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[Title page]

**Aggravation of asthmatic inflammation by chlorine exposure via innate lymphoid cells
and CD11c^{intermediate} macrophages**

Short title: Altered innate immunity by chlorine exposure in asthma

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Conflict of interest

The authors declare that they have no relevant conflict of interest.

Abstract

Background

Chlorine is widely used in daily life as disinfectant. However, chronic exposure to chlorine products aggravates allergic T_H2 inflammation and airway hyperresponsiveness (AHR). Innate lymphoid cells (ILCs) in airways contribute to the inception of asthma in association with virus infection, pollution, and excess of nutrient but it is not known whether chronic chlorine exposure can activate innate immune cells. The aim of this study is to evaluate the impact of chlorine inhalation on the innate immunity such as ILCs and macrophages in relation with the development of asthma by using murine ovalbumin (OVA) sensitization/challenge model.

Methods

Six-week-old female BALB/c mice were sensitized and challenged with OVA in the presence and absence of chronic low dose chlorine exposure by inhalation of naturally vaporized gas of 5% sodium hypochlorite solution. AHR, airway inflammatory cells from BALF and the population of ILCs and macrophages in the lung were evaluated.

Results

The mice exposed to chlorine with OVA (Cl+OVA group) showed enhanced AHR and eosinophilic inflammation compared to OVA-treated mice (OVA group). The population of T_H2 cells, ILC2s and ILC3s increased in Cl+OVA group compared to OVA group. CD11c^{int} macrophages also remarkably increased in Cl+OVA group compared to OVA group. The deletion of macrophages by clodronate resulted in reduction of ILC2s and ILC3s population which was restored by adoptive transfer of CD11c^{int} macrophages.

Conclusions

Chronic chlorine inhalation contributes to the exacerbation of airway inflammation in asthmatic airway by mobilizing pro-inflammatory macrophage into the lung as well as stimulating group 2 and 3 ILCs.

Keywords: asthma, chlorine, innate lymphoid cells, T_H2 cells, monocyte-derived macrophages

Abbreviations

RADS: reactive airway dysfunction syndrome

AHR: airway hyperresponsiveness

ROS: reactive oxygen species

TSLP: thymic stromal lymphopoietin

T_H2: type 2 T helper

OVA: ovalbumin

BAL: bronchoalveolar lavage

ILC: innate lymphoid cell

ILC1: group 1 innate lymphoid cell

ILC2: group 2 innate lymphoid cell

ILC3: group 3 innate lymphoid cell

DCs: dendritic cells

FACS: fluorescence-activated cell sorting

Introduction

Chlorine is a chemical that is commonly used for various purposes, including disinfection, water purification, bleaching, plastic production, and pulp and paper production.¹ Chlorine gas is toxic, and acute exposure to highly concentrated chlorine gas can induce acute respiratory dysfunction, such as pulmonary edema, obstructive or restrictive lung abnormalities, and reactive airway dysfunction syndrome.² These symptoms occur within a day of exposure and last for more than 3 months.³ Affected patients show obstructive patterns on spirometry and airway hyperresponsiveness (AHR).^{1,3} These respiratory effects may be due to airway epithelial damage and inflammation caused by chlorine-induced reactive oxygen species (ROS).⁴ However, most people are usually exposed to low concentrations of chlorine in everyday life, and such chronic exposure may contribute to persistent inflammation.

Sodium hypochlorite (NaOCl) solution is widely used as a bleaching agent, biocide, and water purifier in homes, buildings, and swimming pools.⁵ Chlorine gas naturally vaporizes from NaOCl solution, and its inhalation may affect the airway. The risk of chronic low-dose chlorine exposure has not been properly assessed because it does not cause any remarkable serious symptoms.^{2,5} However, there have been reports of an increased prevalence of asthma in individuals exposed to chronic low-dose chlorine, such as swimmers, swimming pool workers, and cleaning staff.⁶⁻¹⁰ This suggests that long-term exposure to chlorine gas even at low concentrations may affect the respiratory system. While the effects of high concentrations of acute chlorine exposure have been extensively studied, there have been only a few studies on the effects of low-dose chronic chlorine exposure on asthmatic airways.

In our previous study, we performed animal experiments using NaOCl solution and demonstrated that chronic exposure to chlorine gas vaporized from the NaOCl solution

aggravated AHR and airway inflammation in OVA-induced asthmatic mice by stimulating components of the innate immune system, such as IL-1 β and IL-33.⁵

Although the innate immune response lacks antigen specificity and memory, it can rapidly respond to external stimuli.¹¹ To date, most studies on the mechanism of asthma have been focused on adaptive immune responses.¹² However, as mentioned above, the innate immune system is also deeply involved in the development and aggravation of asthma. Based on this knowledge and our previous research results, we hypothesized that innate lymphoid cells and macrophages involved in the innate immune system of the airway are related to the mechanism of asthma aggravation induced by chronic exposure to low-dose chlorine.

Materials and Methods

Mice

Female wild-type BALB/c mice were purchased from Orient Bio, Inc. (Seoul, Korea). The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Laboratory Animal Resources at Seoul National University (SNU-16-0322-4-2).

Allergen sensitization/challenge and chlorine exposure

Mice were divided into four groups, which were treated as follows (n = 6, each group): (1) exposed to phosphate buffered saline (PBS group), (2) exposed to low-dose chlorine for 4 weeks (Cl group), (3) sensitized and challenged with OVA (OVA group), (4) exposed to low-dose chlorine for four weeks and sensitized/challenged with OVA (Cl+OVA group). Allergen sensitization was performed by intraperitoneal injection of 75 μ g of OVA (Sigma, St. Louis, MO, USA) along with 2 mg of aluminum hydroxide (Sigma) on days 1 and 14. The mice were challenged with 50 μ g of OVA via the intranasal route on days 28, 29, and 30. To

simulate low-dose chlorine exposure, gas naturally vaporizing from a 5% sodium hypochlorite solution (NaOCl; Sigma) was used. A container filled with 3 mL of the 5% NaOCl solution was placed in the mouse cage for 8 hours per day on five days in each week for four weeks.⁵

Measurement of AHR and collection of bronchoalveolar lavage fluid

On day 31, AHR was measured with a barometric plethysmographic chamber (OCP 3000; Allmedicus, Anyang, Korea), and the Penh (enhanced pause) value was monitored for 3 min. On day 32, bronchoalveolar lavage (BAL) fluid and lung tissue were collected from sacrificed mice. BAL cell slides were stained with Diff-Quik (Sysmex Co., Kobe, Japan), and at least 300 cells per slide were evaluated to obtain a differential leukocyte count.

Cell analysis by flow cytometry

Single-cell suspensions were preincubated with an Fc γ R-specific blocking mAb (2.4G2; BD Biosciences, Franklin Lakes, NJ) and washed before staining. For fluorescence-activated sorting of cells from mouse lungs, we used the following antibodies: Percp-cy5.5-conjugated anti-CD45; PE/cy7-conjugated anti-CD127; PE-conjugated anti-IL-5, anti-IL-13, anti-IL-17, and anti-IFN- γ ; FITC-conjugated anti-F4/80; and APC-cy7-conjugated anti-CD11c, which were purchased from eBioscience (San Diego, CA). Alexa Fluor 700-conjugated anti-CD4 was purchased from BioLegend (San Diego, CA).

To stain innate lymphoid cells (ILCs), lineage-negative cells (Lin⁻), which were CD3⁻, CD4⁻, CD8⁻, CD11b⁻, CD11c⁻, CD19⁻, F4/80⁻, Fc ϵ RI⁻, and CD49b⁻, were sorted, using CD127 as a surface marker. ILC1s and ILC3s were defined as the CD45⁺Lin⁻CD127⁺INF- γ ⁺ and CD45⁺Lin⁻CD127⁺IL-17⁺ lymphoid cell populations, respectively. ILC2s were defined as the CD45⁺Lin⁻CD127⁺IL-5⁺IL-13⁺ lymphoid cells. Macrophages were sorted as the

CD45⁺F4/80⁺ cells, and dendritic cells were sorted as the CD45⁺F4/80⁻CD11c⁺ cells. Subpopulations of macrophages were identified by CD11c expression levels. To detect type 2 T helper (T_H2) cells, CD4⁺IL-5⁺IL-13⁺ cells were gated. Flow cytometry was performed with a BD LSR II, BD LSRFortessa, and BD LSRFortessa X-20, and the data were analyzed by using FlowJo (version 10.2) software (TreeStar, Ashland, OR).

