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Cigarette Smoke Extract Contributes to the Inception and Aggravation of Asthmatic Inflammation by Stimulating Innate Immunity

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Abstract

Purpose: Smoking is a risk factor for the development of asthma and worsens the long-term prognosis of asthma. This study investigated the effect of cigarette smoke extract (CSE) on innate immune cells such as innate lymphoid cells (ILCs) and macrophages in a murine model of induced asthma. *Methods:* Six-week-old female BALB/C mice were exposed to ovalbumin (OVA) via an intranasal route with or without CSE for 8 weeks to establish a chronic murine asthma model. Airway hyperresponsiveness (AHR), airway inflammatory cells from bronchoalveolar lavage fluid, and the population of $CD4^+$ T cells, ILCs, and macrophages in the lungs were studied to evaluate the effect of chronic CSE exposure on asthma. *Results:* Mice intranasally exposed to CSE along with OVA treatment (CSE/OVA) had significantly enhanced AHR, eosinophilic inflammation, increased IL-13 and IL-17 producing CD4+ T cells compared to mice intranasally exposed to OVA only. On the contrary, the frequency of $Foxp3^+$ in $CD4^+$ T cells was reduced in the CSE/OVA group. CSE enhanced the dendritic cell (DC) population, especially $MHCII^+$ DC with antigen-presenting capacity. Among ILCs, the CSE/OVA group showed a significant increase of IL-13-producing type 2 ILCs, but not interferon-γ^+ ILC1s and IL-17^+ ILC3s. Among macrophages, alveolar macrophage and Ym-1 and FIZZ1 positive M2 macrophage populations were significantly induced by CSE exposure alone and when combined with OVA treatment. *Conclusion:* In this study, we showed that long-term exposure to cigarette smoke contributes to the inception and aggravation of asthmatic inflammation by enhancing DCs, ILC2, and M2 alveolar macrophage populations in the mouse model.

Keywords

Asthma
Cigarette smoking
Innate immunity
Macrophages

1. Introduction

Asthma is a chronic airway inflammatory disease characterized by airway hyperresponsiveness (AHR) and reversible airway obstruction. The most typical phenotype of asthma is the type 2 allergic inflammation mediated by type 2 helper T cells (Th2), which results in the release of cytokines such as interleukin (IL)-4, IL-5, and IL-13, leading to pathophysiology such as eosinophilia [1]. However, asthma involves many triggers, resulting in a diverse and complex phenotype. Allergic asthma can be classified into allergic and non-allergic asthma based on the production of allergen-specific IgE antibodies and has recently been divided into Th2 high and Th2 low asthma. Th2 low is characterized by neutrophilic airway inflammation and is induced by environmental and host factors such as cigarette smoke, air pollution, viral infections, and obesity [2]. Research is needed to understand the phenotype of non-allergic asthma and the mechanisms of action of the different environmental factors that induce it in order to provide appropriate treatment for the different asthma phenotypes [3].

Long-term exposure to tobacco smoke causes abnormalities in the immune system, leading to pathological changes in the airways and lungs, such as inflammatory responses and airway remodeling [4]. Although it is a major risk factor for the development of chronic obstructive pulmonary disease (COPD), several studies have shown that tobacco smoke also contributes to the development and exacerbation of asthma. Increased eosinophilic inflammation and Th2 inflammatory responses have been observed in mouse models of asthma exposed to cigarette smoke, and secondhand smoke has been reported to increase neutrophilic inflammation and Th17 immune responses in asthmatic patients, suggesting that cigarette smoke contributes to asthma exacerbation [5,6]. Studies have focused on adaptive immunity to explain the overexpression of Th17 cells and IL-17 by cigarette smoke [7,8].

However, innate immune cells are also known to play a role in the development of asthma. Innate lymphoid cells (ILCs) are innate immune cells that play an important role in the initiation of inflammatory responses, from the differentiation of T cells to the activation of adaptive immune responses, and, similar to Th cells, secrete cytokines and are involved in immune diseases, including asthma [9]. In mouse models of asthma, ILC2 activation and increased secretion of type 2 cytokines have been reported to be involved in the development and exacerbation of allergen-induced asthma [9]. It has also been reported that all sub-types of ILCs, ILC1, ILC2, and ILC3, are increased in sputum from asthmatic patients and that ILCs contribute to the phenotype of asthma by regulating the polarisation of macrophages [10]. This suggests that studies on the relationship between cigarette smoke and innate immune cells are needed to understand the pathogenesis of cigarette smoke-induced asthma. In this study, we aimed to determine the effects of cigarette smoke extract (CSE) on asthma and its effects on innate immune cells, including ILCs and macrophages, by nasal administration of CSE to a mouse model of ovalbumin (OVA)-induced asthma.

