

Cigarette smoke extract induces thymic stromal lymphopoietin expression, leading to T_H2-type immune responses and airway inflammation

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Background: Both active and passive smoking are considered to be risk factors for asthma development. However, the precise mechanisms involved remain elusive. Recently, thymic stromal lymphopoietin (TSLP) has been shown to play a key role in the development of T_H2-type allergic inflammation in patients with asthma.

Objective: The aim of this study was to investigate whether there was a causal relationship between cigarette smoke exposure and TSLP expression in the lung.

Methods: We examined the effects of repeated intranasal exposure of cigarette smoke extract (CSE) on TSLP mRNA and protein expression in the mouse lung by means of real-time PCR, Western blotting, and immunohistochemistry. We also examined the effects of intranasal exposure of CSE plus ovalbumin (OVA) on T_H2-type immune responses and lung pathology.

Results: Repeated exposure of CSE induced TSLP mRNA and protein expression, which was inhibited by treatment with antioxidative N-acetylcysteine and by TNF- α receptor I deficiency. In addition, the intranasal exposure of CSE simultaneously with OVA induced OVA-specific T_H2-type immune responses and airway inflammation, which were inhibited by the blockade of the TSLP activity.

Conclusion: CSE induced TSLP expression in the mouse lung in an oxidative stress-dependent and TNF- α receptor I-dependent manner, and when challenged simultaneously with an antigen, CSE promoted the development of airway inflammation in association with T_H2-type immune responses. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Cigarette smoke, thymic stromal lymphopoietin, asthma, T_H2

Abbreviations used

CSE: Cigarette smoke extract
mEAR2: Eosinophil-associated RNase2
NAC: N-acetylcysteine
OVA: Ovalbumin
TSLP: Thymic stromal lymphopoietin

Many epidemiologic and animal studies have suggested that active and passive cigarette smoking are risk factors in the development of and increase in the severity of asthma.¹⁻⁴ For instance, passive exposure to cigarette smoke during early life increases the subsequent risk of allergy and asthma.¹ Although direct toxicity, oxidative damage, the recruitment of inflammatory cells, and increased epithelial permeability have been suggested to be involved in the development of and increase in the severity of asthma by cigarette smoke,⁵ the precise molecular mechanisms underlying such observations currently remain unexplained.

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine originally identified as a factor that could support the growth of a mouse B-cell line.^{6,7} Recently, TSLP produced by epithelial cells has been shown to be capable of activating dendritic cells to upregulate OX40 ligand, thus leading to the differentiation of CD4⁺ T cells into T_H2 cells.^{6,7} Therefore TSLP is considered to play a key role in the development of T_H2-type allergic inflammation in asthma or atopic dermatitis.⁶⁻⁸

In contrast to the proallergic action of TSLP, the regulation of TSLP expression in epithelial cells has not yet been fully elucidated. Previous *in vitro* studies suggest that TSLP expression is regulated by vitamin D, retinoic acid, and nuclear factor κ B-activating ligands, such as TNF- α , in human bronchial epithelial cells and keratinocytes.^{8,9} However, the factors controlling TSLP expression *in vivo* remain relatively unknown.

The primary aim of this study was to investigate whether there was a causal relationship between cigarette smoke exposure and TSLP expression in the lung. We hypothesized that cigarette smoke exposure might induce TSLP expression in the lung, thereby promoting the development of asthma. The effects of intranasal exposure of cigarette smoke extract (CSE) on TSLP expression in the mouse lung were examined to test this hypothesis. We also examined the effects of intranasal exposure of CSE plus an innocuous antigen, ovalbumin (OVA), on T_H2-type immune responses and lung pathology in mice. The results

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suggest that cigarette smoke might contribute to the development of asthma, at least in part, by inducing TSLP expression.

METHODS

Mice

Female inbred BALB/c mice, all approximately 6 weeks old, were obtained from Japan SLC (Tokyo, Japan). Homozygous wild-type C57BL/6J and TNF receptor 1-null B6.129-Tnfrsf1a^{tm1Mak}/J mice were purchased from The Jackson Laboratory (Bar Harbor, Me). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Yamanashi.

