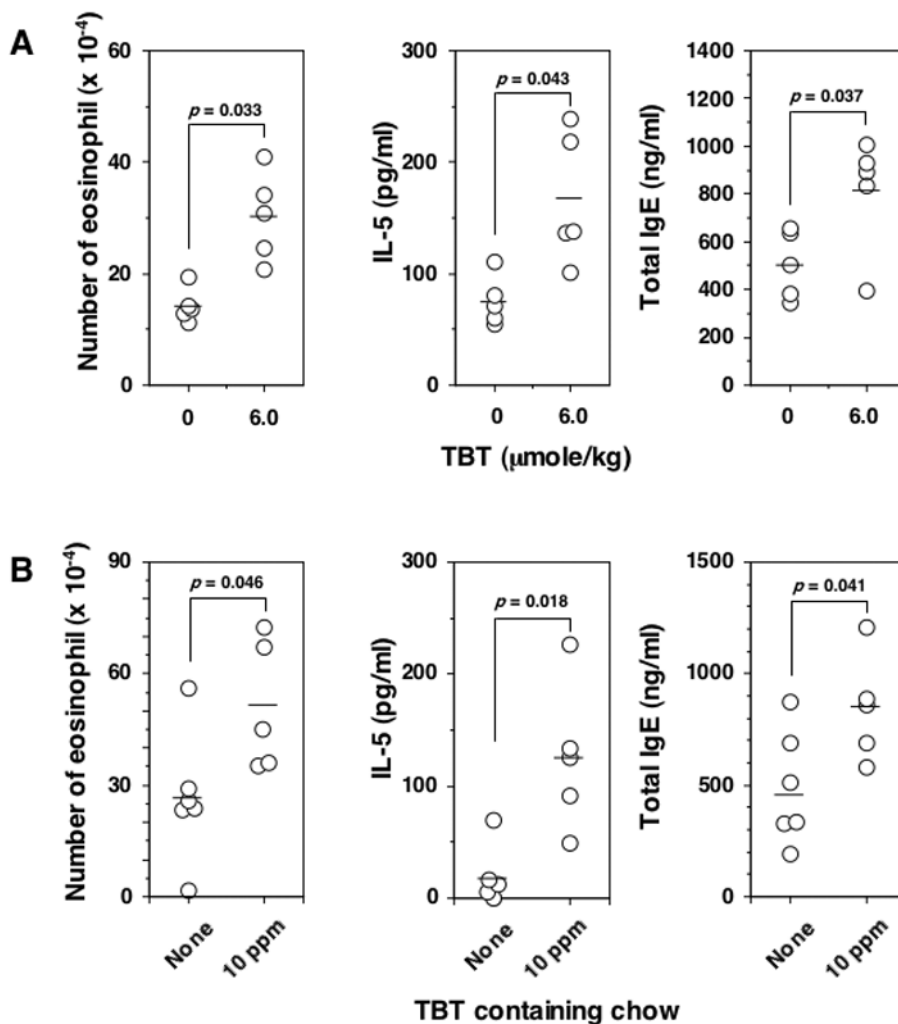


Figure 6. Exacerbation of airway inflammation by TBT. C57BL/6 mice were treated with either corn oil (containing 0.2% DMSO as a control) or TBT (0.6 $\mu\text{mol/kg}$) by oral gavage (A). C57BL/6 mice were fed with TBT-containing chow (10 ppm) for 30 days (B). In these mice, airway inflammation was induced as described in *Materials and methods*. BAL was performed 24 h after the last challenge.

$p < 0.01$). These results suggest that EDC might exacerbate airway inflammation *via* augmentation of Th2 immune responses

Discussion

Studies presented here demonstrate that BP and OP as well as TBT augmented Th2 development and suppressed Th1 development indirectly *via* suppression of IL-12 production and augmentation of IL-10 production of APC. Depletion of intracellular GSH in APC was suggested to be a common mechanism underlying such modulation of APC cytokine production by EDC.

Suppression of Th1 development by these EDC, especially OP, was associated with the promotion of IFN- γ producing Th0 development. Therefore, it is possible that EDC might not suppress Th1-type immune responses. However, the possibility seems unlikely,

since we have previously shown that hydroxymethylglutaryl-coenzyme A reductase inhibitors, statins, suppressed Th1-type immunity *via* suppression of Th1 development with concomitant increase in the generation of Th0 cells [20].