CD11c^{intermediate} macrophage depletion and transfer

To deplete CD11^{int} macrophages, BALB/c mice were treated with liposomal clodronate (Clodrosome, USA). Mice were divided into four groups, which were treated as follows (n = 5, each group): (1) exposed to 200 μ L of empty liposomes (Control), (2) exposed to 200 μ L of liposomal clodronate intravenously (Clodronate group), (3) exposed to empty liposomes and sensitized/challenged with OVA (OVA group), and (4) exposed to 200 μ L of liposomal clodronate for 24 h prior to each OVA challenge (OVA+Clodronate group) according to previously described protocols.^{13,14} In addition, CD45⁺F4/80⁺CD11c^{int} macrophages from OVA-sensitized and challenged mice were sorted with a FACS Aria II (BD Biosciences), and these cells (5×10^5 /mouse) were adoptively transferred by intranasal instillation into BALB/C mice 24 h after treatment with liposomal clodronate.

Statistical analysis

When comparing two groups, the Mann-Whitney test was used for nonparametric data and the unpaired *t* test was used for parametric data. To analyze the correlation between ILCs and macrophages in lung tissue, Spearman's *r* correlation test was performed for nonparametric data. Values are expressed as mean $\pm$ standard deviation (SD). *P* values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA).

Results

Chronic low-dose chlorine exposure aggravates allergen-induced AHR and inflammation

To examine the effect of chlorine exposure on AHR and lung inflammation, we investigated four groups of mice (each with $n = 6$): PBS (control), chlorine only (Cl group), OVA only (OVA group), and both chlorine and OVA exposed (Cl+OVA group; Figure 1A). The results showed that AHR was significantly further increased in the Cl+OVA group compared to OVA group ($P < 0.05$), but Cl group did not show AHR (Figure 1B). The total number of inflammatory cells in BAL fluid was significantly higher in the Cl+OVA group than in the OVA group ($P < 0.05$), with significantly higher numbers of macrophages, eosinophils, and neutrophils (each with $P < 0.05$, Figure 1C). However, the number of CD11c⁺ F4/80⁺ dendritic cells in lung tissue was not higher in the Cl+OVA group than in the OVA group (Figure 1D).

Increased numbers of ILC2s, ILC3s, and T_H2 cells in the lung tissue of low-dose chlorine exposed asthmatic mice

The numbers of T_H2 cells were significantly higher in the lung tissue of mice in the OVA group than in mice in the PBS group ($P < 0.01$), and, as expected, this increase was much greater in the Cl+OVA group ($P < 0.001$; Figure 2A and 2B). Next, to investigate the effects of chronic low-dose chlorine exposure on the distribution of ILCs, we detected the INF- γ ⁺ cells (ILC1s), IL-5⁺IL-13⁺ cells (ILC2s), and IL-17⁺ cells (ILC3s) in the lung tissues. The absolute number of ILC2s in the Cl and OVA groups was not significantly different from the number in the PBS group. However, the number of ILC2s in the Cl+OVA group was significantly higher (1.87-fold) than the number in the OVA group ($P < 0.01$; Figure 2C). The number of ILC3s was also significantly higher in the Cl+OVA group than in the OVA group

($P < 0.01$), whereas there was no significant difference in the numbers of ILC1s (Figure S1 A).

Increased CD11c^{int} macrophages in chronic low-dose chlorine-exposed asthmatic mice

Macrophages play an important role in the innate immune system. Therefore, we examined macrophages in the mouse lung tissue by using anti-F4/80 and anti-CD11c antibodies (Figure 3A). A significant increase in total F4/80⁺ macrophages was observed in the Cl+OVA group compared with the OVA group ($P < 0.05$; Figure 3B). Next, we sorted macrophages by CD11c expression level into CD11c^{negative}, CD11c^{intermediate}, and CD11c^{high} groups. A previous study showed that CD11c was not expressed in interstitial macrophages but was highly expressed in alveolar macrophages.¹⁵ According to a literature search, CD11c^{int} macrophages are more likely to be monocyte-derived macrophages that migrate to inflamed tissues through extravasation.^{16,17} In our experiments, the CD11c⁻ and CD11c^{high} macrophages did not differ significantly between the OVA and Cl+OVA groups. However, the CD11c^{int} macrophages were significantly higher in the Cl+OVA group than in the OVA group ($P < 0.05$; Figure 3B).

We also performed a correlation analysis to evaluate the association between CD11c^{int} macrophages and ILCs. A significant correlation was observed between CD11c^{int} macrophages and ILC2s ($P = 0.008$) and ILC3s ($P < 0.01$; Figure S1 B) but not ILC1s (Figure 3C).

Decreased T_H2 cells, ILC2s, and ILC3s induced by deletion of CD11c^{int} macrophages in OVA-induced asthmatic mice

To determine the effect of CD11c^{int} macrophages on ILCs and the phenotype of asthma, we depleted these macrophages in asthma murine model by intravenously injecting liposomal clodronate into OVA-sensitized mice before OVA challenge (each group with $n = 5$; Figure

4A). We confirmed that the number of total F4/80⁺ macrophages was significantly decreased in OVA-induced asthmatic mice following treatment with liposomal clodronate compared to the control ($P < 0.01$; Figure 4C). Next, we investigated the changes in the macrophage subpopulations caused by clodronate administration in OVA-induced asthmatic mice. There were significantly fewer CD11c^{int} macrophages in the OVA+Clodronate group than in the OVA group ($P < 0.01$), and CD11c^{hi} macrophages were also decreased by clodronate treatment, but this decrease was lesser than that for the CD11c^{int} macrophages (Figure 4D). The number of eosinophils in BAL fluid was significantly lower in the OVA+Clodronate group than in the OVA group ($P < 0.01$; Figure 4B). Next, we examined the changes in other immune cells, such as T_H2 cells and ILCs, after deletion of the CD11c^{int} macrophages (Figure 5A). The results showed a significant decrease in T_H2 cells, ILC2s (each with $P < 0.05$; Figure 5B), and ILC3s ($P < 0.05$; Figure S2) in the OVA+Clodronate group when compared to the OVA group.

Adoptive transfer of CD11c^{int} macrophages restored ILC2s and ILC3s

In the next step, to clarify the effect of CD11c^{int} macrophages on eosinophils, T_H2 cells, and ILCs, we performed an experiment in which CD11c^{int} macrophages were adoptively transferred to clodronate-injected OVA-induced asthmatic mice via the intranasal route (each group with $n = 5$; Figure 6A). The number of eosinophils in BAL fluid, which was reduced following clodronate injection, was restored by adoptive transfer of CD11c^{int} macrophages ($P < 0.05$; Figure 6B). T_H2 cells, which were significantly decreased in OVA+Clodronate mice, were also restored by transfer of CD11c^{int} macrophages, but this change was not statistically significant (Figure 6C and D). ILC2s (1.51-fold; Figure 6D) and ILC3s (1.73-fold; Figure S3) were both significantly restored by transfer of CD11c^{int} macrophages (ILC2, $P < 0.05$; ILC3, $P < 0.01$).

Discussion

Chronic exposure to low-dose chlorine gas was shown to exacerbate allergic T_H2 inflammation and AHR by activating the inflammasome danger signaling pathways.⁵ However, its effect on innate immune cells, such as ILCs and macrophages, had not been explored. The present study revealed that ILCs and macrophages are crucial players in the aggravation of allergic inflammation caused by chronic exposure to low-dose chlorine gas using a murine asthma model.