Preparation of CSE

CSE was prepared from a major cigarette brand in Japan (tar content, 10 mg), as previously described,¹⁰ and stored at -20°C until use. Briefly, following the method of the International Organization for Standardization, mainstream smoke was prepared from individual cigarettes by using a constant vacuum flow (10.5 L/min), and thereafter, it was collected in a glass bottle (volume, 2 L) containing PBS. The smoke from 20 cigarettes was dissolved in 500 mL of PBS with vigorous shaking for 5 minutes, and then the CSE was prepared.

In vivo CSE exposure

The mice were challenged intranasally with 40 μL of CSE or PBS daily for a total of 7 days or challenged intratracheally with 30 μL of CSE or PBS to investigate the effects of CSE on TSLP expression. The mice were challenged intranasally with 40 μL of CSE or PBS with or without OVA (2.5 mg/mL; Sigma-Aldrich, St Louis, Mo) 5 times per week for 8 weeks to investigate the effects of CSE, OVA, or both on $\text{T}_\text{H}2$ -type immune responses and lung pathology. Three hours after the final CSE challenge, OVA challenge, or both (otherwise indicated), the pulmonary and systemic circulation of the mice was rinsed with PBS, and the mice were killed for the subsequent analysis (real-time PCR, Western blotting, histology, antigen recall response, and serum OVA IgE determination). For some experiments, 150 mg/kg per mouse N-acetylcysteine (NAC; Sigma-Aldrich) or rat anti-mouse TSLP-neutralizing antibody or control rat IgG2a (15 mg/kg per mouse; R&D, Inc, Minneapolis, Minn) was administered intraperitoneally every week during the exposure period of CSE, OVA, or both until death. The dosage of the antibody (15 mg/kg) was determined based on the findings of previous experiments ($n = 6$ in each group).¹¹

Quantitative real-time RT-PCR

The right lung was homogenized with TissueLyser II (QUIAGEN, Inc, Valencia, Calif), and total RNA was extracted with the RNeasy Mini kit (QUIAGEN, Inc). cDNA was then synthesized from 2 μg of total RNA with the Reverse Transcriptase System (Applied Biosystems, Foster City, Calif). A real-time PCR analysis for mouse TSLP, TNF- α , eosinophil-associated RNase2 (mEAR2),¹² GATA3, IL-13, heme oxygenase-1, and glyceraldehyde-3-phosphate dehydrogenase was performed by using the AB7500 real-time PCR system (Applied Biosystems). The number of DNA molecules was then calculated from the DNA mass by using DNA standards and the molecular weight and expressed relative to glyceraldehyde-3-phosphate dehydrogenase.

Western blotting

The left lung was homogenized with TissueLyser II (QUIAGEN, Inc). The whole lung extracts (10 μg) were subjected to immunoblotting with anti-TSLP antibody (R&D, Inc) or β -actin antibody (Cell Signaling Technology, Inc, Danvers, Mass), as previously described.¹³

Histology

Mouse lung tissue sections were stained with hematoxylin and eosin. For immunohistochemistry, the lung sections were stained with anti-TSLP antibody

or control IgG antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) through the use of peroxidase-based VECTASTAIN ABC kits with DAB substrate (Vector Laboratories, Burlingame, Calif), as previously described.¹⁴ The nuclei were counterstained with hematoxylin. The sections were photographed with a digital color-CCD camera (BX50; Olympus, Tokyo, Japan).

Quantification of histologic examination

The number of TSLP-positive cells in the lung sections was determined microscopically in a blinded manner. Briefly, a minimum of 200 cells in the bronchial epithelium was counted in high-power fields ($\times 400$). The percentage of TSLP-positive cells among the total bronchial epithelial cells was expressed (as a percentage). At least 4 specimens of TSLP-stained sections from 1 mouse were selected. The mean percentage was calculated, and then the mean percentage was calculated in 4 mice.

