Allergen endotoxins induce T-cell–dependent and non–IgE-mediated nasal hypersensitivity in mice

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Background: Allergen-mediated cross-linking of IgE on mast cells/basophils is a well-recognized trigger for type 1 allergic diseases such as allergic rhinitis (AR). However, allergens may not be the sole trigger for AR, and several allergic-like reactions are induced by non–IgE-mediated mechanisms.

Objective: We sought to describe a novel non–IgE-mediated, endotoxin-triggered nasal type-1-hypersensitivity-like reaction in mice.

Methods: To investigate whether endotoxin affects sneezing responses, mice were intraperitoneally immunized with ovalbumin (OVA), then nasally challenged with endotoxin-free or endotoxin-containing OVA. To investigate the role of T cells and mechanisms of the endotoxin-induced response, mice were adoptively transferred with *in vitro*–differentiated OVA-specific T_H2 cells, then nasally challenged with endotoxin-free or endotoxin-containing OVA.

Results: Endotoxin-containing, but not endotoxin-free, OVA elicited sneezing responses in mice independent from IgE-mediated signaling. OVA-specific T_H2 cell adoptive transfer to mice demonstrated that local activation of antigen-specific T_H2 cells was required for the response. The Toll-like receptor 4-myeloid differentiation factor 88 signaling pathway was indispensable for endotoxin-containing OVA-elicited rhinitis. In addition, LPS directly triggered sneezing responses in OVA-specific T_H2-transferred and nasally endotoxin-free OVA–primed mice. Although antihistamines suppressed sneezing responses, mast-cell/basophil-depleted mice had normal sneezing responses to endotoxin-containing OVA. Clodronate treatment abrogated endotoxin-containing OVA-elicited rhinitis, suggesting the involvement of monocytes/macrophages in this response.

Conclusions: Antigen-specific nasal activation of $CD4^+$ T cells followed by endotoxin exposure induces mast cell/basophil-independent histamine release in the nose that elicits sneezing responses. Thus, environmental or nasal residential bacteria may exacerbate AR symptoms. In addition, this novel phenomenon might explain currently unknown mechanisms in allergic(-like) disorders. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Allergic rhinitis, $CD4^+$ T cell, endotoxin, histamine, hypersensitivity, IgE, monocyte/macrophage, nonallergic rhinitis, LPS*

Type 1 hypersensitivity reactions are a major pathological response in allergic diseases such as allergic rhinitis (AR), allergic asthma, and allergic anaphylaxis.[1] Classical type 1 hypersensitivity reactions are mediated by mast cells and basophils armed with antigen-specific IgE.[1,2] Cross-linking of IgE on mast cells/basophils by cognate antigens induces degranulation and the release of chemical mediators including histamine, which elicits bronchoconstriction and vasodilation and increases vascular permeability.[2] Although the IgE-mast-cell–mediated pathway is closely linked to allergic disorders, several type-1-hypersensitivity-like symptoms are induced by non–IgE-mediated mechanisms.[3-5]

The early-phase responses of AR (eg, sneezing) are mainly induced by allergen-mediated IgE cross-linking on mast cells.[6] However, the allergen-IgE pathway may not be the sole trigger, because some patients with AR do not respond to anti-IgE therapy.[7] Currently, non–IgE-mediated trigger(s) of AR symptoms are entirely unknown. AR diagnosis is based on rhinitis symptoms: sneezing, nasal discharge, and nasal clotting, and the presence of systemic or local (nasal) allergen-specific IgE.[8-10] Patients with rhinitis without allergen-specific IgE are diagnosed as suffering from nonallergic rhinitis (NAR) and, in most cases, triggers of the symptoms are unclear.[11] Although NAR is not considered an allergy, some patients with NAR show nasal infiltration of inflammatory cells including neutrophils, eosinophils, and mast cells that suggests local activation of innate and/or adaptive immunity.[12-15] In addition, histamine, a major mediator of type 1 allergic reactions, is involved in the disease symptoms, because antihistamines are often effective in patients with NAR.[11] Thus, the nasal symptoms of NAR with inflammation might be an immune-mediated hypersensitivity reaction. Currently, the underlying pathological mechanisms in these non–IgE-mediated allergic reactions have been rarely studied. Although IgE cross-linking is the classical signal for

