

Original Article

Trichloroethylene enhances TCR-CD3-induced proliferation of CD8⁺ rather than CD4⁺ T cells

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ABSTRACT — Trichloroethylene (TCE) is suspected as a potent immunomodulator that accelerates the development of allergic diseases. We previously reported that TCE promotes ovalbumin (OVA)-induced active cutaneous anaphylaxis, including enhancing antigen-specific serum IgE levels and splenic lymphocyte proliferation. However, the target cells and molecular mechanism through which TCE modulates antigen-specific immune responses remain unclear. To identify a potential underlying mechanism, we investigated whether TCE modulates T cell receptor (TCR)-induced T cell activation and proliferation *in vitro*. TCE enhanced T cell proliferation primed by anti-CD3 antibody, but not concanavalin A, in a dose-dependent fashion. In addition, TCE enhanced anti-CD3-primed proliferation of CD8⁺ rather than CD4⁺ T cells. Consistent with this result, TCE markedly enhanced the Lck phosphorylation mediated by anti-CD3 antibody in CD8⁺ but not CD4⁺ T cells. Furthermore, we analyzed the effect of TCE exposure via drinking water for 2 weeks on splenocyte populations in non-immunized and OVA-immunized mice. In OVA-immunized mice, TCE (3 mg/l) significantly expanded CD3⁺, CD8⁺ and CD4⁺ cell populations, however the effect at the lower concentration was significant only in the CD8⁺ populations, whereas TCE had no effect on these cells population in non-immunized mice. These findings suggest that TCE enhances TCR-CD3-induced proliferation of CD8⁺ rather than CD4⁺ cells and disrupts various activities of peripheral T cells.

Key words: Trichloroethylene (TCE), T cell proliferation, Lck phosphorylation, CD8⁺ T cells

INTRODUCTION

Trichloroethylene (TCE) is a synthetic solvent that is used in dry cleaning and in various industries to remove oils and greases from metal. TCE has shown toxicity for the nervous system, liver, and kidneys, and suspected carcinogen (Barton and Clewell, 2000; Gist and Burg, 1995; Scott and Chiu, 2006). TCE also is known as an immunomodulator. Occupational TCE exposure caused increases in both IL-2 and IFN- γ levels but reduced IL-4 levels (Iavicoli *et al.*, 2005). In animal experiments, TCE enhanced autoimmune responses, including activation of Th1 cells and inhibition of apoptosis of CD4⁺ T cells, in autoimmune-prone MRL^{+/+} mice (Blossom *et al.*, 2008; Gilbert *et al.*, 2006; Griffin *et al.*, 2000a, 2000b). In addition to these effects, we previously reported that TCE enhanced mast cell activation and subsequently caused immediate allergic reactions in rats (Seo *et al.*, 2008a, 2008b). More recently, we revealed that

TCE exposure through drinking water promotes ovalbumin (OVA)-induced active cutaneous anaphylaxis (ACA), including enhancing OVA-specific serum IgE levels and splenic lymphocyte proliferation, in mice (Kobayashi *et al.*, 2010). These observations suggest that antigen-specific immune responses modulated by TCE result from promotion of antigen-specific T cell activation and proliferation. How TCE achieves these effects remains unclear.

Generally, antigen-specific T cell activation is triggered by signals from antigen-presenting cells (APCs) through the binding of a processed antigenic peptide within the major histocompatibility complex (MHC) molecule to the T cell receptor (TCR) (Huang *et al.*, 1999; Mannie *et al.*, 1998; Taams *et al.*, 1999). In addition, the CD4 and CD8 co-receptors play important roles in T cell activation, especially under conditions of limiting antigen concentration (Janeway, 1988; Veillette *et al.*, 1988). Binding of the extracellular domain of the co-receptors to invariant membrane-proximal regions of the MHC mol-

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ecules allows simultaneous association of both the TCR and CD4 or CD8 with the same MHC class II or class I molecule, respectively, thereby increasing the avidity of the antigen-MHC recognition process (Doyle and Strominger, 1987; Norment *et al.*, 1988). Furthermore the cytoplasmic domains of the CD4 or CD8 co-receptors contribute to signaling through their association with the tyrosine kinase Lck (Alexander, 2000; Chan *et al.*, 1994; Holdorf *et al.*, 2002); this association and Lck phosphorylation are important for the optimal antigen-specific response of T cells.