Antigen-recall response

Total splenocytes (3×10^6 cells/well) were incubated in 24-well plates for 72 hours in RPMI 1640 medium (Invitrogen-Gibco, Carlsbad, Calif) containing 10% FCS, 2-mercaptoethanol, and antibiotics in the presence or absence of 100 $\mu\text{g}/\text{mL}$ OVA. Culture supernatants were then harvested, and the amounts of IL-4 and IL-13 were determined by using mouse IL-4 and IL-13 ELISA kits (R&D, Inc), according to the manufacturer's instructions.

Serum OVA-specific IgE

OVA-specific IgE concentrations in the mouse serum were measured with the DS mouse IgE ELISA (OVA) kit (DS Pharma Biomedical Co, Ltd, Osaka, Japan). The minimum sensitivity of this assay is 2.7 ng/mL.

Data analysis

The data are summarized as the mean $\pm$ SD. One-way ANOVA with the *post-hoc* test (with a Bonferroni correction) was used for the statistical analysis of the results. A *P* value of less than .05 was considered significant.

RESULTS

CSE exposure induces TSLP expression in the mouse lung in an oxidative stress-dependent and TNF- α receptor 1-dependent manner

To investigate whether a causal relationship exists between cigarette smoke exposure and TSLP expression in the lung, we first examined the effects of cigarette smoke exposure on TSLP expression in the mouse lung. BALB/c mice were intranasally exposed to CSE every day for a total of 7 days (days 0-6), and expression of TSLP mRNA and protein in the mouse lung was examined by means of real-time PCR, Western blotting, and immunohistochemistry. TSLP mRNA expression gradually increased in the lungs of mice exposed to CSE in a time-dependent manner (Fig 1, A). Consistent with these findings, the expression of TSLP was detected at the protein level in the lungs of mice exposed to CSE on day 7 (Fig 1, B), and bronchial epithelial cells appeared to be the main cell type to produce TSLP protein (Fig 1, C and D). These results indicated that exposure of CSE to the mouse lung induced TSLP expression.

To address how TSLP is induced in mice exposed to CSE, we examined the effects of antioxidative NAC on the CSE-induced TSLP expression. NAC abolished the CSE-induced TSLP mRNA and protein expression in the mouse lung (Fig 1, C and D, and Fig 2, A). TNF- α is known to be induced by cigarette smoke through oxidative stress, and TNF- α mRNA increased in the mouse lung exposed to CSE (Fig 2, B). In addition, expression