Chlorine is highly soluble in water, and once inhaled, it is hydrolyzed to hypochlorous acid (HOCl) and the reactive anion hypochlorite (ClO⁻) in the aqueous mucus layer covering the airway epithelium, and both can produce ROS.^{4,18} Previous animal studies showed damaged epithelium, increased BAL protein concentrations, AHR, and infiltration of inflammatory cells, such as neutrophils and macrophages, following exposure to chlorine gas at 50–800 ppm.^{4,19-23} However, it is not common for humans to be exposed to high concentrations of chlorine in everyday life, except accidental exposure. Humans can recognize chlorine at concentrations of 0.1-0.3 ppm, and complain of mucosal irritation at up to 15 ppm.² Inhalation of naturally vaporized chlorine gas from sodium hypochlorite solutions that are widely used for sterilization and bleaching, may affect the airway and cause respiratory symptoms.⁵ There have been some reports showing that low-dose chlorine exposure is associated with atopic dermatitis, allergic contact dermatitis, and contact urticaria.²⁴⁻²⁷

Since the airway is in close contact with the external environment, it functions in the primary defense against various harmful substances, microorganisms, and allergens; therefore, the innate immune system in the airway is more well-developed than in other organs.²⁸ This study investigated the effects of chronic exposure to low-dose chlorine on the development or aggravation of asthma, with a particular focus on the changes in the innate immune system. Our previous study showed that AHR and airway inflammation were exacerbated by chronic

low-dose chlorine exposure in OVA-induced asthmatic murine airways.⁵ The study also revealed that the levels of T_H2 cytokines, such as IL-4 and IL-5, were elevated in the Cl+OVA group, as was IL-1 β , which is mainly expressed in macrophages and monocytes and matured by toll-like receptors or NLRP3 inflammasome signaling.²⁹⁻³¹ In addition, we previously observed an increase in alarmins, such as IL-33 and TSLP, which are known to stimulate ILC2s.³² Therefore, we sought to determine the detailed mechanism underlying the changes in the innate immune system caused by chronic low-dose chlorine exposure.

In this study, we re-confirmed that chronic low-dose chlorine exposure aggravates AHR and airway inflammation in OVA-induced asthmatic mice. Next, we examined the changes in ILCs, and the results showed that ILC2s and ILC3s were more remarkably increased in the Cl + OVA group than in the OVA group, whereas no significant changes occurred in the Cl group. Chlorine is not an allergen but an irritant.⁴ Although low dose chlorine exposure does not result in an apparent airway pathology, it potentiates the damage of airway epithelium when combined with allergic inflammation by enhancing the expression of IL-33.⁵ In this study, we revealed that enhanced allergic airway inflammation by low dose chlorine exposure is contributed by increased ILCs and CD11c^{int} macrophages. ILC2s and ILC3s appear to play key roles in the aggravation of asthmatic airway induced by low-dose chronic chlorine exposure.

ILC2s, which were recently discovered in both mice and humans, are components of the innate immune system that are involved in the induction and maintenance of type 2 inflammation.^{33,34} ILC2s mainly exist at locations where the host meets the environment, that is, in the lung, intestine, and skin.³⁵ Functioning as a sentinel on the front with the environment, these cells connect the innate and adaptive immune responses of type 2 immunity to external stimuli.^{11,36} ILC2s activated by external stimuli produce the type 2 cytokines IL-4, IL-5, and IL-13 and promote type 2 inflammation and eosinophilic airway

inflammation.³⁴ Goblet cell hyperplasia, mucus production, airway smooth muscle contractility, and collagen deposition can also occur.³⁷ These findings show that ILC2s are involved in allergic asthma.³⁷ In 2014, it was shown that ILC2-deficient mice exhibit less severe lung inflammation and eosinophilic infiltration as well as lower IL-13 production than wild-type mice following intranasal administration of allergens, such as papain or dust mites.³⁸ Since this discovery, the association of ILC2 with asthma and the underlying mechanism have been under investigation. ILC2s have also been an active research topic in chronic sinusitis with nasal polyps, lung fibrosis, atopic dermatitis, and eosinophilic esophagitis.^{37,39} In contrast, ILC3s have been shown to be involved in non-allergic asthma and associated with obesity and psoriasis.³⁹

When airway epithelial cells are stimulated by protease allergens, fungi, or viruses, epithelial-driven cytokines called alarmins, such as IL-33, IL-25, and TSLP, are generated in the airway epithelium, which activates ILC2s.³⁷ In our previous study, we showed that IL-33 and TSLP levels were significantly higher in the Cl+OVA group than in the OVA group, and histologic examination showed increased inflammatory cell infiltration in the Cl+OVA group as well as a significantly higher mucus index score in PAS staining in the Cl+OVA group than in the OVA group.⁵ In the current study, we first showed significantly larger ILC2 and ILC3 cell populations following exposure to low-dose chlorine in OVA-induced murine asthmatic airways, whereas chloride exposure alone did not lead to any changes in ILCs. These findings suggest that ILCs may be a missing link between epithelial damage-induced cytokines and airway inflammation in the aggravation of asthma caused by chronic low-dose chlorine exposure.

Recent studies reported the role of ILC2s in the persistence and exacerbation of asthma in humans. The ILC2s levels in blood, BAL fluid, and induced sputum were higher in asthmatic subjects than in the controls.^{40,41} Furthermore, increased numbers of ILC2s and higher IL-33

levels in BAL fluid were significantly associated with poor lung function in asthmatics.⁴²⁻⁴⁴

There have also been reports that asthma severity and control levels are associated with the numbers of ILC2s.^{41,45} Patients with severe asthma had significantly higher numbers of ILC2s in blood and sputum than patients with mild asthma,⁴¹ and patients with uncontrolled or only partly controlled asthma also had significantly higher numbers of ILC2s than patients with well-controlled asthma and healthy controls.⁴⁵ These high numbers of ILC2s were reversed when symptoms were controlled.⁴⁵ Although ILCs are known to be associated with several inflammatory diseases, no significant increase in ILC1s was observed in the CI, OVA, or CI+OVA groups. Although ILC1s do not appear to be associated with allergic inflammation, they are known to function in the control of intracellular pathogens in the gut and are associated with Crohn's disease in the gut, and COPD in the lung.³⁷

Macrophages are one of the major cellular components of the innate immune system.¹¹ Recently, it was shown that macrophages are more abundant in the induced sputum of asthmatic patients than in that of healthy controls, and ILC2s promoted the polarization of alveolar macrophages to M2 macrophages, or alternatively activated macrophages, especially in eosinophilic asthma.^{40,46} However, to the best of our knowledge, the association between ILCs and interstitial macrophages has not yet been elucidated.

There are two types of tissue-resident macrophages in the lung, alveolar and interstitial macrophages.⁴⁷ Alveolar macrophages are detected in the lung beginning in embryogenesis and survive for a long time, and supplementation from bone marrow-derived monocytes is minimal.^{17,48} Alveolar macrophages usually function in tissue homeostasis and surfactant clearance, and when bacteria or viruses invade, they play a role in pathogen phagocytosis and T cell recruitment.⁴⁹ In addition to tissue-resident macrophages, other macrophages are newly supplied through the bloodstream in certain situations, and are called inflammatory lung macrophages, exudate lung macrophages, or monocyte-derived macrophages.⁴⁷ When the

tissue is inflamed, monocytes quickly extravasate into the injured tissue and then differentiate into DCs or macrophages.⁴⁸ These monocyte-derived macrophages have various bactericidal activities; they are capable of phagocytosis, produce nitric oxide, and promote local inflammatory responses. Ultimately, they act as defenders, but they are multi-faceted cells, and their roles have not been fully investigated.⁴⁹

In this study, CD11c^{int} macrophages were newly identified as regulators of ILC2 function. In 2007, Moon et al. demonstrated that CD11b⁺ CD11c^{int} macrophages infiltrate into the lungs of OVA-induced asthmatic mice, and these populations could induce AHR and eosinophilic inflammation.¹⁶ Intriguingly, CD11c^{int} macrophages were significantly increased, along with ILC2s and ILC3s, in this study. ILC2s and ILC3s were significantly decreased by depletion of monocyte-derived macrophages from the bloodstream by intravenous administration of clodronate, and this was reversed by adoptive transfer of CD11c^{int} macrophages. This finding suggests that CD11c^{int} macrophages originating from the circulating monocytes are key for stimulating ILC2s and ILC3s. Our results showed a significant positive correlation between ILC2s and total macrophages or CD11c^{int} macrophages. This suggests that CD11c^{int} macrophages, which are considered to be monocyte-derived macrophages, play an essential role in asthmatic airway inflammation by increasing ILC2s and ILC3s.