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Abbreviations used
AR: Allergic rhinitis
DT: Diphtheria toxin
Ec-OVA: Endotoxin-containing OVA
Ef-OVA: Endotoxin-free OVA
ILC2: Group 2 innate lymphoid cell
i.n.: Intranasally
i.p.: Intraperitoneally
MyD88: Myeloid differentiation factor 88
NAR: Nonallergic rhinitis
OVA: Ovalbumin
TLR4: Toll-like receptor 4
WT: Wild type

mast cell/basophil degranulation,[1,2] many signals induce IgE-independent mast cell degranulation.[4,16] In addition, many cell types other than mast cells/basophils can produce and release histamine by non–IgE-mediated mechanisms.[17-22] These observations prompted us to investigate the causes and mechanisms of non–IgE-mediated type-1-hypersensitivity-like reactions.

LPS, also known as endotoxin, is a component of the gram-negative bacterial cell wall and is a common contaminant in several allergens including household dust[23-25] and animal dander,[26,27] as well as ambient air pollutants.[28] Low-dose LPS is a well-known T_H2-inducing adjuvant in airway application models.[29,30] Furthermore, several allergens contain ligands for Toll-like receptor 4 (TLR4), the LPS receptor, which mediates their allergenicity.[31-33] Even though mast cells also express TLR4, and LPS stimulation of mast cells induces cytokine production, LPS does not elicit mast cell degranulation.[34] Thus, even though LPS can participate in some allergic sensitizations, it is not considered a trigger of effector-phase responses.

In this study, we show the presence of a hitherto unknown non–IgE-mediated nasal allergy-like response in mice. Nasal application of endotoxin-containing ovalbumin (OVA) (Ec-OVA) triggers sneezing, a type-1-hypersensitivity-like reaction, in mice, which is IgE- and mast-cell–independent, but dependent on $CD4^+$ T cells, histamine, and monocytes/macrophages. We propose that the local activation of antigen-specific $CD4^+$ T cells followed by endotoxin exposure can elicit histamine release in the nose that triggers a type-1-hypersensitivity-like reaction. This reaction might contribute to classical and non–IgE-mediated allergic disorders of which the pathological mechanisms are not fully understood.

METHODS

More detailed methods can be found in this article's Methods section in the Online Repository at www.jacionline.org.

Mice

BALB/c wild-type (WT) mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). BALB/c-background DO11.10$^+$[35] and myeloid differentiation factor 88–deficient (*Myd88*$^{-/-}$)[36] mice were maintained at the animal facilities of Hyogo College of Medicine. BALB/c-background *Fcer1a*$^{-/-}$ and *Rag2*$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Me). BALB/c-background *Tlr4*$^{-/-}$ mice were purchased from OrientalBioService (Kyoto, Japan). BALB/c-background Mas-TRECK mice were provided by Dr Masato Kubo (Division of Molecular Pathology, Research Institute for Biological Sciences, Tokyo University of Science, Tokyo, Japan). These mice were maintained under specific pathogen-free conditions. All mouse experiments were performed with the approval of, and in accordance with, the guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine (No. A11-235, No. 14-007, and No. 28028).

Removal of endotoxin from OVA

Endotoxin-free ovalbumin (Ef-OVA) was generated from commercially available Ec-OVA. Overall, 1 mL of OVA solution (20 mg/mL) was mixed with 10 μL of Triton X-114 by vortexing. Samples were placed on ice for 5 minutes, then incubated at 37°C for 5 minutes. After centrifugation (9000*g* for 7 seconds), the upper aqueous phases were collected. The procedure was repeated 3 times. Residual detergent in the aqueous phase was removed by Pierce Detergent Removal Spin Columns (Pierce Biotechnology, Rockford, Ill). The endotoxin levels in Ec-OVA and Ef-OVA were determined by Pierce LAL Chromogenic Endotoxin Quantitation Kit (Pierce Biotechnology). Endotoxin levels in OVAs used in this study were 100 to 207 EU/mg (Ec-OVA) and 1.74 EU/mg (Ef-OVA), where 1 EU is equivalent to 0.1 ng of LPS from *Escherichia coli* E111:B4.