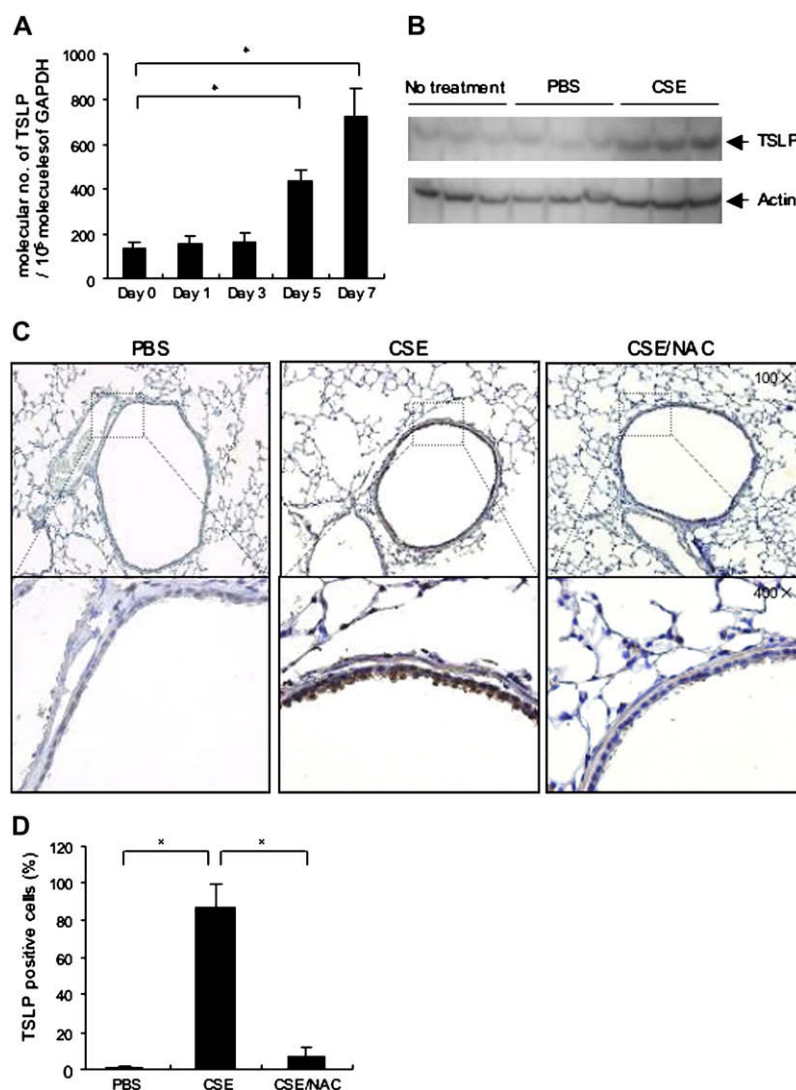


FIG 1. CSE exposure induces TSLP expression in the mouse lung. **A-C**, BALB/c mice were intranasally challenged with CSE or PBS every day for a total of 7 days (days 0-6). The mouse lungs were removed on day 7 (3 hours after the final challenge), and real-time PCR (**A**), Western blot analysis (**B**), and immunohistochemical staining (**C**) for TSLP were performed. Quantitative analysis of the TSLP immunoreactivity is shown in **D**. Representative results of 3 independent experiments are shown. * $P < .05$ in comparison with the corresponding control. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

of an oxidative stress marker, heme oxygenase-1 mRNA,¹⁵ indeed increased in the mouse lung exposed to CSE, which was inhibited by NAC (**Fig 2, C**). These results suggest that the CSE-induced TSLP expression depends on oxidative stress.

We also found that TSLP mRNA expression was induced at 3 hours after a single intratracheal challenge of CSE in the lungs of mice (**Fig 2, D**). Because TNF- α regulates the TSLP production in bronchial epithelial cells *in vitro*⁸ and CSE strongly induced the TNF- α mRNA expression in the mouse lung (**Fig 2, B**), the effects of TNF- α receptor I (TNFR1) deficiency on the rapid induction of TSLP by means of intratracheal CSE challenge were also examined in TNF- α receptor I-deficient mice. CSE-induced TSLP mRNA expression was abolished in TNFR1-deficient mice (**Fig 2, E**). Given that the treatment of mice with NAC inhibited CSE-induced mRNA expressions of TNF- α , as well as TSLP (**Fig 2, B**), these results suggest that CSE induces oxidative stress,

resulting in TNF- α expression, which is thought to play a crucial role in the subsequent TSLP expression in the mouse lung.

Intranasal exposure of CSE simultaneously with OVA induces OVA-specific T_H2 responses and airway inflammation in mice

BALB/c mice were intranasally exposed to CSE simultaneously with OVA 5 times per week for 8 weeks and then were evaluated for local and systemic T_H2-type immune responses and lung pathology to determine whether the CSE-induced TSLP expression in the lung affects the development of T_H2-type immune responses. A histologic examination at 2, 4, and 8 weeks during the CSE challenge, OVA challenge, or both revealed that the mice exposed to CSE or OVA alone did not show any pathologically overt inflammation in the lungs (**Fig 3**). In contrast, the