Although both ILCs and macrophages are important cells in the innate immune system, their interaction is not fully understood. It has been suggested that macrophages residing in adipose tissue may be maintained in an M2 phenotype by type 2 cytokines, such as IL-4 and IL-13, secreted from ILC2s or eosinophils.⁵⁰ However, no report has shown that macrophages directly affect ILCs. In the current study, we clearly showed that CD11c^{int} macrophages, probably monocyte-derived macrophages from the bloodstream, are vital for the maintenance

of ILC2s and ILC3s in the OVA-induced murine model of asthma, and these cells seem to influence each other.

This study has 2 major limitations; first, we have conducted the deletion and adoptive transfer of CD11c^{int} macrophages only in the OVA group not in the Cl+OVA group. However, despite the relatively lower level of ILCs and CD11c^{int} macrophages in the OVA group than the Cl+OVA group, our experiment clearly demonstrated significant differences in ILC2 and ILC3 levels according to the presence of CD11c^{int} macrophages. We therefore believe that these type of macrophages play a key role in the enhancement of ILC2s and ILC3s when allergic inflammation occurs. Second, we present descriptive findings but did not revealed a mechanistic evidence of how CD11c^{int} macrophages induce ILC2s and ILC3s. Additional experiments are needed to reveal the underlying mechanism of these findings.

In conclusion, when mice with OVA-induced asthma were exposed to chlorine gas vaporized from NaOCl solution, significant increases in both AHR and the total number of inflammatory cells in BAL fluid, especially macrophages and eosinophils, occurred. In addition, ILC2s and ILC3s were significantly increased, as were CD11c^{int} macrophages, which are a specific population of macrophages. We confirmed, via depletion and adoptive transfer in the OVA-induced murine model of asthma, that the influx of CD11c^{int} macrophages, which are considered to be monocyte-derived, to the inflamed tissues was essential for the maintenance of ILC2s and ILC3s, and subsequently eosinophils and T_H2 cells.

Our results demonstrate that chronic low-dose chlorine exposure aggravates asthma, and the innate immune response, including the increases in ILC2s, ILC3s, and monocyte-derived macrophages, is important for its pathophysiology. These findings are expected to be widely applicable in studies of the development and aggravation of asthma caused by other external triggers, such as particulate matter.

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Figure legends

Figure 1. The effect of chlorine exposure on lung inflammation and AHR in OVA-induced asthmatic mice

A, Experimental protocol of the study. **B**, Methacholine-induced AHR was measured 24 h after the last intranasal challenge. **C**, The number of inflammatory cells including eosinophils in BAL fluid. **D**, CD11c⁺ F4/80⁻ dendritic cell frequency (percentage of CD45⁺ cells) in the lung tissue. Mann-Whitney test was used to compare differences between two groups. Values represent means $\pm$ SDs. * indicates $P < 0.05$. i.p.; intraperitoneal, i.n.; intranasal, OVA; ovalbumin, NaOCl; sodium hypochlorite, MBPT; methacholine bronchial provocation test, PBS; phosphate buffered saline, Cl; exposure to chlorine, NS; not significant.

Figure 2. The effect of chlorine exposure on T_H2 cells and ILCs in OVA-induced asthmatic mice

A, Representative T_H2 cell (upper) and ILC2 (lower) gating strategy from the lung tissue of PBS group, Cl group, OVA group, and Cl+OVA group (each with n = 6). **B**, Comparison of the numbers of T_H2 cells and ILC2s between the control group and the experimental group. Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.01$ and ** $P < 0.001$. NS; not significant.

Figure 3. The effect of chlorine exposure on macrophage populations in OVA-induced asthmatic mice

A, Representative gating strategy of total macrophage (left) and its subpopulation depending on CD11c levels (right) from the lung tissue of OVA group. **B**, Comparisons of total macrophage, CD11c negative (CD11c⁻) macrophage, CD11c intermediate (CD11c^{int}) macrophage, and CD11c high (CD11c^{hi}) macrophage frequencies (percentage of CD45⁺ cells)

between the control group and the experimental group. Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$. **C**, Correlation plot between ILC2 and CD11c^{int} macrophage in the lung tissue of PBS group (blank circle), Cl group (light grey), OVA group (dark grey), and Cl+OVA group (black) (total n = 24). Spearman's r correlation test was used to measure association between two variables. ** $P < 0.01$.

Figure 4. The effect of liposomal clodronate injection on eosinophils and macrophages

A, Experimental protocol of the study. **B**, The number of eosinophils in BAL fluid. **C**, Comparisons of total macrophage frequency (percentage of CD45⁺ cells) between the control group and the experimental group. **D**, Comparisons of CD11c⁻, CD11c^{int}, and CD11c^{hi} macrophage frequencies (percentage of F4/80⁺ cells) with or without clodronate administration between the control group and the OVA group. Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$ and ** $P < 0.01$. i.p.; intraperitoneal, i.n.; intranasal, i.v.; intravenous, CTRL; control.

Figure 5. The effect of liposomal clodronate injection on T_H2 cells and ILC2s

A, Representative T_H2 cell (upper) and ILC2 (lower) gating strategy from the lung tissue of PBS group, Clodronate group, OVA group, and OVA+Clodronate group (each with n = 5). **B**, Comparison of T_H2 cell (percentage of CD4⁺ T cells) and ILC2 (percentage of lineage⁻ IL-7R⁺ cells) frequencies between the control group and the experimental group. Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$ and ** $P < 0.01$. CTRL; control.

Figure 6. The effect of adoptive transfer of CD11c^{int} macrophages on eosinophils, T_H2 cells and ILC2s in liposomal clodronate treated OVA-induced asthmatic mice

A, Experimental protocol of the study. **B**, The number of inflammatory cells including eosinophils in BAL fluid. **C**, Representative gating strategy of T_H2 cell and ILC2 from the lung tissue of the OVA group, OVA + Clodronate group, and OVA + Clodronate + $CD11c^{int}$ transfer group (each with $n = 5$). **D**, Comparison of T_H2 cell (percentage of $CD4^+$ T cells) and ILC2 (percentage of lineage $^-$ IL-7R $^+$ cells) frequencies between the control group and the experimental group. Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$. i.p.; intraperitoneal, i.n.; intranasal, i.v.; intravenous, NS; not significant.

Figure S1. The effect of chlorine exposure on ILC1s and ILC3s in OVA-induced asthmatic mice

A, The numbers of ILC1s (left) and ILC3s (right) in the lung tissue from the control and experimental group. Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$ and ** $P < 0.01$. NS; not significant. **B**, Correlation plot between ILC3 and $CD11c^{int}$ macrophage in the lung tissue of PBS group (blank circle), Cl group (light grey), OVA group (dark grey), and Cl+OVA group (black) (total $n = 24$). Spearman r correlation test was used to measure association between two variables. ** $P < 0.01$.

Figure S2. The effect of liposomal clodronate injection on ILC3s

ILC3 frequencies (percentage of lineage $^-$ IL-7R $^+$ cells). Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$.

Figure S3. The effect of adoptive transfer of $CD11c^{int}$ macrophages on ILC3s in liposomal clodronate treated OVA-induced asthmatic mice

ILC3 frequencies (percentage of lineage $^-$ IL-7R $^+$ cells). Mann-Whitney test. Values represent means $\pm$ SDs. * $P < 0.05$ and ** $P < 0.01$.

Figure 1.