Mouse models

For the OVA immunization model, mice were injected intraperitoneally (i.p.) with a mixture of Ef-OVA (30 μg/200 μL/mouse) and alum (1 mg/200 μL/mouse) in PBS on days −18, −11, and −4. Five days after the final immunization (day 1), mice were intranasally (i.n.) administered 20 μL of PBS, Ec-OVA, or Ef-OVA (1 mg protein/dose in PBS) for 4 consecutive days. For cell-transfer models, mice were injected intravenously with naive or *in vitro*–differentiated OVA-specific T_H2 cells from DO11.10$^+$CD4$^+$ T cells (4-6 × 10^6 cells) on day −1. Two days after the transfer (day 1), mice were i.n. administered 20 μL of PBS, Ec-OVA, or Ef-OVA (1 mg protein/dose in PBS) for 4 consecutive days. Memory T_H2 cells were generated in mice transferred with DO11.10$^+$ T_H2 cells, and these were then left untreated for 6 weeks. In some experiments, mice were i.n. administered 20 μL of LPS (0.5, 5, or 50 ng/mL in PBS). To deplete mast cells and basophils, Mas-TRECK and control WT mice were injected i.p. with diphtheria toxin (DT) (250 ng/200 μL/mouse) on days −6, −5, −4, −3, −2, 1, and 3. Monocyte/macrophage depletion was achieved by intraperitoneal injection of clodronate liposome (100 μL/mouse) on day 3, 6 hours after the nasal challenge. For antihistamine treatment, mice were injected i.p. with diphenhydramine (1 or 2 mg/200 μL/mouse) or fexofenadine (5 mg/200 μL/mouse) in PBS 90 minutes before nasal challenge with OVA. Immediately after each nasal challenge, the frequency of sneezing was counted for 10 minutes. The mice were sacrificed 24 hours after the final nasal challenge, and noses were dissected for analyzing infiltrating inflammatory cells.

Statistics

Two-tailed Student *t* test and 1-way ANOVA followed by Tukey test were used to determine the statistical significance between 2 groups and among more than 2 groups, respectively. Two-way ANOVA followed by Bonferroni test was used for analyzing statistics of time-course experiments. *P* values of less than .05 were considered statistically significant.

RESULTS

Ec-OVA elicits IgE-independent rhinitis

We investigated whether AR-like symptoms can be elicited by non–IgE-mediated mechanisms. WT and *Fcer1a*$^{-/-}$ mice, deficient for FcεRI and thus deficient for IgE-mediated allergic reactions,[9,37] were i.p. immunized with Ef-OVA 3 times (days −18, −11, and −4), then i.n. challenged with 1 mg of Ef-OVA (endotoxin level, 1.74 EU/mg) or Ec-OVA (endotoxin level,