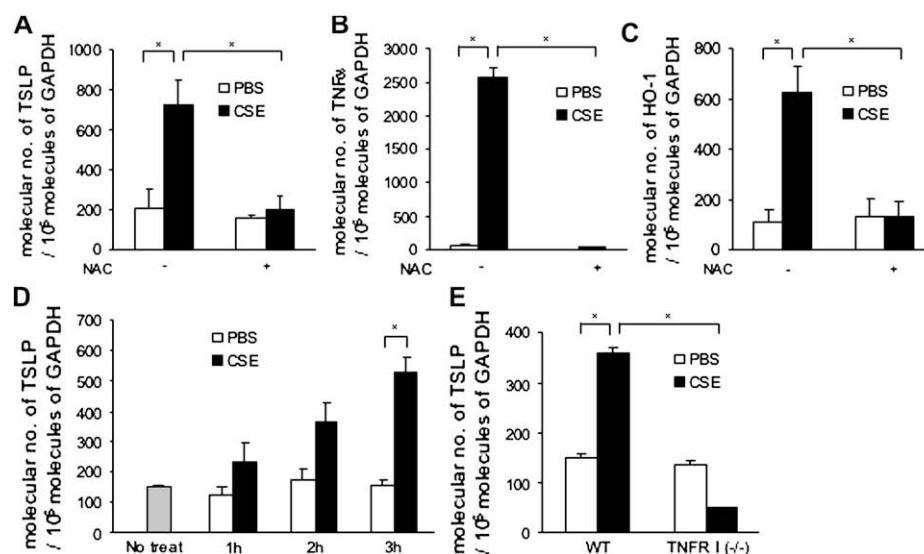


FIG 2. CSE-induced TSLP expression in the mouse lung depends on oxidative stress and TNF- α receptor I (*TNFR1*). **A–C**, BALB/c mice were intranasally challenged with CSE or PBS every day for a total of 7 days (days 0–6) with or without treatment with 150 mg/kg NAC. The mouse lungs were removed on day 7 (3 hours after the final challenge), and real-time PCR for TSLP (Fig 2, A), TNF- α (Fig 2, B), heme oxygenase 1 (*HO-1*; Fig 2, C), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was performed. **D and E**, BALB/c mice (Fig 2, D) or TNFR1-null mice and control C57BL/6 mice (Fig 2, E) were intratracheally challenged with CSE, and the mouse lungs were removed at the indicated times (Fig 2, D) or 3 hours after the challenge (Fig 2, E), and real-time RT-PCR was performed for TSLP and GAPDH. Representative results of 3 independent experiments are shown. * $P < .05$ in comparison with the corresponding control.

mice exposed to CSE plus OVA showed significant leukocyte infiltration in the lungs at 2 weeks during the challenge (Fig 3, A). The leukocyte infiltration into the lung was thereafter gradually increased at 4 and 8 weeks (Fig 3, B and C). Consistent with these findings, mEAR2¹² mRNA expression increased only in the mice exposed to CSE plus OVA at 8 weeks (Fig 4, A). In addition, the T_H2 cell-specific transcriptional factor GATA3 mRNA expression increased in the lungs of the mice exposed to CSE plus OVA along with IL-13 mRNA but not in the lungs exposed to CSE or OVA alone at 8 weeks (Fig 4, B and C). Furthermore, OVA-specific IgE production was only increased in the sera of mice exposed to CSE plus OVA at 8 weeks (Fig 4, D). Consistently, OVA-induced IL-4 and IL-13 production was observed in the spleen cells obtained from the mice exposed to CSE plus OVA at 8 weeks (Fig 4, E and F). Importantly, the blockade of TSLP activity with anti-TSLP antibody, but not control antibody, during the exposure period of CSE, OVA, or both abolished these T_H2-type immune responses and airway inflammation (Fig 3, C, and Fig 4). These results suggest that TSLP plays a key role in the development of CSE plus the antigen-induced T_H2-type immune responses and airway inflammation.