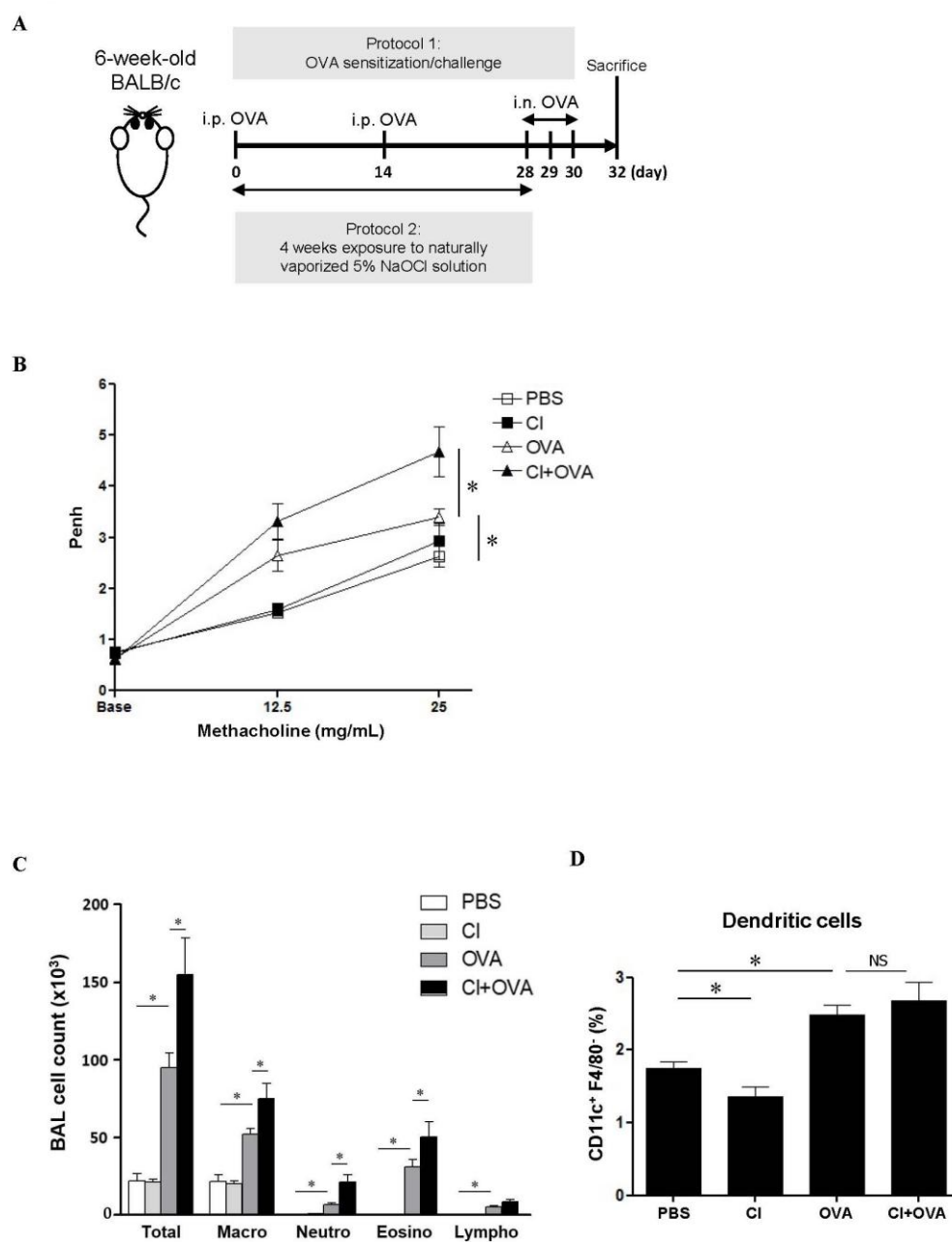


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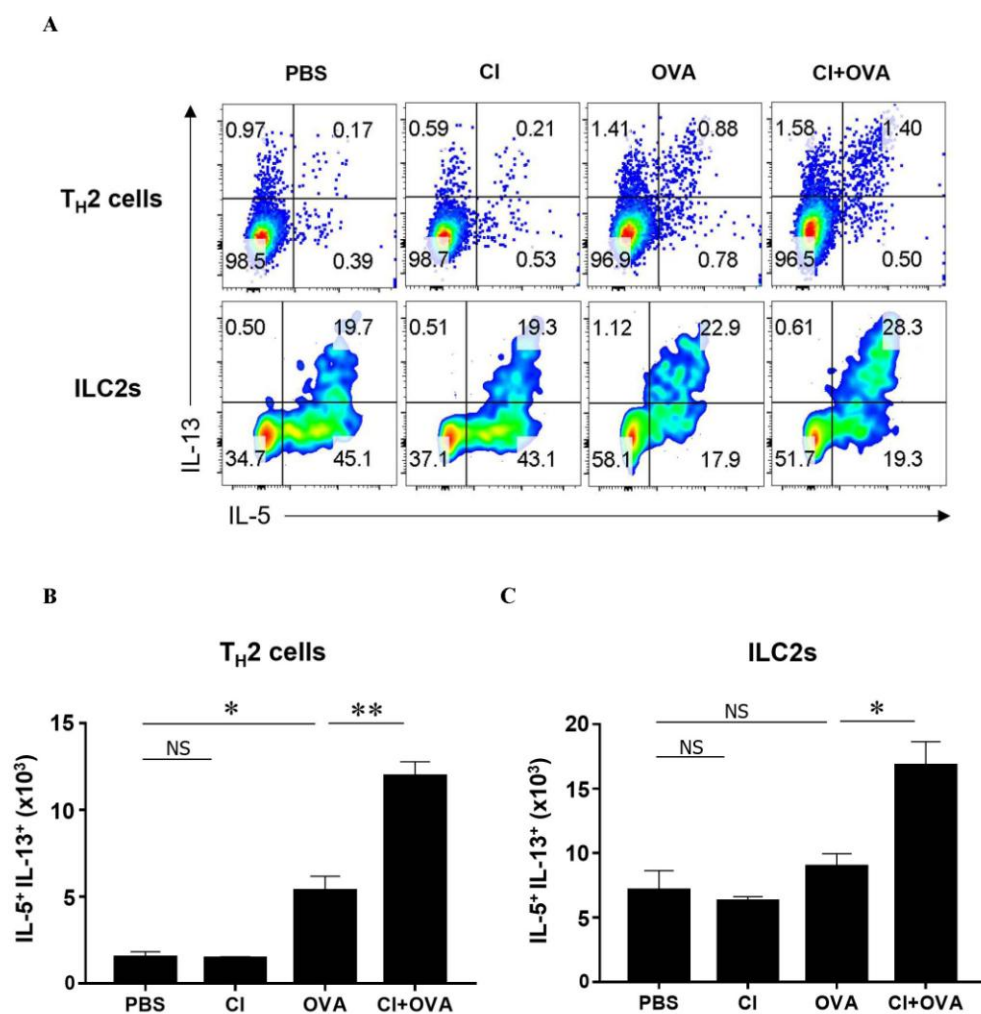