2. Materials and methods

2.1. Creation of an asthma mouse model and cigarette smoke extract

3R4F Kentucky Research Cigarettes (University of Kentucky Research Cigarettes, USA) were used. Each cigarette was combusted for 8–10 min using a variable-flow peristaltic pump (Fisherbrand Variable-Flox Peristaltic Pumps, China), and eight filtered cigarettes were dissolved in 10 mL of phosphate-buffered saline (PBS), and CSE was prepared by filtering particulates with a microfilter (0.75 μm) (Figure 1A) [11].

Six-week-old female BALB/C mice were purchased (Orient Bio Inc., Seongnam, Korea) and housed under specific pathogen-free conditions. They were divided into four groups: a normal group receiving PBS alone, a normal group receiving CSE alone, an OVA-induced asthma group, and an OVA-induced asthma group exposed to CSE. CSE (5%) or OVA (100 μg) was administered intranasally five times a week for

a total of eight weeks (Figure 1B). Six mice were set up in each group, and a total of 24 mice were used in the experiment, and the results were replicated. All procedures were performed under the IACUC approval (SNU-200429-5-1) of the Institute for Laboratory Animal Resources, Seoul National University.

2.2. Measurement of airway hyperresponsiveness

The OCP3000 (Allmedicus, Korea) was used to measure methacholine-induced AHR 24 hours after the last intranasal dose. AHR was measured by whole-body plethysmography, a non-invasive method, in which mice were kept free from injury. Methacholine concentrations of 6.25, 12.5, 25, and 50 mg/mL were inhaled using a nebulizer for 3 minutes, and Penh (enhanced pause) was measured at 30-second intervals for 3 minutes at each concentration [12].

2.3. Analysis of inflammatory response

Mice were sacrificed 24 hours after measuring AHR, and bronchoalveolar lavage fluid was collected by administering 2 mL of PBS into the airways. A cell pellet of bronchoalveolar lavage fluid was obtained and resuspended in 100 μL PBS, and the total number of cells was determined using a hemocytometer. Twenty μL of bronchoalveolar lavage fluid was diluted with an additional 80 μL of PBS, and cells were fixed on slides by centrifugation using a cytospin (Shandon CytoSpin III, Shandon, UK). The cells attached to the slides were stained with Diff-quick (Sysmex Co., Kobe, Japan), and a total of 300 or more cells were counted under a light microscope to distinguish macrophages, lymphocytes, neutrophils, and eosinophils, and the distribution of each immune cell was determined [13].

2.4. Flow cytometry

The harvested lung tissues were cut into small pieces using a razor blade and placed in 5 mL RPMI1640 medium containing 10% type 4 collagenase (Worthington Biochemical Corporation, USA) for 90 min at 37°C, and the collagen-bonded tissues were filtered through a 40 μM sterile cell strainer to isolate single cells. Red blood cells were removed by treatment with erythrocyte lysis solution, and cell counts were taken. For macrophage flow cytometry, 1×10^6 cells were seeded into tubes and treated with an FcγR-specific blocking monoclonal antibody to block Fc receptors and stained for cell surface markers [13]. For dendritic cells and macrophages staining, FITC-conjugated anti-CD86, PE/Cy7-conjugated anti-CD206, PE-conjugated anti-F4/80, APC-conjugated anti-MHC II (I-Ab), BV421-conjugated anti-SiglecF, BV510-conjugated anti-Ly6C, BV650-conjugated anti-CD45, BV711-conjugated anti-CD11c, BV785-conjugated anti-CD11b, and the following antibodies (Biolegend, USA) were used.

For T cell, ILC flow cytometry, 1×10^6 cells were seeded in 96-well plates and stimulated by treatment with PMA (100 ng/mL; Sigma, USA), Ionomycin (1 μg/mL; Sigma, USA), and GolgiStop (1 μL/mL; BD Biosciences, USA), then cells were harvested and subjected to Fc receptor blockade and cell surface marker staining. Cell membrane and nuclear membrane permeabilization was then performed followed by cytokine and transcription factor staining. For T cell ILC staining, BV650-conjugated anti-CD45, FITC-conjugated anti-lineage (CD3ε, CD11c, CD19, CD49b, FcεRa, F4/80, CD11b) or anti-CD3, BV785-conjugated anti-CD4, PE-Cy7-conjugated anti-CD90, PE-conjugated anti-CD8, PerCP/Cy5.5-conjugated anti-IFN-γ or anti-Foxp3, APC-conjugated anti-IFN-γ or anti-IL-5, PE-conjugated anti-IL-13, BV421-conjugated anti-CD25 or anti-IL-17A antibodies (Biolegend, USA) were used.