To identify a potential mechanism of TCE-induced immunomodulation, we here focused on TCR-mediated signal transduction for T cell activation and investigated the effect of TCE on both T cell proliferation and TCR-primed phosphorylation of Lck.

MATERIALS AND METHODS

Chemicals and animals

TCE was purchased from Nacalai Tesque (Kyoto, Japan). Penicillin-streptomycin solution was purchased from Invitrogen (Grand Island, NY, USA). All other reagents and solvents were of analytical grade. Male BALB/c mice (7 to 9 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and allowed to acclimate for a week after their arrival. They were maintained under a 12:12-hr light:dark cycle at $25 \pm 2^\circ\text{C}$ and 40% to 60% relative humidity, with free access to food and drinking water. The experiments were performed according to guidelines for the care and use of experimental animals written by the Japanese Association for Laboratory Animal Science in 1987. This study was approved by the Ethics Committee of Gifu Pharmaceutical University.

Cell preparation and culture

Mouse primary splenocytes and lymph node cells were cultured as previously described (Hayashi *et al.*, 1999; Kobayashi *et al.*, 2010). Briefly, splenocytes and lymph node cells were removed aseptically and teased into single-cell suspensions in RPMI 1640 medium (Wako Pure Chemical, Osaka, Japan) supplemented with penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), 2-mercaptoethanol (50 μM), and 10% horse serum. CD4⁺ and CD8⁺ cell populations were purified from the splenocytes by using MACS technology and mouse CD4 (L3T4) microbeads (Miltenyi Biotec, Auburn, CA, USA), mouse CD8a (Ly-2) microbeads (Miltenyi Biotec), and LS columns (Miltenyi Biotec). These cells were cultured with or without each compound in the presence or absence of each stimulant at 37°C , 5% CO_2 .

Proliferation assay

Cell proliferation in culture was measured by [³H]-thymidine incorporation. Cells (5×10^5 cells/well) were stimulated with or without immobilized monoclonal rat anti-mouse CD3 antibody (Clone 17A2; R&D Systems, Minneapolis, MN, USA) or concanavalin A (Con A; Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of TCE or fenofibrate (Sigma-Aldrich) in 96-well plates for 96 hr. Each culture was pulsed with 30 kBq [³H]-thymidine (American Radiolabeled Chemicals, St. Louis, MO, USA) for 16 hr before harvesting, and the radioactivity incorporated into the cells was quantified by liquid scintillation counting (Hayashi *et al.*, 1999; Nakanishi *et al.*, 2006; Nakanishi *et al.*, 2002).

Western blotting

CD4⁺ and CD8⁺ cells (10^6 cells/well) were stimulated with or without anti-mouse CD3 antibody in the presence or absence of TCE in 24-well plates for 24 hr and then harvested. Lysates from the harvested cells were prepared by using ice-cold lysis buffer (1% NP-40, 20 mM Tris [pH 8.0], 137 mM NaCl, 5 mM MgCl_2 , and 10% glycerol) supplemented with protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Nacalai Tesque). Protein samples (20 μg) underwent sodium dodecyl sulphate-polyacrylamide electrophoresis by using Any kD Mini-Protean TGX Gels (Bio-Rad Laboratories, Hercules, CA, USA) and were transferred to a polyvinylidene difluoride membrane. Rabbit anti-Lck polyclonal antibody (Upstate, Lake Placid, NY, USA) and rabbit anti-phospho-Lck (Tyr⁵⁰⁵) polyclonal antibody (Cell Signaling, Beverly, MA, USA) were used as primary antibodies for immunoblotting. Peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a secondary antibody, and the immunoreactive bands were visualized with 0.05% 3,3'-diaminobenzidine, 0.05% nickel ammonium sulfate, and 0.015% H_2O_2 in PBS. For quantitative analysis, band intensities were determined by using Image J software (National Institutes of Health, Bethesda, MD, USA).