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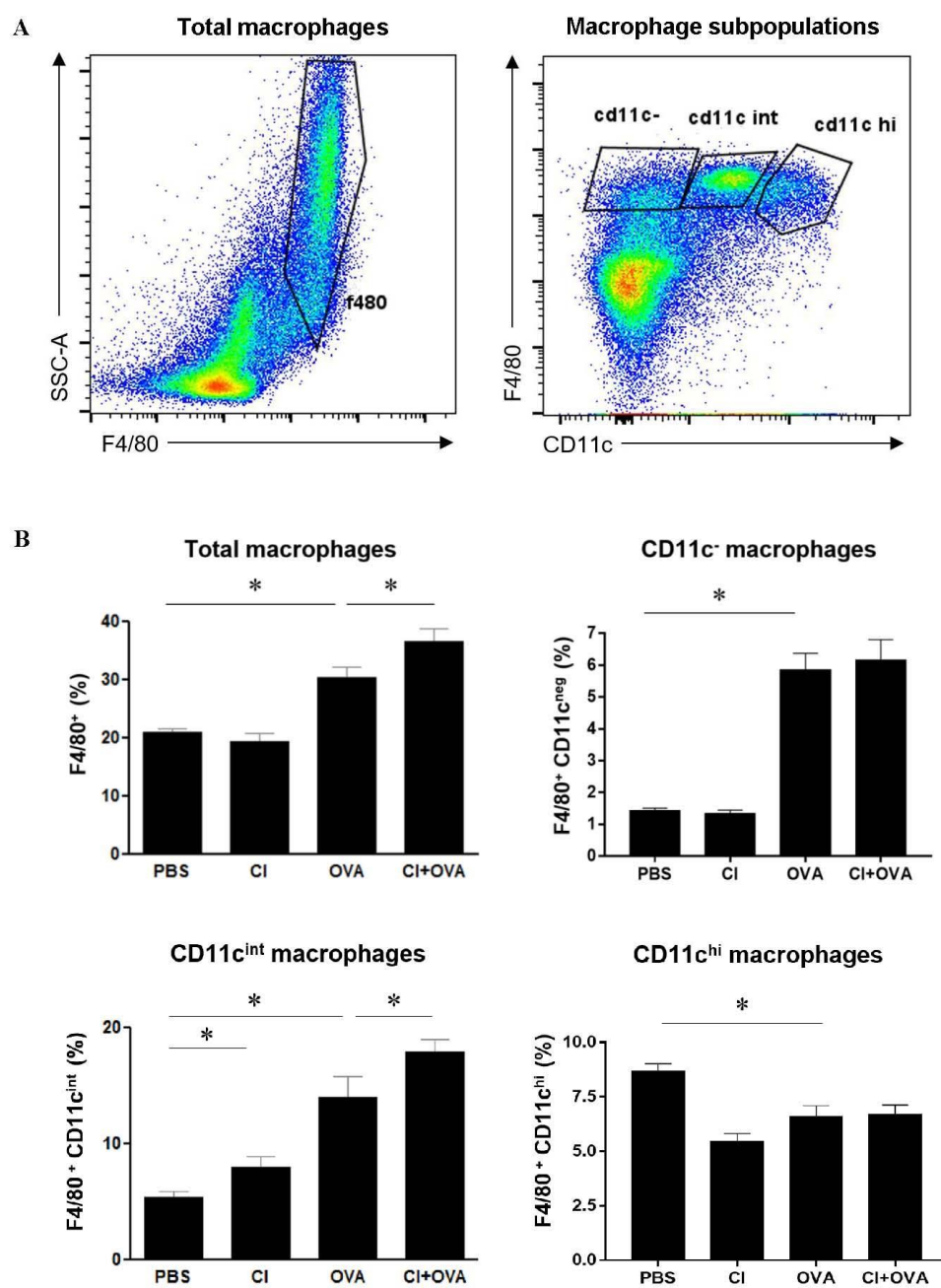


Figure 3.

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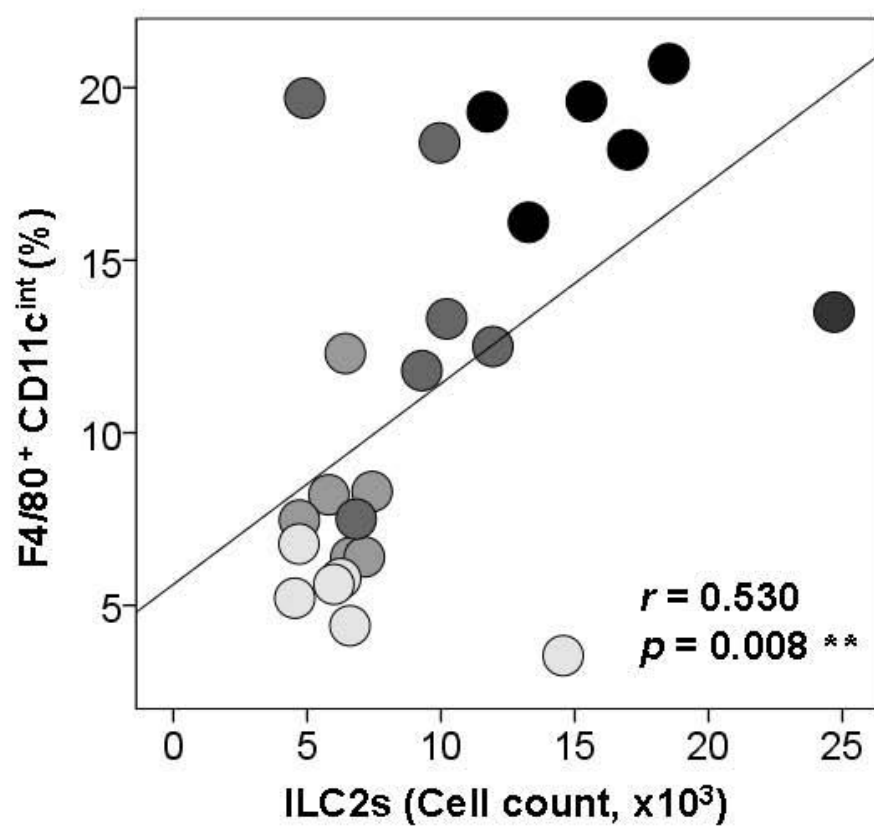


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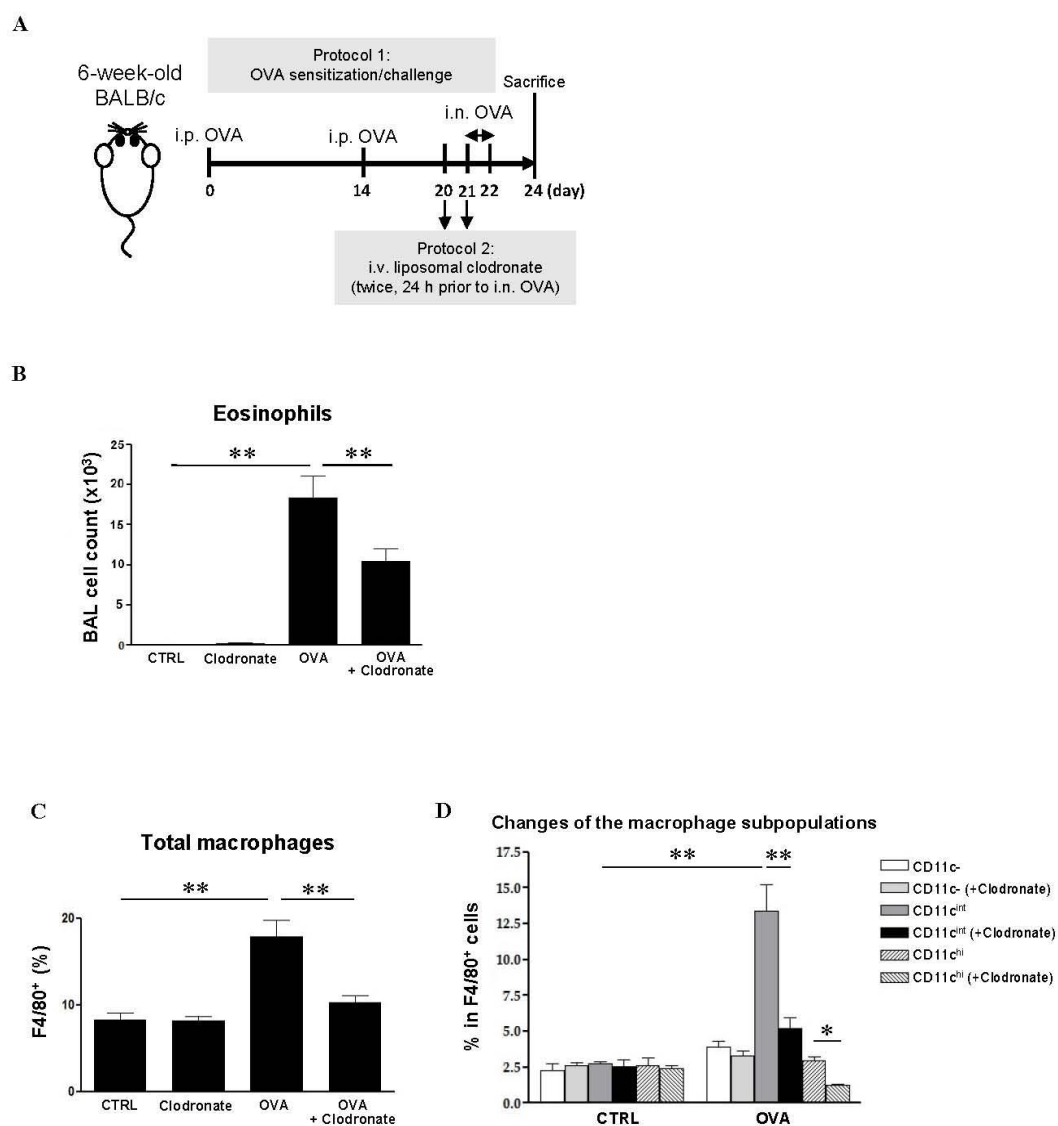