DISCUSSION

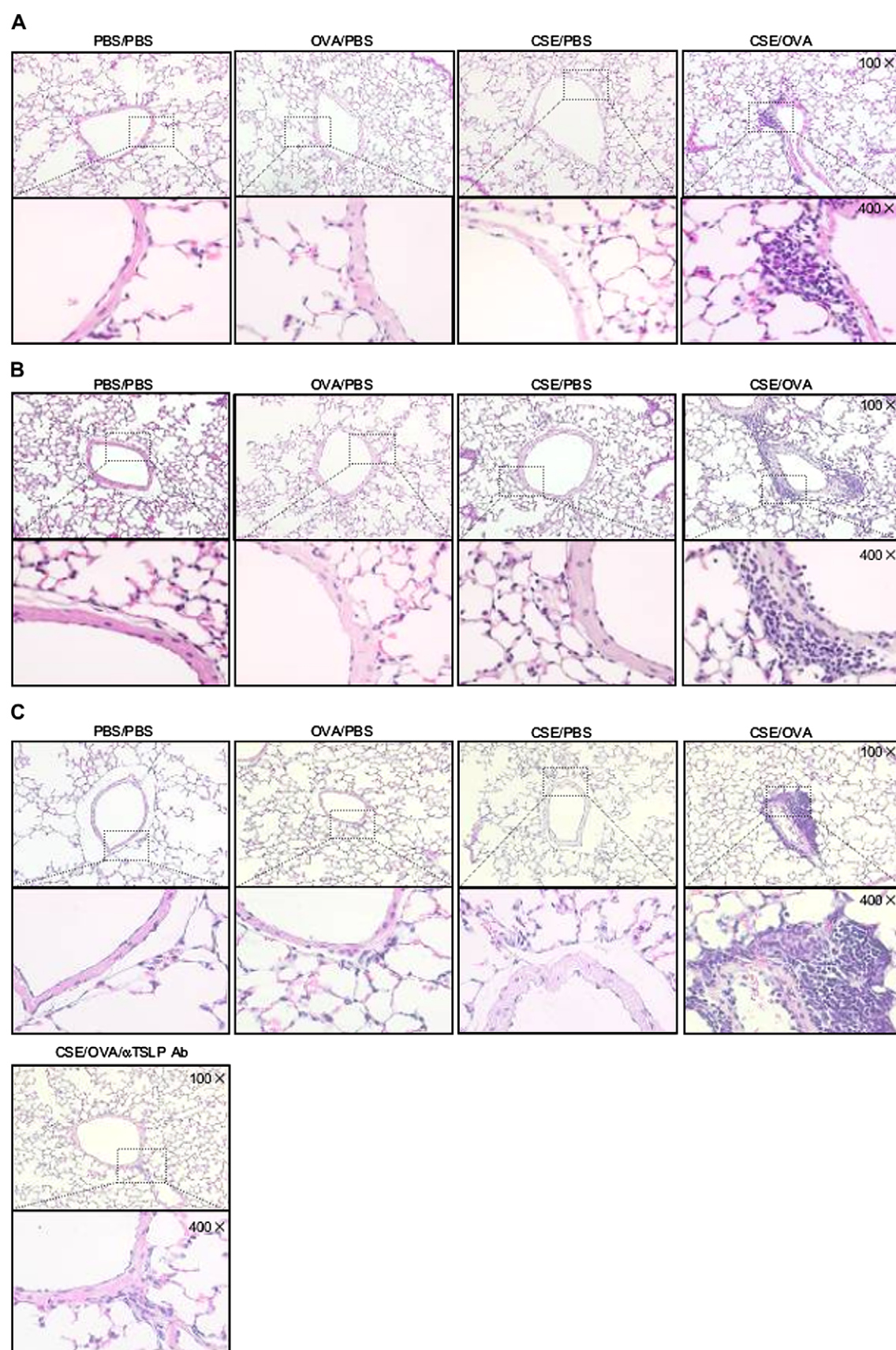
Exposure to cigarette smoke is strongly correlated with asthma severity and development,^{1–4} although it remains a matter of debate whether there is a causal relationship between cigarette smoke exposure and asthma development. In this study we showed that exposure to CSE induced the expression of TSLP, a master switch of T_H2-type allergic inflammation,^{6–8} in the mouse lung. Importantly, when simultaneously challenged with an innocuous antigen (OVA), CSE induced the development of

airway inflammation associated with the T_H2-type responses, depending on TSLP activity. Therefore cigarette smoke exposure might promote the development of asthma, at least in part, through the induction of TSLP. Most recently, Zhang et al¹⁶ reported TSLP expression in the lung tissue from patients with chronic obstructive pulmonary disease, which might be compatible with our current findings.

It is well accepted that cigarette smoke induces oxidative stress in the lung, which is inhibited by such antioxidative reagents as NAC.¹⁷ It is clear from the current results (Figs 1 and 2) that oxidative stress is necessary for CSE-induced TSLP expression in the mouse lung. However, it remains unclear whether oxidative stress alone is sufficient or whether it requires TNF- α or other types of gene expression for TSLP induction in the mouse lung.