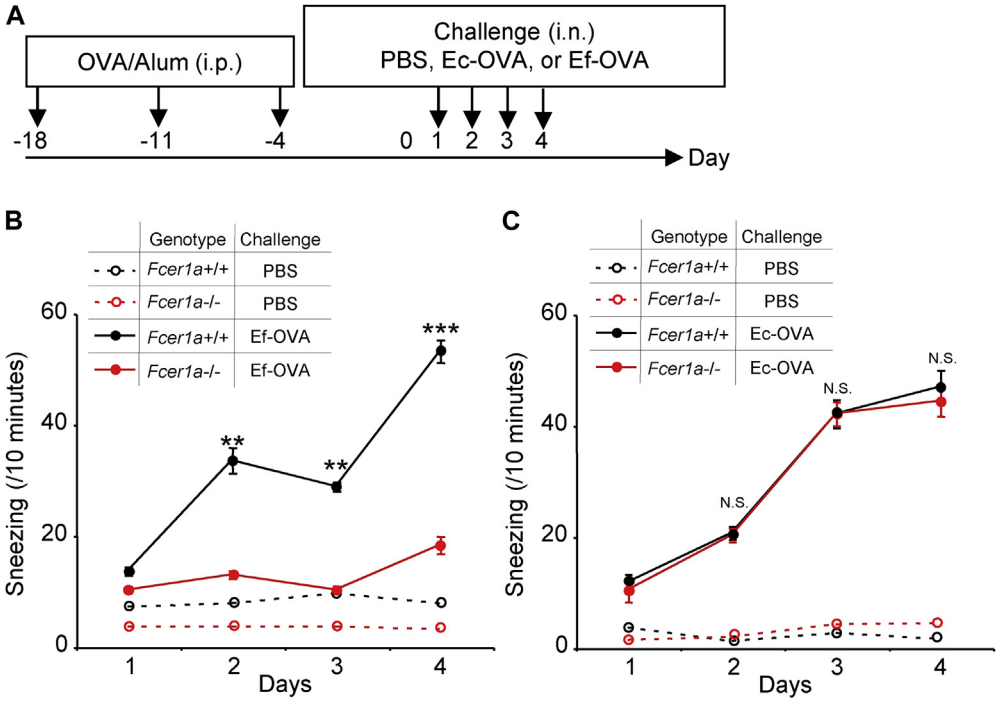


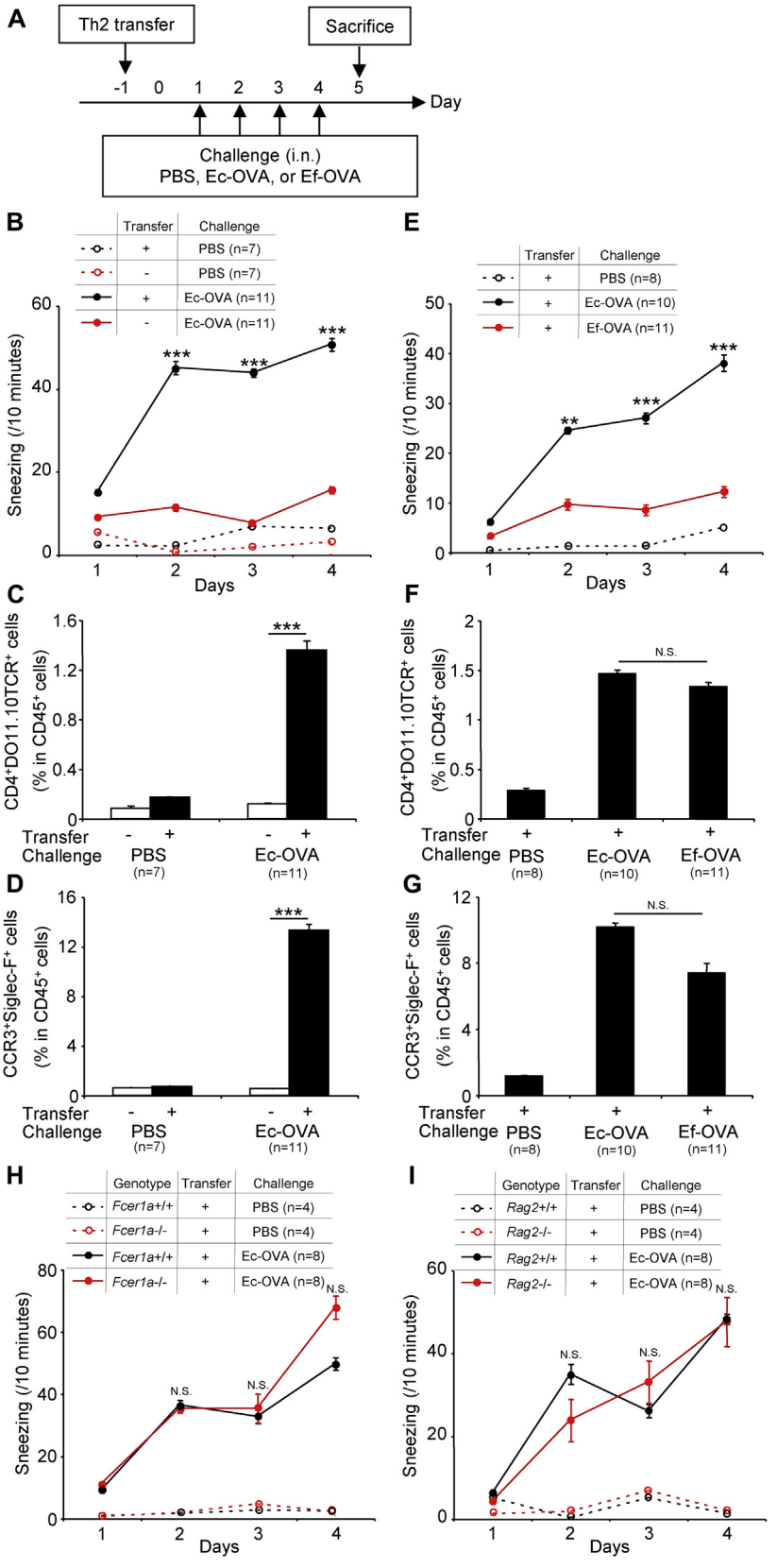
FIG 1. Ec-OVA induces FcεRI-independent sneezing. **A,** Experimental schema. *Fcer1a*$^{+/+}$ or *Fcer1a*$^{-/-}$ mice were i.p. immunized with OVA. **B,** Mice were then i.n. challenged with PBS or Ef-OVA. **C,** OVA-sensitized mice were i.n. challenged with PBS or Ec-OVA. **B** and **C,** Number of sneezes was counted for 10 minutes immediately after each i.n. challenge. Pooled data from 2 independent experiments are shown (mean, SEM, n = 5 [PBS groups], n = 8 [Ef-OVA groups], n = 7 [Ec-OVA groups]). *N.S.*, Not significant. $^{**}P < .01$; $^{***}P < .001$.