TCE exposure, OVA immunization, and flow cytometric analysis

TCE exposure and ovalbumin (OVA; albumin from chicken egg white, grade V, 98% purified, Sigma-Aldrich) immunization was performed as previously described (Kobayashi *et al.*, 2010). Mice were divided into groups of six and received free access to tap water containing 0, 0.03, or 3 mg/l TCE (solutions prepared fresh daily) for 2 weeks (beginning on day 0). In addition, the mice were immunized intraperitoneally on days 0 and 7 with 1 μg

OVA and 5 µg aluminum hydroxide (alum; Wako Pure Chemical) in 0.2 ml phosphate-buffered saline (PBS); non-immunized mice were injected with PBS without OVA-alum. At day 14, splenocytes were prepared from the mice. The percentages of the splenocytes subpopulations were analyzed by FACScan (BD Bioscience, Franklin Lakes, NJ, USA) by using BD Mouse T Lymphocyte Subset Antibody Cocktail, with isotype controls (PE-Cy7-CD3e, PE-CD4, and FITC-CD8; BD Bioscience).

Statistical analysis

All data from the control and treatment groups were obtained from the same numbers of replicated experiments. All experiments were performed independently two or three times. Data are shown as mean $\pm$ 1 S.D. Statistical analyses were performed by using one-way ANOVA followed by Bonferroni's post-hoc procedure using SPSS15.0J software (Chicago, IL, USA). *P* values less than 0.05 or 0.01 were considered to be statistically significant.

RESULTS AND DISCUSSION

TCE enhances TCR-CD3-mediated T cell proliferation

To assess the effect of TCE on T cell proliferation, two stimulants were used: Con A, which is a tradition mitogenic lectin that acts as a polyclonal T cell stimulant, and immobilized anti-CD3 antibody, which is a more physiologically relevant approach to stimulating T cells that mimics stimulation by APCs. Compared with that in vehicle-only controls, the amount of [³H]-thymidine incorporated into splenocytes was significantly increased in the presence of 100 nM TCE (*P* < 0.05, Fig. 1A) and in lymph node cells at 1 nM TCE (*P* < 0.01, Fig. 1B). In contrast, TCE had no effect on the proliferation of unstimulated splenocytes and lymphocytes (Figs. 1A, B) or Con A-induced lymphocytes (Fig. 1C). Con A activates T lymphocytes by binding to cell membrane glycoproteins through not only Ca²⁺ influx and/or protein kinase C-dependent pathway but also the independent pathway with activation of Na⁺/H⁺ exchange (Grinstein *et al.*, 1987; Fischer *et al.*, 1988). These results suggest that TCE acts as a potential T cell stimulator to accelerate the TCR-CD3-mediated signaling pathway but not that of Con A.

TCE is a peroxisome proliferator-activated receptor α (PPAR α) agonist and causes hepatotoxicity and hepatic tumors through activation of this receptor (Laughter *et al.*, 2004; Nakajima *et al.*, 2000; Walgren *et al.*, 2000; Zhou and Waxman, 1998). To investigate whether PPAR α

activation affects TCE-induced enhancement of TCR-CD3-mediated T cell proliferation, we assessed whether fenofibrate (a typical PPAR α agonist) altered the T cell proliferation induced by anti-CD3. However fenofibrate had no effect on [³H]-thymidine incorporation in lymphocytes (Fig. 2).