The detailed kinetics of lung TSLP mRNA expression in mice exposed to CSE remains to be determined. Although we found rapid induction of TSLP mRNA expression at 3 hours after CSE challenge (Fig 2, D), we did not detect an increase in TSLP mRNA expression at 24 hours (1 day) after CSE challenge (Fig 1, A). This might suggest the complex regulation of TSLP mRNA expression. Alternatively, the differences in the route of CSE administration to mice (intratracheal versus intranasal) might affect the pattern of TSLP mRNA expression in the mouse lung.

Mice exposed to OVA alone did not experience airway inflammation (Fig 3). One explanation for this is respiratory tolerance, as observed in previous studies.¹⁸ The current results suggest that CSE exposure might make it possible to overcome the normal inhalational tolerance against inert proteins, such as OVA, and CSE acts as a kind of adjuvant. These findings are consistent with previous studies showing CSE to be a risk factor for asthma development.^{1–4} Alternatively, the lack of inflammation in mice exposed to OVA might be explained by the relatively



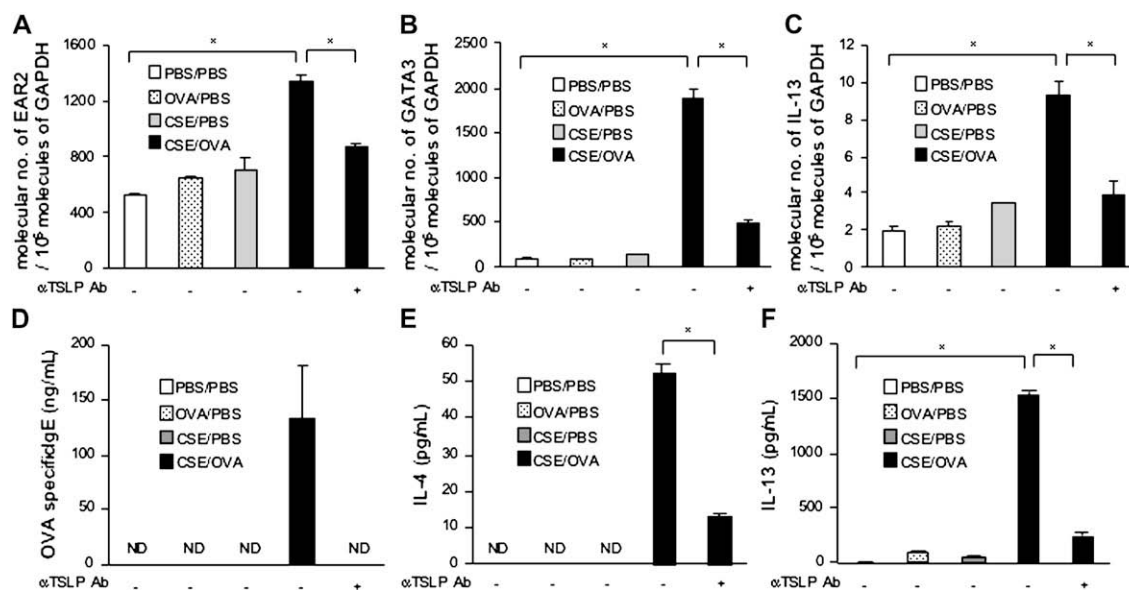


FIG 4. Intranasal exposure of CSE simultaneously with OVA induces OVA-specific T_H2 -type immune responses in mice. BALB/c mice were intranasally challenged with CSE, OVA, or both (or PBS) 5 times per week for 8 weeks with or without treatment with 15 mg/kg per mouse anti-TSLP antibody or control antibody every week. The mice were killed at 3 hours after the final CSE challenge, OVA challenge, or both for further analysis. **A-C**, RNA was extracted from the mouse lungs, and real-time RT-PCR was performed for mEAR2 (Fig 4, A), GATA-3 (Fig 4, B), IL-13 (Fig 4, C), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **D**, The mouse sera were collected, and the concentrations of OVA-specific IgE antibody were measured by means of ELISA. **E and F**, Spleen cells were collected and stimulated with 100 μ g/mL OVA for 72 hours *in vitro*. The concentrations of IL-4 (Fig 4, E) and IL-13 (Fig 4, F) in the culture supernatants were measured by means of ELISA. Representative results of 3 independent experiments are shown. * $P < .05$ in comparison with the corresponding control.

short-term exposure to OVA (8 weeks) or the route of OVA administration (intranasal, but not aerosolized, challenge).