2.5. Statistical analysis

All data were expressed as mean ± standard error of the mean. Differences between groups were calculated by *t*-test for statistical significance. $P < 0.05$ was considered statistically significant.

3. Result

3.1. Effects of cigarette smoke on airway inflammatory response and airway hyperresponsiveness

Bronchoalveolar lavage fluid inflammatory cell analysis was performed to determine the effect of CSE exposure on inflammatory cell infiltration. CSE alone significantly increased total inflammatory cells, macrophages, neutrophils, and lymphocytes compared to the control group. The OVA group was also characterized by a significant increase in total inflammatory cells, neutrophils, and lymphocytes, as well as an increase in eosinophils, compared to the control group. The OVA/CSE combined exposure group showed a significant increase in total inflammatory cells, macrophages, neutrophils, and eosinophils compared to the OVA group ($P < 0.05$) (Figure 1C).

Airway hyperresponsiveness showed no difference between groups at baseline, but at a concentration of 50 mg/mL of methacholine, a significant increase in Penh value was observed in the OVA/CSE combined exposure group compared to CSE alone and OVA group ($P < 0.05$) (Figure 1D).

3.2. Effects of cigarette smoke on helper T cells and regulatory T cells

When comparing Th2 cell fractions, there was no relative increase in the OVA group compared to the control group, but there was a significant increase in the CSE alone and OVA/CSE combined exposure groups, with the largest change in the OVA/CSE combined exposure group ($P < 0.01$) (Figure 2A).