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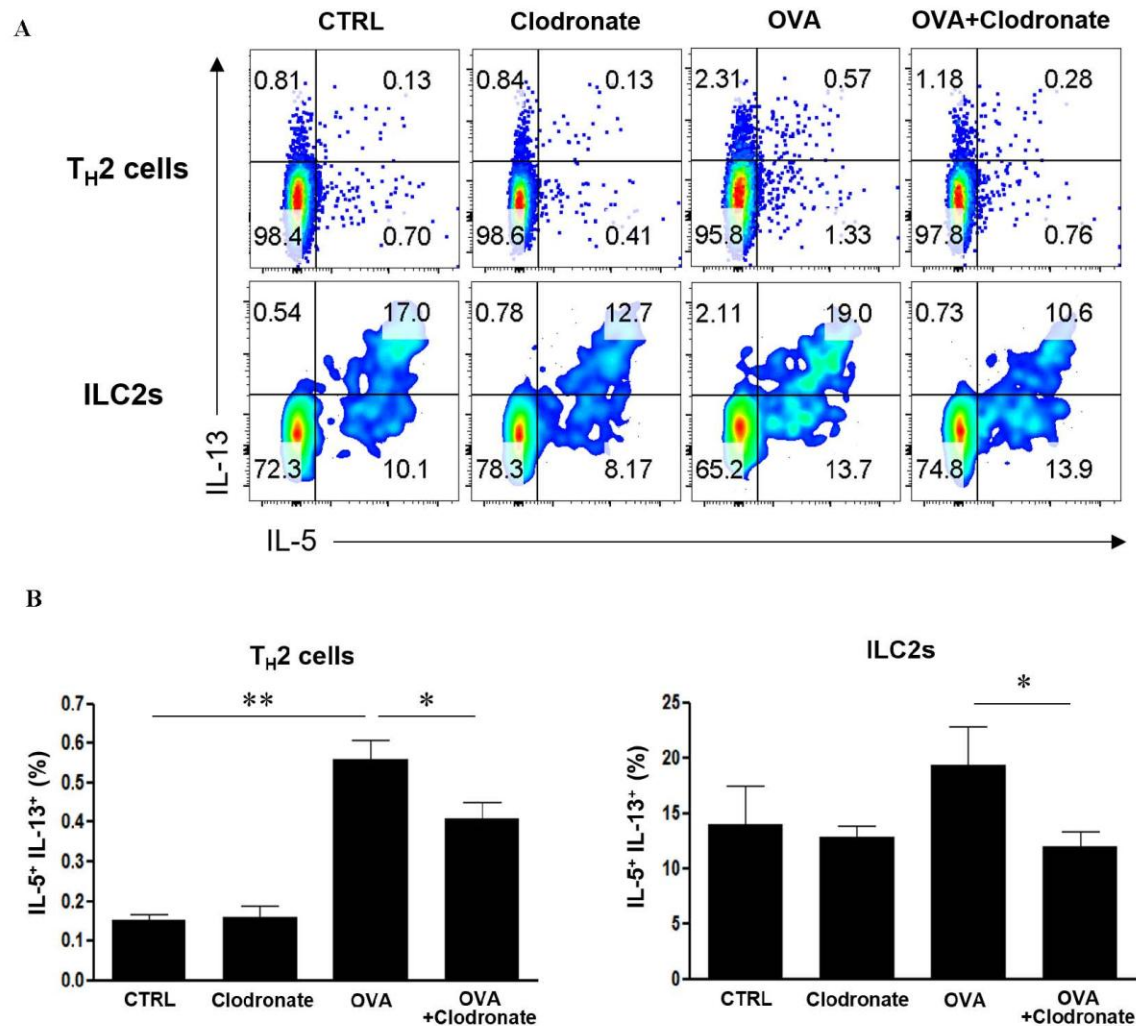