It is well accepted that long-term exposure to cigarette smoke induces chronic obstructive pulmonary disease and emphysema, with tissue neutrophilia in human subjects and also in mice.¹⁹ However, we did not observe neutrophilic inflammation in the mice exposed to CSE alone or CSE plus OVA (Fig 3). This might be explained by the relatively short-term exposure to CSE (8 weeks) according to the route of CSE administration (intranasal challenge but not aerosolized challenge) or by the chemical content of CSE used in this study. In fact, Moerloose et al²⁰ reported that BALB/c mice briefly exposed to aerosolized cigarette smoke did not experience any overt airway inflammation.

The mice exposed to CSE plus OVA showed significant airway inflammation (Fig 3). However, it should be noted that the airway inflammation observed in these mice was relatively mild when compared with that observed in the mice sensitized to OVA and then intranasally challenged with OVA (data not shown). This might be due to the insufficient ability of short-term CSE exposure to destroy inhalation tolerance. Because of the weak inflammatory reaction, it was difficult to characterize the inflammatory cells pathologically in the mouse lung. However, the increase in

eosinophil- and T_H2 cell-specific molecular markers, such as mEAR2, GATA3, or IL-13, with the increase in OVA-specific serum IgE and splenic IL-4 and IL-13 production supports the notion that the mice exposed to CSE plus OVA indeed showed skewed local and systemic T_H2 responses.

CSE alone had the capacity to induce TSLP expression in the mouse lung (Figs 1 and 2). However, it remains unclear as to why the mice exposed to CSE alone did not show any T_H2 responses. We speculate that CSE might induce epithelial injury and facilitate allergen entry into the submucosal environment. Consequently, this could increase the allergen's contact with dendritic cells and the likelihood of allergic T_H2 -type immune responses through TSLP expression. Therefore although CSE alone had the capacity to induce TSLP expression in the mouse lung (Figs 1 and 2), CSE might not trigger the subsequent acquired immune responses without the coexistence of allergens.

In addition, it should be noted that only overexpression of TSLP (eg, transgenic mice expressing TSLP in the lung or skin or exogenous administration of a large amount of TSLP) could induce T_H2 -type responses *in vivo* in the previous studies,^{6,7} and it remains unclear whether induction of endogenous TSLP expression alone can lead to T_H2 -type responses *in vivo*.

FIG 3. Intranasal exposure of CSE simultaneously with OVA induces airway inflammation in mice. BALB/c mice were intranasally challenged with CSE, OVA, or both (or PBS) 5 times per week for 8 weeks with or without treatment with 15 mg/kg per mouse anti-TSLP antibody or control antibody every week. The mouse lungs were removed at 2 (A), 4 (B), and 8 (C) weeks during the CSE challenge, the OVA challenge, or both, and then the tissue sections were stained with hematoxylin and eosin. Representative results of 3 independent experiments are shown.

The current results rely on the exposure of CSE, but not cigarette smoke, to mice. Given consideration about some missing components during the preparation of CSE (eg, water-insoluble substances), it is therefore considered to be important to investigate whether the effects of CSE and cigarette smoke exposure on TSLP induction differ in future studies.

In summary, the current results suggest that cigarette smoke might promote the development of asthma, at least in part, by inducing TSLP expression. To our knowledge, this is the first study to provide a molecular insight into the mechanisms underlying the potential role of cigarette smoke in asthma development. These findings might therefore help to explain the increased incidence of asthma associated with cigarette smoke in many epidemiologic studies.

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Clinical implications: The induction of TSLP by cigarette smoke might play a key role in the development of asthma associated with cigarette smoke exposure.

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