EDC are chemicals in environment that interfere with endocrine system *via* mimicking or antagonizing sex hormones [21]. Sex hormones have been shown to modulate both Th1 and Th2 immunity in various mouse models, and implicated in the sex-based difference in incidence of allergic diseases [22, 23]. However, it appeared that hormonal activities were not relevant to the effect of these EDC on the Th development, since all three EDC regardless of their estrogenic (BP and OP [24, 25]) or androgenic (TBT [15]) activities showed similar promotion of Th2 polarization. Our prediction was partly supported by recent studies showing that OP promotes Th2 development independently of estrogen receptor [26, 27].

In contrast to the effect of BP and OP on Th1 development, TBT slightly but statistically significantly suppressed Th1 development even when naive CD4⁺ T cells were primed in the absence of APC. Since it has been shown that TBT induces apoptosis in CD4⁺ T cells and Th1 cells were more susceptible to apoptosis than Th2 cells [28, 29], it is possible that Th1 cells developed in cultures driven by immobilized anti-CD3 plus anti-CD28 were selectively depleted during primary cultures. Although TBT seemed to affect Th1 development partly by direct effect on CD4⁺ T cells, the effect of EDC on the promotion of Th2 polarization appeared to be largely indirect, since marked enhancement of Th2 development by any of these three EDC was not observed when naive CD4⁺ T cells were primed with plate-coated anti-CD3 plus anti-CD28 in the absence of TDS.

Cytokines, such as IL-6, IL-10, and IL-12, which are produced by APC interacting with naive CD4⁺ T cells, play critical roles in directing either Th1 or Th2 development [3]. IL-10 in concert with IL-6 has been shown to be essential for the Th2 development [6, 7, 16]. We showed that levels of IL-10 in cultures where TDS were stimulated by naive CD4⁺ T cells in the presence of BP and OP as well as TBT were significantly higher than those in the absence of EDC. The possibility that EDC augmented CD4⁺ T cells to produce IL-10 seemed unlikely, since we could not detect IL-10 mRNA expression in T cells (data not shown). Furthermore, EDC also enhanced IL-10 production of TDS stimulated with LPS.

In contrast to the effect of EDC on IL-10 production, the IL-12 production of APC, which plays a critical role in the Th1 development, was significantly suppressed by EDC. Although IL-10 has been reported to suppress IL-12 production of APC [30], the augmented IL-10 production by EDC seemed not to be responsible for the suppression of IL-12 production, since the addition of anti-IL-10 did not recover IL-12 production of TDS cultured in the presence of EDC (data not shown). These results together with the result that deprivation of IL-10 inhibited the effect of EDC on Th development indicate that augmented IL-10 and suppressed IL-12 production of APC by EDC are responsible for the promotion of Th2 polarization.

The modulation of IL-10 and IL-12 production of APC by BP, OP, and TBT was indicated to be, at least in part, due to their ability to deplete intracellular GSH levels in APC. GSH is a major intracellular redox buffer constituting nearly 90% of the intracellular non-protein thiols [31] that determine intracellular redox status. Accumulating evidence indicates that the cellular redox status tightly regulates activities of transcription factors such as NF- κ B and enzymes such as p38 MAPK involved in signal transduction for the activation of cytokine genes [32]. It has been shown that reductive redox status, *i.e.* high intracellular GSH levels, facilitates IL-12

production *via* activation of p38 MAPK [33], whereas oxidative redox status, *i.e.* low intracellular GSH levels, augments IL-10 production *via* activation of NF- κ B [9].