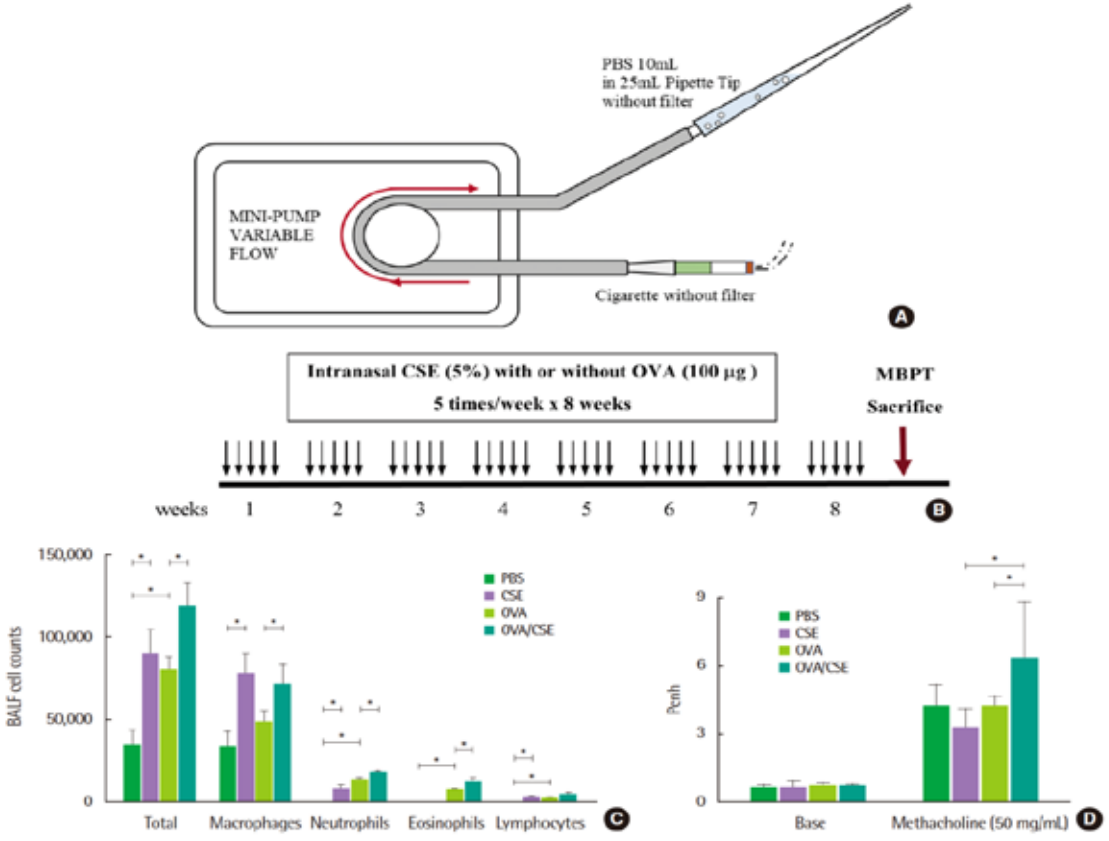

Figure 1. An experiment protocol for the evaluation of the effect of CSE and changes in asthmatic inflammation and airway hyperresponsiveness by CSE. (A) A schematic diagram of CSE preparation. (B) The protocol of the long-term effects of cigarette smoke in murine OVA-induced asthmatic model. (C) The number of inflammatory cells in BALF. (D) Methacholine hyperresponsiveness was measured 24 hours after the last challenge. CSE, cigarette smoke extract; PBS, phosphate-buffered saline; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; MBPT, methacholine bronchial provocation test. *t*-test, $*P < 0.05$, and $n = 6$.

$CD4^+IFN\text{-}\gamma^+$ Th1 cells were significantly reduced in the OVA group but significantly increased in the combined OVA and CSE exposure compared to OVA alone, and $CD4^+IL\text{-}17^+$ Th17 cells were also increased in the combined OVA and CSE exposure ($P < 0.05$) (Figure 2B).

Regulatory T cells (Treg) showed a significant decrease in CSE alone and OVA/CSE combined exposure compared to the control and OVA groups, respectively ($P < 0.01$) (Figure 2C).

3.3. Effects of cigarette smoke on innate lymphoid cells

The expression of $IL\text{-}5^+$ ILC2 and $IL\text{-}13^+$ ILC2 was significantly increased in the OVA/CSE combined exposure group compared to the OVA group ($P < 0.01$) (Figure 3A). No significant changes in $IFN\text{-}\gamma^+$ ILC1 and $IL\text{-}17^+$ ILC3 were detected by CSE exposure (Figure 3B).

3.4. Effects of cigarette smoke on dendritic cells and macrophages

CSE alone or OVA alone significantly increased $CD11c^+$ DCs compared to the control group, and a significant increase in $CD11c^+$ DCs was also observed in the OVA/CSE combination group compared to OVA alone ($P < 0.05$) (Figure 4A). $CD11C^+MHCII^+$ DCs were significantly increased in the CSE alone and OVA groups compared to the control group ($P < 0.05$) (Figure 4B).