TCE affects the proliferation of CD8⁺ rather than CD4⁺ cells

We then addressed which type of T cells, CD4⁺ or CD8⁺, was affected by TCE. TCE enhanced anti-CD3-mediated [³H]-thymidine incorporation of CD8⁺ cells in a concentration-dependent manner, with significant induction compared with that in controls at concentrations of 1 nM or greater (*P* < 0.01, Fig. 3A). However, TCE significantly induced the proliferation of CD4⁺ cells only at 1 nM (*P* < 0.05, Fig. 3B). In addition, the induction by TCE in CD8⁺ cells was greater than that in CD4⁺ cells.

TCE increases TCR-CD3-mediated phosphorylation of Lck in CD8⁺ cells

Lck, a Src family kinase, associates with the CD4 and CD8 co-receptors and plays a major role in TCR-CD3 signaling (Alexander, 2000; Chan *et al.*, 1994; Holdorf *et al.*, 2002), which upregulates the Tyr⁵⁰⁵ phosphorylation of Lck (Kim and White 2006; Luo and Sefton, 1990). We therefore assessed the effect of TCE on Tyr⁵⁰⁵ phosphorylation of Lck mediated by TCR-CD3 stimulation. In both CD4⁺ and CD8⁺ cells, the total Lck level did not differ between TCE treatment and vehicle control groups. However, Tyr⁵⁰⁵ phosphorylation of Lck in CD8⁺ cells was increased by TCE treatment at 1 nM or more (Fig. 4A), whereas TCE had little effect on that in CD4⁺ cells (Fig. 4B). These results suggest that TCE accelerates the TCR-CD3-mediated signaling pathway in CD8⁺, but not CD4⁺, T cells. Although CD4 and CD8 both function as co-receptors and bind to Lck, their structures are quite distinct (Chen *et al.*, 2009; Garcia and Adams 2005; Leahy, 1995). The considerable differences in TCE-mediated immunomodulation between CD4⁺ and CD8⁺ T cells might reflect the different structures of these receptors.

TCE increases the number of splenic CD8⁺ T cells after antigen immunization

TCE's remarkable enhancement of the TCR-CD3-mediated proliferation of CD8⁺ rather than CD4⁺ T cells *in vitro* prompted us to examine whether TCE increases the proportion of CD8⁺ T cells primed by antigen immunization *in vivo*. To better understand TCE-induced immunomodulation, we assessed the effect of TCE exposure on the rates at which splenocyte subpopulations are primed