Using human and mouse primary cell cultures, it has been shown that all, BP, OP, and TBT, enhance reactive oxygen species generation, which might result in the depletion of intracellular GSH [34–36]. In the present study, we have demonstrated that intracellular GSH levels in TDS used as APC were clearly decreased by BP, OP, and TBT at the concentrations that induced Th2 polarization. We have also shown that the suppression of IL-12 production and augmentation of IL-10 production of APC by BP, OP, and TBT, which were responsible for the promotion of Th2 polarization, were associated with decrease in intracellular GSH levels. Furthermore, the addition of NAC, which replenishes the cysteine required for synthesis and restoration of the depleted GSH [17], cancelled, at least in part, the effect of these EDC on the APC cytokine production, which was also associated with the normal Th development. These results agree quite well with previous observations that depletion of intracellular GSH in APC by reagents such as ethanol, diethyl malate, cyclophosphamide, or *L*-buthionine-[*S,R*]-sulfoximine suppressed IL-12 and augmented IL-10 production of APC [8–10].

Although available results on human exposure to BP, OP and TBT are limited, human exposure to TBT has been documented *via* consumption of TBT-containing meat and fish products. TBT has been reported to be detectable in human blood at concentrations of 25.1 ± 47.3 [18] and 64.5 ± 32.9 nM [19]. It is noteworthy that the concentrations reported to be detectable in human blood were shown to promote Th2 polarization *in vitro* in the present experiments. Furthermore, we showed that oral administration of TBT exacerbated airway inflammation in a murine model of allergic asthma with concomitant enhancement of Th2-type immunity. Epidemiological studies indicated an increase in the prevalence of allergic diseases in environmentally polluted areas, suggesting that environmental factors may be one of the causes of this disease increase [13]. Our present results might provide insight into the contribution of EDC to the increase in prevalence of Th2-driven allergic diseases.

Accumulating evidence suggests that oxidative stress that depletes intracellular GSH plays a crucial role in the pathogenesis of asthma [11]. While it has been reported that reduced levels of GSH in the epithelial lining fluid were found in asthmatic lungs compared with those in normal subjects [37], results on GSH levels in APC in asthmatic patients are not available to date. However, it is possible that the GSH levels in APC in the patients with asthma are decreased, since TGF- β 1, which acts as a potent inhibitor of GSH synthesis [38], has been shown to be systemically elevated in asthmatic patients [39].

In this regard, it is noteworthy that systemic administration of reagents that replenish GSH, such as NAC or 2-oxothiazolidine-4-carboxylic acid, has been shown to suppress Th2-type immune response, and to reduce airway inflammation and hyper-reactivity in animal models of asthma [40, 41]. These results are in line with our present results that EDC that deplete intracellular GSH in APC promote Th2 polarization and that one EDC, TBT, exacerbates airway inflammation in a murine model of asthma. Therefore, it seems highly likely that depletion of intracellular GSH in APC by EDC might be the underlying mechanism for the promotion of Th2 polarization and subsequent induction and/or exacerbation of allergic diseases. In addition, these results also suggest that testing for GSH-depleting activities of chemicals might provide a new option for screening environmental chemicals with pro-allergic activities.

Materials and methods

Mice

Female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan) and used at 7–8 wk of age. IL-10^{-/-} mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). The experiments were approved by the Ethics Review Committee for Animal Experimentation of Mie University School of Medicine.

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 solutions of BP, OP, NP, OCS, DBP, DCHP (all purchased from Sigma Chemical Co.), and TBT (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) were 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. NAC (Sigma Chemical Co.) was dissolved in culture medium, and pH was adjusted to 7.4 by the addition of NaOH.

Cells

FACS-sorted CD4⁺CD44^{low} T cells were prepared from spleens of C57BL/6 mice as described previously [16]. In some experiments, CD62L⁺ T cells were isolated from negatively selected splenic CD4⁺ T cells followed by positive selection of magnetic labeled CD62L⁺ cells by MACS column according to the manufacturer's instruction. These CD4⁺CD44^{low} or CD62L⁺CD4⁺ T cells were used as naive CD4⁺ T cells. TDS were prepared from syngeneic mouse spleen as described previously [16] and used as APC after the treatment with 50 µg/mL mitomycin C (Kyowahakko Co., Tokyo, Japan).