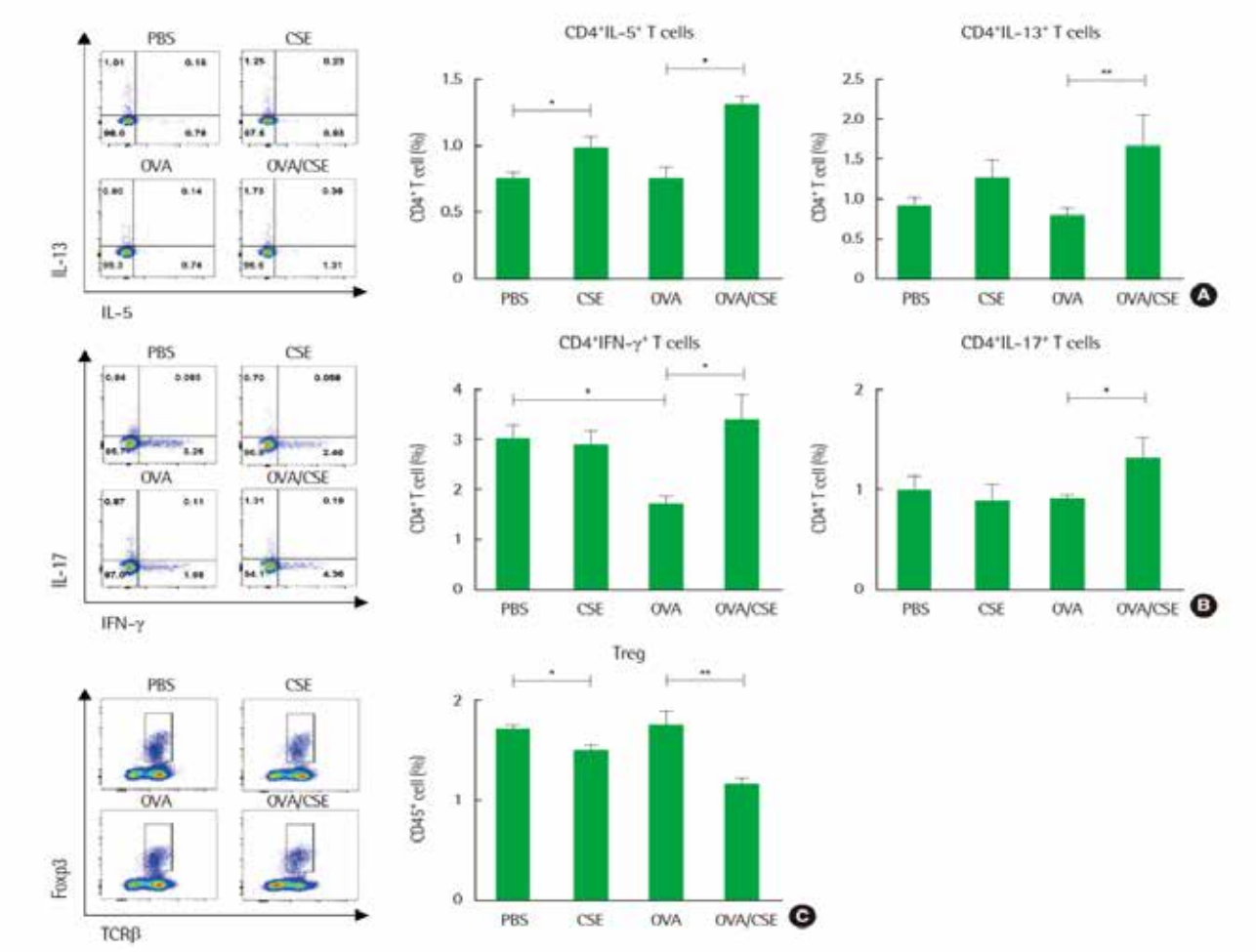

Figure 2. Effect of CSE on $CD4^+$ T cells and Tregs in the lung tissues of each group. (A, B) A flow cytometric representation of $IL\text{-}5^+$, $IL\text{-}13^+$, $IL\text{-}17^+$, and $IFN\text{-}\gamma^+$ T cells, and percentages of each population in $CD4^+$ T cells. (C) A flow cytometric representation of $Foxp3^+TCR\beta^+$ T cells, and percentages of each population in $CD45^+$ cells. PBS, phosphate-buffered saline; CSE, cigarette smoke extract; IL, interleukin; OVA, ovalbumin; IFN, interferon; TCR, T-cell receptor; Treg, regulatory T cell. *t*-test, $*P < 0.05$, $**P < 0.01$, and $n = 6$.