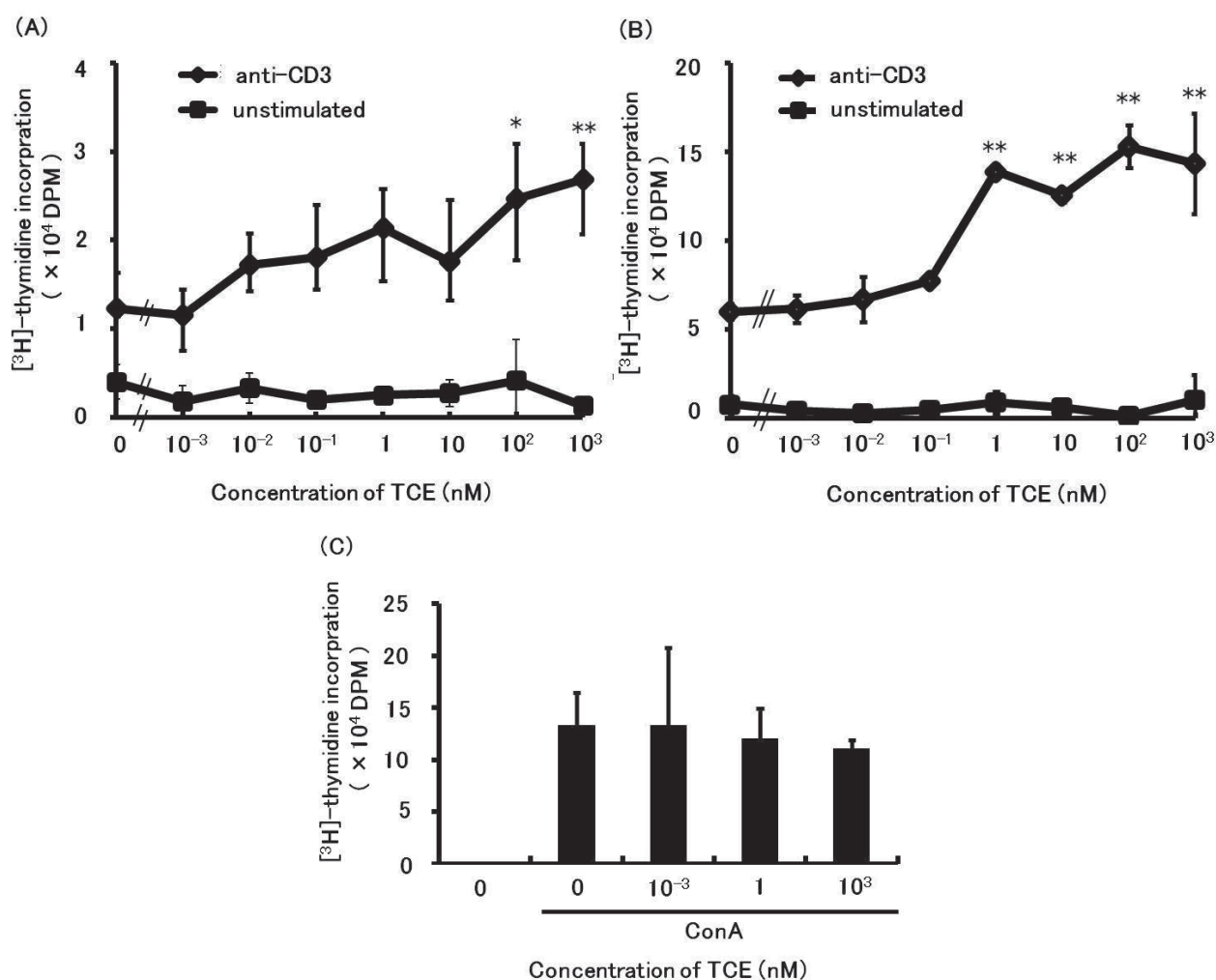


Fig. 1. Effect of TCE on T cell proliferation mediated by anti-CD3 antibody or ConA. (A) Splenocytes and (B) lymph node cells (5×10^5 cells/well) were stimulated with anti-CD3 in the presence or absence of TCE for 96 hr or (C) lymph node cells (5×10^5 cells/well) were stimulated with or without Con A in the presence or absence of TCE. Each culture was pulsed with 30 kBq $[^3\text{H}]\text{-thymidine}$ for 16 hr before harvest, and the radioactivity incorporated into the cells was quantified by liquid scintillation counting. Data are expressed as mean $\pm$ 1 S.D. ($n = 4$ or 5). Statistical differences (*, $P < 0.05$; **, $P < 0.01$) compared with the value for the control group were determined by one-way ANOVA.

by OVA immunization *in vivo*. Rates of body weight gain and average water intake did not differ between mice in control and TCE exposure groups (data not shown). The average TCE doses for the 0.03 and 3 mg/l exposure groups were 0.12 and 12 $\mu\text{g/d}$, respectively.