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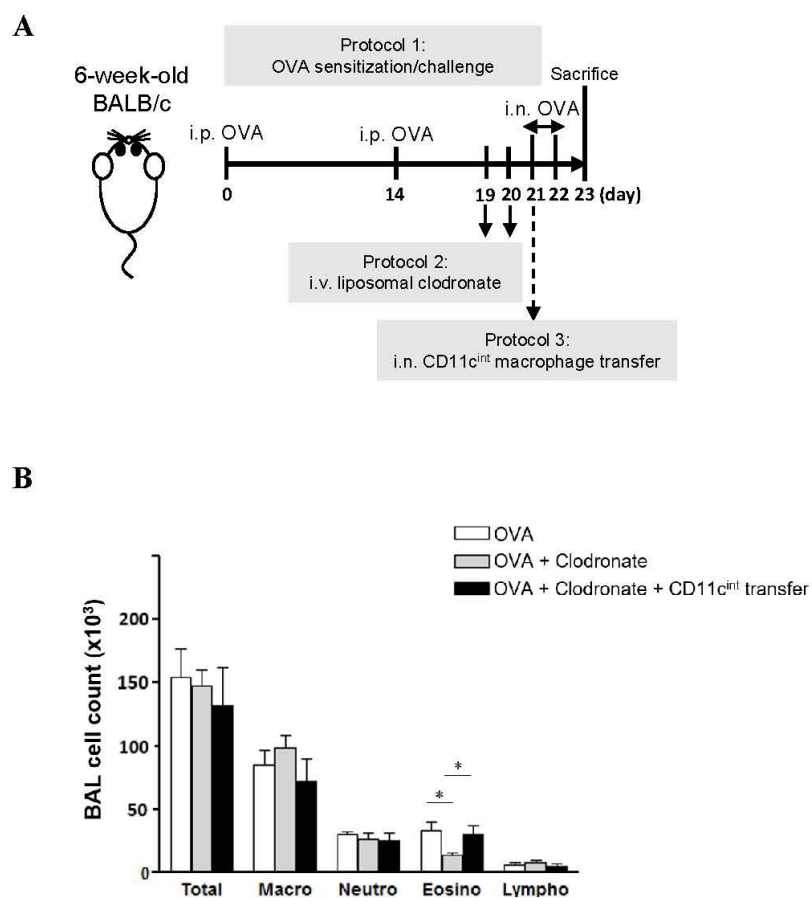
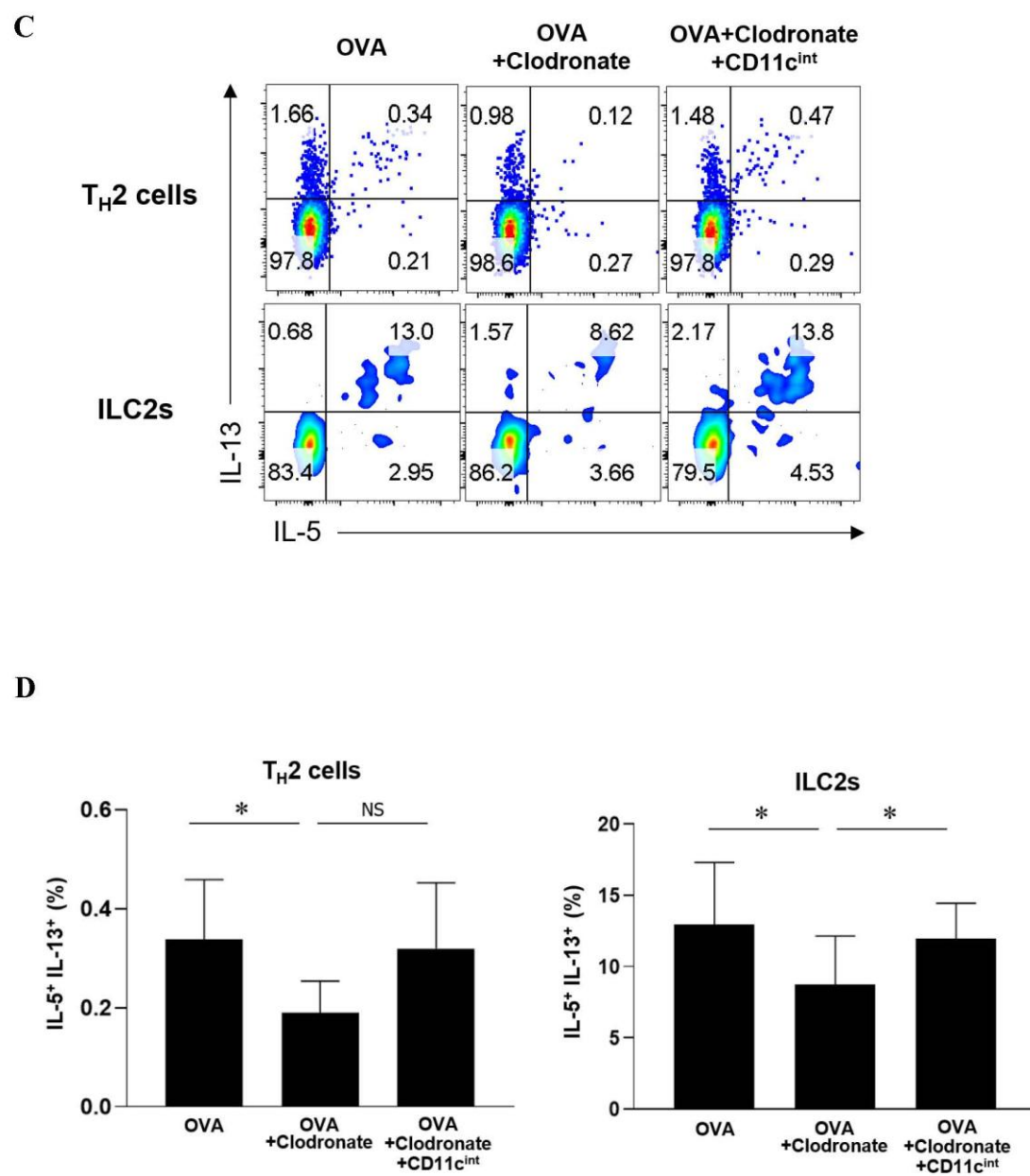
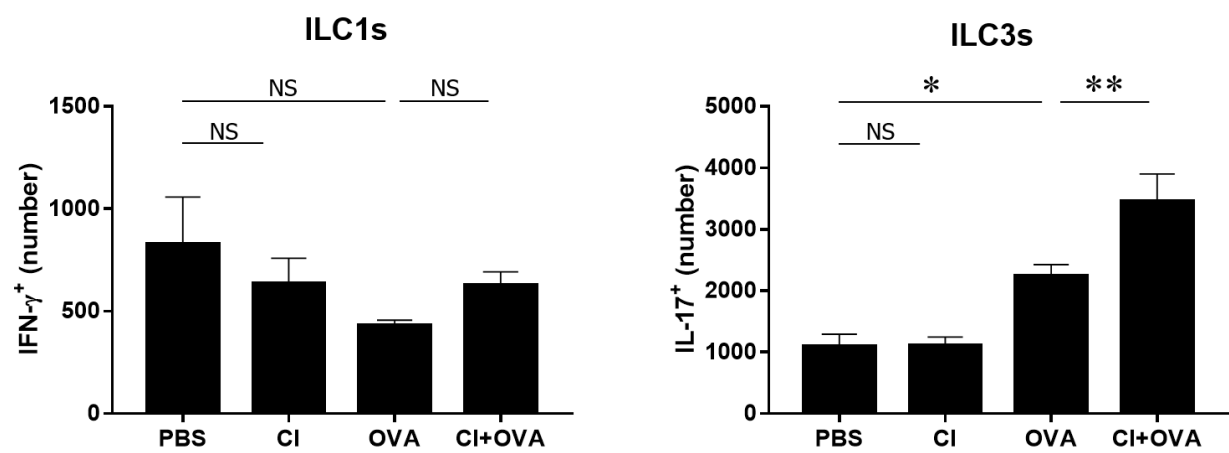


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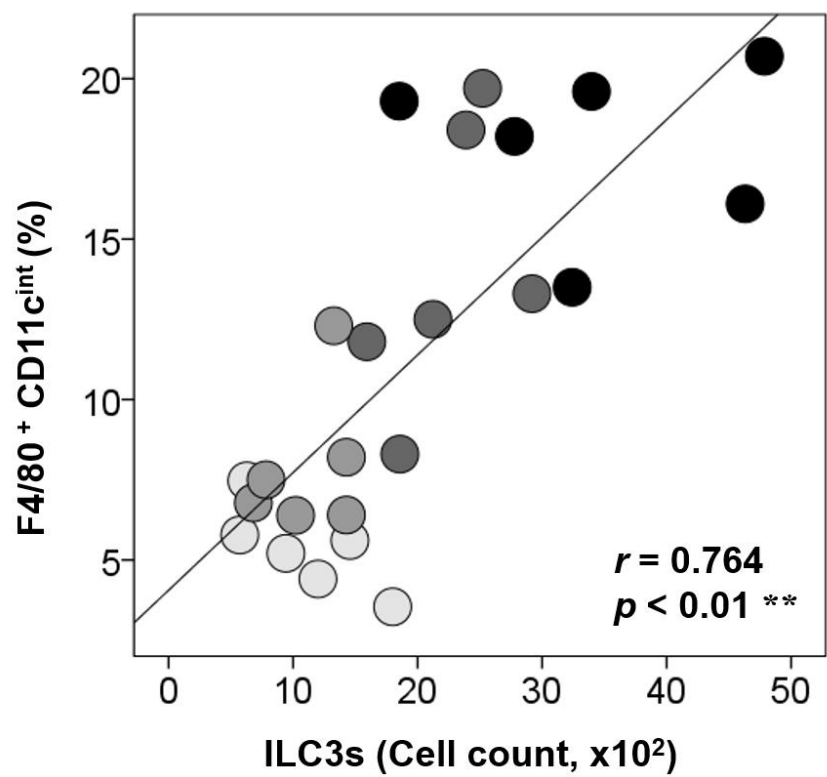


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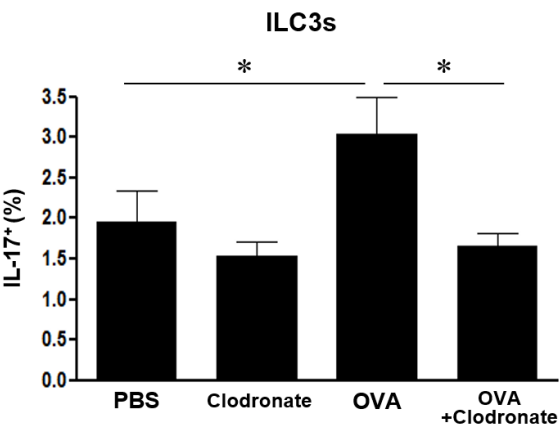
A



B



related to Figure 5. = Figure S2.



related to Figure 6. = Figure S3.