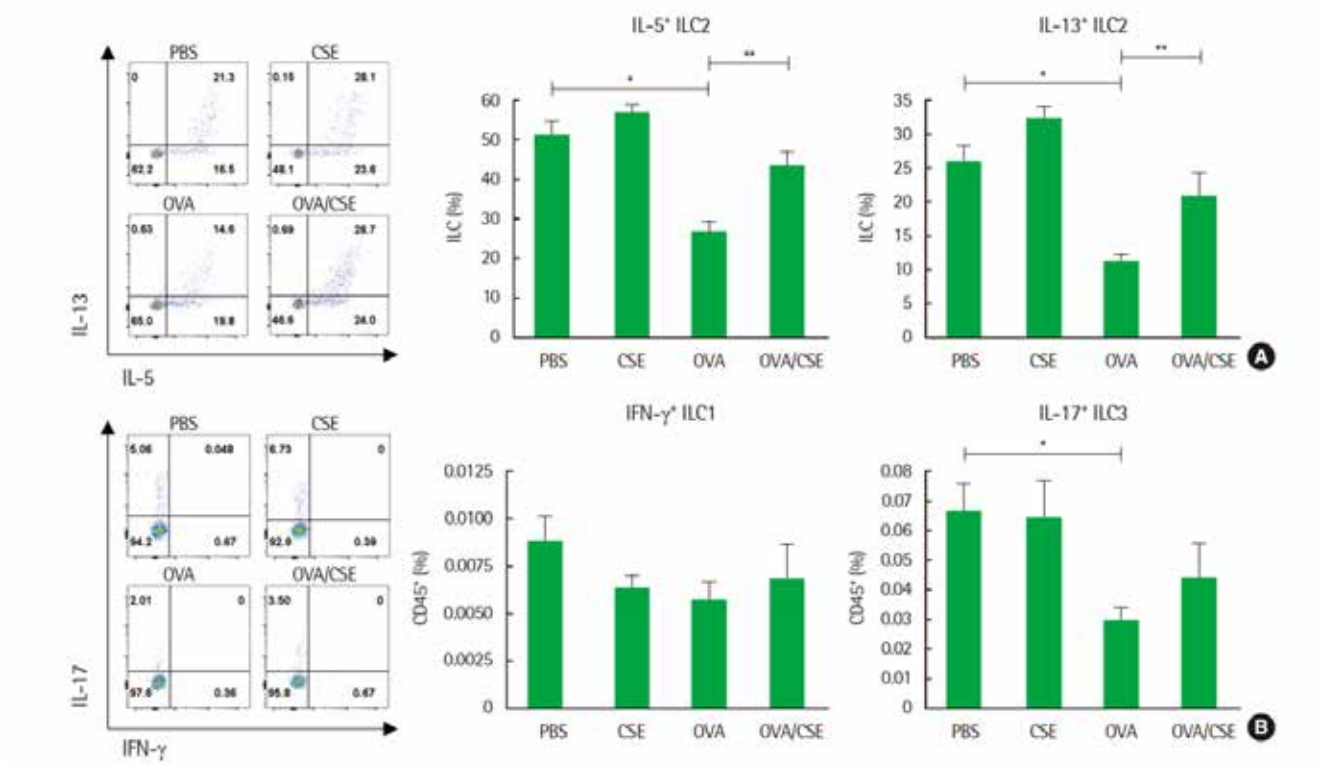

Figure 3. Effect of CSE on ILCs in the lung tissues of each group. (A) A flow cytometric representation of IL-5$^+$ and IL-13$^+$ ILCs, and percentages of each population in ILCs. (B) A flow cytometric representation of IL-17$^+$ and IFN-γ$^+$ ILCs, and percentages of each population in CD45$^+$ cells. PBS, phosphate-buffered saline; CSE, cigarette smoke extract; IL, interleukin; OVA, ovalbumin; IFN, interferon; ILC, innate lymphoid cell. *t*-test, **P* < 0.05, ***P* < 0.01, and *n* = 6.

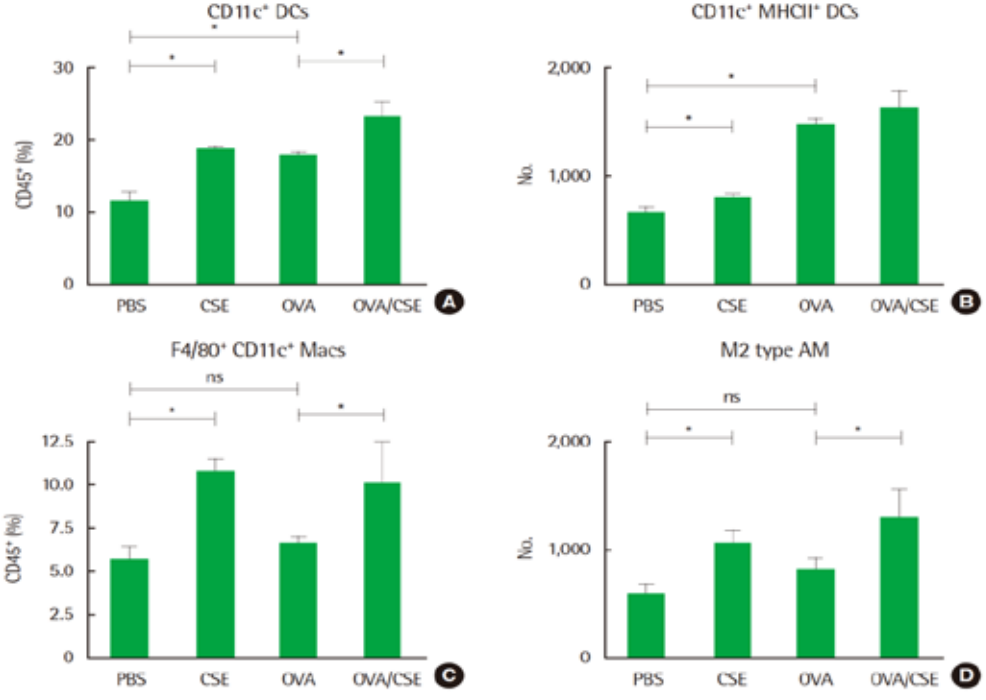

Figure 4. Effect of CSE on DCs and alveolar macrophages in the lung tissues of each group. (A) The percentages of CD11c$^+$ DCs in CD45$^+$ cells. (B) The counts of CD11c$^+$MHCII$^+$ dendritic cells. (C) The percentages of F4/80$^+$CD11c$^+$ alveolar macrophages in CD45$^+$ cells. (D) The counts of CD206$^+$ M2 alveolar macrophages. PBS, phosphate-buffered saline; CSE, cigarette smoke extract; IL, interleukin; OVA, ovalbumin; DC, dendritic cell; AM, alveolar macrophage. *t*-test, **P* < 0.05, and *n* = 6.