Flow cytometric analysis of splenocytes from OVA-immunized mice exposed to TCE at 3 mg/l, beginning concurrently with OVA immunization, revealed a statistically significant increase in the percentage of CD3^+ cells compared with that of OVA-immunized control mice

($P < 0.05$, Table 1). Consistent with *in vitro* results, the percentage of splenic CD8^+ cells was significantly higher in OVA-immunized mice receiving TCE at 0.03 mg/l or more than in OVA-immunized control mice ($P < 0.05$, Table 1). Although TCE exposure at 3 mg/l significantly increased the percentage of CD4^+ cells in the spleens of OVA-immunized mice ($P < 0.05$, Table 1), the overall increase was smaller than that of CD8^+ cells. Contrary to these findings, TCE had no obvious effect on the proportions of various splenocyte subpopulations in non-immu-

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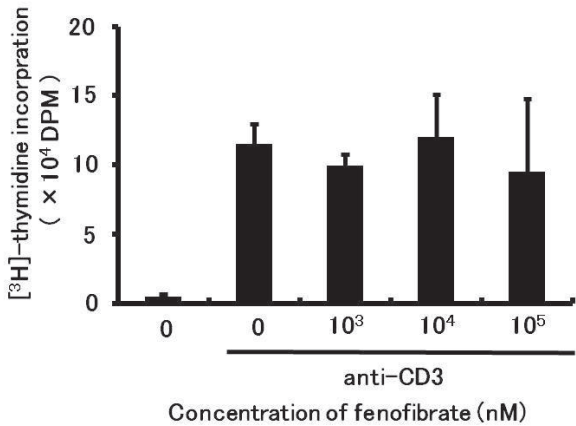


Fig. 2. Effect of TCE on T cell proliferation after Con A stimulation and the effect of a PPAR α agonist on T cell proliferation mediated by anti-CD3 antibody. Lymph node cells (5×10^5 cells/well) were stimulated with or without anti-CD3 in the presence or absence of fenofibrate for 96 hr. The radioactivity incorporated into the cells was detected as described for Fig. 1. Data are expressed as mean $\pm$ 1 S.D. ($n = 4$).

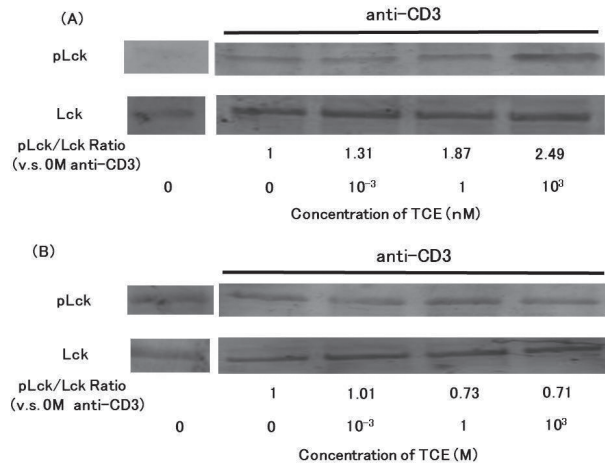


Fig. 4. Effect of TCE on Tyr⁵⁰⁵ phosphorylation of Lck in CD4⁺ and CD8⁺ cells. (A) CD8⁺ and (B) CD4⁺ cells (1×10^6 cells/well) were stimulated with or without anti-CD3 in the presence or absence of TCE for 24 hr and then harvested. Lysates from harvested cells were prepared by using ice-cold lysis buffer. The expression levels of total Lck and pLck (Tyr⁵⁰⁵) in protein samples (20 μ g) were detected by Western blotting.