In vitro priming of naive CD4⁺ T cells

Priming of naive CD4⁺ T cells (2×10^5 cells/2 mL/culture) was conducted using 1 µg/mL anti-CD3 (145-2C11) together with mitomycin C-treated splenic APC (3×10^6 cells/2 mL/culture) in a well of 24-well plate (Iwaki Glass Co., Tokyo, Japan). In some cases, soluble anti-CD3 and APC were replaced by immobilized (2 µg/mL) anti-CD3 and anti-CD28 (37.51). To these cultures, various concentrations of EDC were added. Cultures that received only vehicle alone served as controls. In addition, some cultures were supplemented with 5 µg/mL anti-IL-10 (JES5-2A5; PharMingen) or 0.24 mM NAC. The CD4⁺ T cells primed for 4 days were expanded into four wells with fresh medium containing 2.5 ng/mL IL-2 (rhIL-2; Ajinomoto Co., Kawasaki, Japan) and cultured for another 2 days. Six days after priming, the CD4⁺ T cells were recovered and restimulated with PMA plus ionomycin in the presence of 5 µg/mL brefeldin A followed by intracellular cytokine staining as described [16].

Induction and analysis of airway inflammation

C57BL/6 mice were sensitized by i.p. injection of 10 µg OVA and 1.125 mg aluminium hydroxide (Imject Alum; Pierce, Rockford, IL) 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 0.1 µmol/kg TBT by oral gavages on days 0, 2, 4, 7, 9, and 11 (total 6 µmol/kg). On days 14–16, the mice received 10 µg OVA dissolved in 50 µL of saline intranasally under anesthesia with pentobarbital. In C57BL/6 mice that had been fed with TBT-containing chow (10 ppm) for 30 days, airway inflammation was also induced as above. On day 17, BALF and serum were collected as described [16]. The supernatant and cells of BALF were subjected to cytokine ELISA and differential cell counts as described [16]. Supernatants from spleen cells of these mice cultured at 2×10^6 cells/mL in the presence of 200 µg/mL of OVA for 48 h were also assayed for IFN-γ by ELISA.

Cytokine and Ig ELISA

IL-5 in BALF was assayed by ELISA using paired mAb specific for the corresponding cytokine as described previously [16]. ELISA for IL-10 and IL-12 p40 were conducted using commercial kits (Endogen, Woburn, MA). Lower detection limits of these assays were as follows: IL-5, 50 pg/mL; IL-10, 100 pg/mL; IL-12 p40, 11 pg/mL. Total serum IgE levels were determined by ELISA using paired mAb specific for mouse IgE and IgE standard (BD PharMingen). Lower detection limit of IgE was 10 ng/mL.

Semiquantitative RT-PCR

Total cellular RNA of the cells from the primary naive CD4⁺ T cell-TDS culture or that of TDS stimulated with LPS (20 µg/mL) was reverse-transcribed into cDNA and semiquantitative analyses of IL-6, IL-10, IL-12 p40, and IL-12p35 mRNA accumulation were performed as described previously [16]. Quantitative analysis was performed using NIH Image software and the results were presented as relative mRNA levels

obtained by the ratio of cytokine PCR product to hypoxanthine phosphoribosyltransferase PCR product. The results are expressed as means $\pm$ SE of triplicate samples.

Assay for intracellular GSH

Intracellular GSH level was evaluated using high-performance chromatography with a gold electrode developed by us [42]. Briefly, TDS (2×10^6 cells/mL) cultured for 18 h in the presence of graded concentrations of EDC were washed twice with PBS, followed by homogenization in ice-cold PBS containing 0.1% v/v Triton X-100 and centrifugation at 18 500 $\times$ g. In an aliquot of the supernatant, protein concentration was assessed using BCA protein assay kit (Pierce). Then 5% w/v trichloroacetic acid was added to the supernatant, and the supernatant centrifuged at 18 500 $\times$ g. The supernatant was then diluted with 0.1 N HCl and the total GSH concentration was determined on an HPLC-ECD (Eicon, Kyoto, Japan) using authentic GSH (Kohjin Co. Ltd. Tokyo, Japan) as a standard.

Statistics

Statistic analyses were performed with two-tailed Student's *t*-test and one-way ANOVA followed by pair-wise comparisons using the Dunnett's *post-hoc* procedure. A *p* value of <0.05 was considered significant. All experiments were performed at least three times.

Acknowledgements: This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan; the Japan Chemical Industry Association; and Mie Medical Research Foundation.

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