$F4/80^+CD11c^+$ alveolar macrophages were significantly increased in CSE alone and OVA/CSE combined exposure groups compared to control and OVA groups, respectively ($P < 0.05$) (Figure 4C). In particular, $CD206^+$ M2 macrophages showed a significant increase in response to CSE exposure in the CSE alone and OVA/CSE combination groups ($P < 0.05$) (Figure 4D).

4. Discussion

In this study, we used an OVA-induced asthma mouse model to confirm that cigarette smoke stimulates the innate immune system, including ILC2 and M2 alveolar macrophages, and contributes to the development and exacerbation of asthma.

In this study, 5% CSE was exposed to the airways through the nasal passages to create a smoking-like model, which was repeated for 8 weeks to determine the effects of long-term cigarette smoke exposure on the innate immune system. This study used an OVA-only nasal administration model instead of an OVA/alum intraperitoneal administration protocol to confirm the contribution of CSE-induced innate immune activation to the development of asthma. Typically, mouse models used in asthma research use either OVA or house dust mite (HDM) as the allergen. HDM stimulates the pattern recognition receptor (PRR) via dectin-2 to induce IL-33 expression in airway epithelial cells, which promotes ILC2 differentiation and induces a typical type 2 response [14]. On the other hand, OVA is difficult to induce a significant asthmatic phenotype when administered directly into the airways because it does not stimulate the PRR to initiate an immune response; therefore, in mouse models using OVA, sensitization to OVA is induced by intraperitoneal injection after mixing with a pro-Th2 adjuvant such as aluminum hydroxide (alum), which enhances the adaptive immune system via the inflammasome [15]. To demonstrate the contribution of CSE to asthma inception, this study used an OVA-alone intranasal administration model rather than the more commonly used asthmatic mouse model. Previous studies have identified a role for CSE in asthma using the OVA-alone intranasal model [16]. They reported that intranasal administration of 100 μg of OVA five times per week for a total of eight weeks did not induce a clear asthmatic phenotype, but the co-administration of CSE-induced thymic stromal lymphopoietin (TSLP) expression in epithelial cells did, resulting in a Th2 response and asthmatic airway inflammation. In this study, as expected, intranasal administration of OVA alone did not produce typical asthma, but CSE co-administration activated the innate immune system and significantly enhanced airway inflammation and AHR. In other words, repeated exposure of the airway immune system to a harmless protein without PRR stimulation does not produce an immune response, but exposure to CSE stimulates the innate immune system, especially innate lymphocytes, and macrophages, which activates the immune system and causes sensitization to OVA, suggesting that asthma develops. In general, the effects of tobacco smoke on the immune system are broad and multifactorial, and the mechanisms of action are complex [17]. In general, reactive oxygen species in tobacco smoke damage the airway lining epithelial cells and promote inflammation by causing DNA damage and activating pro-inflammatory genes [4]. Long-term exposure to tobacco smoke is known to modulate T cells and other cell signaling pathways that lead to the activation of immune cells [4]. Tobacco smoke is a major risk factor for the development of COPD [18]. In asthma-COPD overlap syndrome (ACOS), a condition in which pathological features of asthma and COPD coexist, studies have shown a higher rate of progression to ACOS in asthmatics exposed to tobacco smoke and air pollution [19,20]. As such, tobacco smoke is a risk factor for airway disease and has been reported to contribute to the development and severity of asthma [9,10]. Exposure to tobacco smoke affects the proportion of subsets of T cells, with $CD4^+$ T cells and memory

T cells being particularly increased in smokers [21]. CSE is known to contribute to the development and severity of COPD by inducing an increase in Th1/Th17 immune responses, but it also contributes to allergy sensitization and asthma by exacerbating Th2 immune responses [21]. In a patient study, it was reported that children with asthma living in a secondhand smoke environment had a decreased proportion of Treg and an increased proportion of Th17 cells, contributing to asthma exacerbation [6]. In this study, as previously reported, an increase in Th1, Th2, and Th17 cells and a decrease in Treg cells were observed in a mouse model of asthma exposed to cigarette smoke, suggesting that tobacco-induced perturbation of the adaptive immune system is associated with asthma exacerbation. Asthmatic patients with a history of smoking had increased asthma severity, including worsening symptoms of emphysema and airway obstruction and decreased lung function, and increased IgE [22]. Although this study did not directly measure OVA-specific IgE, flow cytometry confirmed the finding of increased DCs following CSE exposure, suggesting that tobacco may promote the sensitization process.