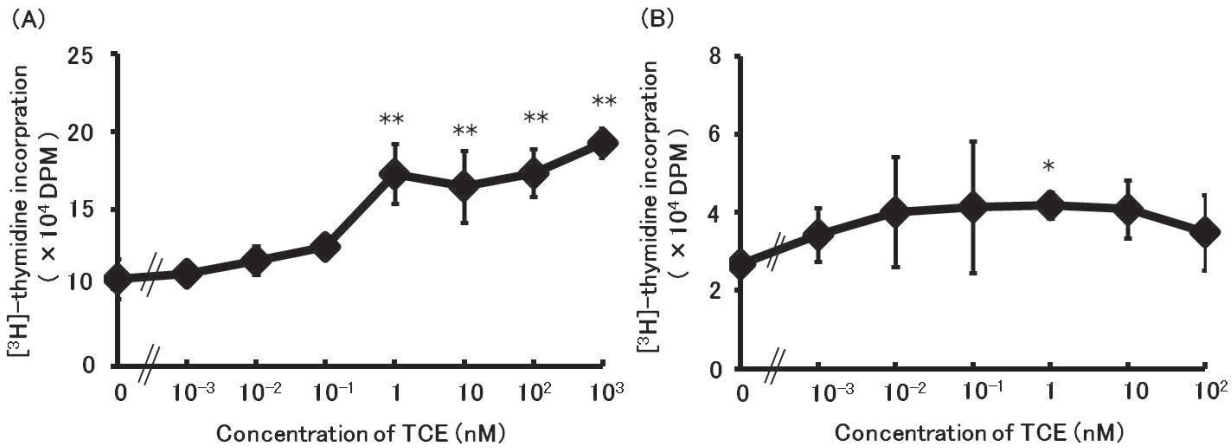


Fig. 3. Effect of TCE on CD4⁺ and CD8⁺ cell proliferation. (A) CD8⁺ and (B) CD4⁺ cells (5×10^5 cells/well) were purified and stimulated with anti-CD3 in the presence or absence of TCE. The radioactivity incorporated into the cells was detected as described for Fig. 1. Data are expressed as mean $\pm$ 1 S.D. ($n = 4$). Statistical differences (*, $P < 0.05$; **, $P < 0.01$) compared with the value for the control group were determined by one-way ANOVA.

nized mice. These results suggest that TCE affects the proportion of peripheral T cells primed by antigen immunization and increases CD8⁺ rather than CD4⁺ populations in particular.

Exposure to high levels of TCE accelerates the development of autoimmune responses in autoim-

mune-prone MRL^{+/+} mice (Blossom *et al.*, 2008; Gilbert *et al.*, 2006; Griffin *et al.*, 2000a, 2000b). This TCE-induced autoimmune response was associated with an increase in activated CD4⁺ T cells, which produce Th1-like cytokines such as IFN- γ . CD8⁺ T cells regulate Th1 immune responses against pathogens and alloanti-

Table 1. The effect of TCE on splenocyte cell differentiation

Concentration of TCE (mg/l)	Non immunization			OVA immunization		
	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD3 ⁺	CD4 ⁺	CD8 ⁺
0	49.54 ± 5.77	33.25 ± 4.58	17.54 ± 1.38	46.75 ± 2.74	28.72 ± 0.98	16.34 ± 1.87
0.03	46.27 ± 2.38	34.67 ± 1.57	14.78 ± 1.00	51.88 ± 3.22	32.18 ± 2.00	19.90 ± 1.71*
3	48.67 ± 0.71	34.93 ± 1.06	17.00 ± 0.64	57.80 ± 4.86*	35.16 ± 3.52**	22.35 ± 2.22**

The data was expressed as means ± 1 S.D. (n = 5). Statistical difference for control group **P* < 0.05, ***P* < 0.01.

gens and play a role in the development of contact hypersensitivity (Dubois *et al.*, 2010; Holmes *et al.*, 1997; Kish *et al.*, 2005), airway hyperreactivity and inflammation (Miyahara *et al.*, 2004), and delayed-type allergic reactions. In the current study, we demonstrated that the proportion of CD8⁺, rather than CD4⁺, T cells increases significantly after TCE exposure of antigen-immunized mice. The potential immunomodulatory function of TCE may be a key mechanism underlying TCE-induced autoimmune responses.

In conclusion, TCE enhances the TCR-CD3-mediated proliferation of CD8⁺ T cells to facilitate Lck phosphorylation and increases the proportion of CD8⁺ T cells primed by antigen immunization. TCE exposure might cause immune diseases by disrupting peripheral T cell activities.

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