To determine the role of innate immune cells in the activation of an excessive immune response following tobacco smoke exposure, the authors identified the expression and subtypes of innate immune cells in a mouse model of asthma exposed to CSE. We observed a significant increase in innate immune cells, ILC2, and alveolar macrophages, in lung tissue, suggesting that modulation of the innate immune response is one of the mechanisms of asthmatic airway inflammation and airway remodeling induced by cigarette smoke exposure. ILCs are innate immune cells that reside in various tissues and play an important role in maintaining tissue immune homeostasis. ILCs are divided into ILC1, ILC2, and ILC3 subtypes, each of which has a phenotype similar to Th1, Th2, and Th17 cells, with different transcription factors and cytokines expressed by each subtype [23]. ILCs are rapidly activated early in the immune response by signaling molecules such as IL-33 and IL-25 in response to infection and injury, and due to their cytokine-secreting properties, they were expected to play a key role in the pathogenesis of asthma [23]. Indeed, an association with asthma has been confirmed by increased numbers of ILC1, ILC2, and ILC3 in sputum collected from asthmatic patients [10]. Exposure to cigarette smoke causes damage to epithelial cells, resulting in increased permeability of airway epithelial cells and secretion of signaling molecules such as IL-33 and TSLP, which induce activation of ILC2 [21]. Patient studies have shown that cigarette smoke induces an increase in IL-17A and Th17 cells, and indeed a co-occurrence of Th17, ILC3, and neutrophils has been observed in a mouse model of inflammatory bowel disease exposed to CSE [24]. Increased $NRP1^{+}$ILC3 and increased secretion of IL-17A and IL-22 have also been identified in the lungs of smokers and COPD patients, and it has been reported in mouse studies that CSE can induce chronic inflammation by increasing ILC3 [25]. In a mouse model of COPD exposed to cigarette smoke, all three ILC subtypes were increased, with a particularly pronounced increase in ILC1 [26,27]. In this study, we observed a significant increase in $IL\text{-}5^{+}$ ILC2 and $IL\text{-}13^{+}$ ILC2 after prolonged exposure to cigarette smoke in a mouse model of OVA-induced asthma, and, contrary to expectations, a slight but not statistically significant increase in $IFN\text{-}\gamma^{+}$ ILC1 and $IL\text{-}17^{+}$ ILC3. This suggests that the pathogenesis of CSE may be different in different lung diseases, and may be due to differences in tobacco smoke exposure protocols and asthma models used.

Macrophages are also innate immune cells involved in tissue immune homeostasis and can differentiate into two phenotypes, M1 and M2, depending on the surrounding microenvironment. M1 macrophages produce pro-inflammatory cytokines to elicit an immune response against bacteria and viruses, while M2 macrophages secrete anti-inflammatory cytokines and inflammatory mediators, which are involved in airway inflammation and airway remodeling in asthma

patients [28]. Cytokines secreted by ILCs interact with macrophages to polarise them, with ILC2 inducing differentiation into M2 macrophages and ILC1/ILC3 inducing differentiation into M1 macrophages [10]. M1 and M2 macrophages can be distinguished by their surface proteins and secreted cytokines. IRF5, a major transcription factor involved in the differentiation of M1 macrophages, is a marker of M1 macrophage activation in mice and humans and is known to be increased in patients with severe asthma [29]. Ym-1, a protein found in inflammatory zone 1 (FIZZ1), involves the deposition of extracellular matrix, induces lung fibrosis in mice, and is a marker of M2 macrophage activation [28]. It has been reported that FIZZ2 expressed in human airway epithelium and FIZZ1 in mice are highly homologous, and FIZZ2 is involved in human fibrotic lung disease and its expression is particularly increased in asthmatic patients [30]. In this study, we found that alveolar and M2 macrophages were significantly increased in the OVA/CSE group compared to the OVA alone group. This suggests that the increase in ILC2 and M2 macrophages induced by cigarette smoke exposure is one of the pathogenetic mechanisms of asthma and suggests the possibility of a correlation between ILC2 and M2 macrophages.

This study is significant because it identifies a previously under-explored effect of smoking on innate immune cells in asthmatic airways. However, further studies are needed to investigate the role of cigarette smoke exposure-induced increase in ILC2 in the pathogenesis of asthma, and in particular to confirm the correlation between ILC2 and M2 macrophages, as a close link between ILCs and macrophages has been reported. In addition, although this study used OVA to create an asthma mouse model, it is difficult to apply clinically as OVA is not an antigen that causes asthma in humans, and continuous infusion of OVA alone may cause immune tolerance [31].

In conclusion, this study used a mouse model of asthma induced by cigarette smoke extract and OVA to investigate the effects of cigarette smoke on ILCs and macrophages and found that long-term exposure to cigarette smoke may contribute to the development and exacerbation of asthma by inducing the activation of ILC2 and M2 macrophages.

Disclosure statement

The authors declare no conflict of interest.

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