100-207 EU/mg) for 4 consecutive days (days 1-4) (Fig 1, *A*). Ef-OVA–challenged WT mice showed increased sneezing from the second day, whereas *Fcer1a*$^{-/-}$ mice had levels of sneezing similar to those of PBS-challenged mice, indicating that the response was IgE-dependent (Fig 1, *B*). However, when mice were challenged with Ec-OVA, both WT and *Fcer1a*$^{-/-}$ mice showed similar increases in sneezing (Fig 1, *C*). Thus, pure antigens elicit rhinitis only via an IgE-dependent mechanism, whereas endotoxin-containing antigens can elicit non–IgE-mediated rhinitis in mice.

CD4$^+$ T-cell activation is required for inducing Ec-OVA–elicited rhinitis

To investigate the requirement of antigen-specific CD4$^+$ T cells for the induction of Ec-OVA–elicited rhinitis, we used an OVA-specific T_H2 cell adoptive transfer model. DO11.10$^+$ mice-derived naive CD4$^+$ T cells, which express an $OVA_{323\text{-}339}$-specific T-cell receptor, were *in vitro* differentiated into T_H2 cells (OVA-T_H2). The cells were adoptively transferred into naive recipient mice (day −1) followed by intranasal challenge with Ef-OVA or Ec-OVA from 2 days after the transfer (days 1-4) (Fig 2, *A*). Ec-OVA challenge elicited sneezing responses in OVA-T_H2–transferred, but not nontransferred (Fig 2, *B*) or naive OVA-specific CD4$^+$ T-cell–transferred mice (see Fig E1 in this article's Online Repository at www.jacionline.org). Ec-OVA challenge also elicited sneezing in mice that received OVA-T_H2 cells and that were left for 6 weeks to generate memory OVA-specific T_H2 cells (see Fig E2 in this article's Online Repository at www.jacionline.org). OVA-T_H2 cells accumulated in the nose (Fig 2, *C*; see Fig E3, *B*, in this article's Online Repository at www.jacionline.org) concomitantly with nasal eosinophilia, a marker of local T_H2 activation[9,37] (Fig 2, *D*, and Fig E3, *C*) in OVA-T_H2–transferred and Ec-OVA–challenged mice. Ef-OVA challenge failed to elicit sneezing in OVA-T_H2–transferred mice (Fig 2, *E*), but induced nasal OVA-T_H2 accumulation comparable to that with Ec-OVA challenge (Fig 2, *F*). Likewise, nasal eosinophilia was comparably induced in Ef-OVA– and Ec-OVA–challenged mice (Fig 2, *G*). These results demonstrated that the development of antigen-specific T_H2 cells and their local activation were essential for inducing Ec-OVA–elicited rhinitis, but the antigen alone was insufficient to trigger sneezing. Group 2 innate lymphoid cells (ILC2s) are implicated in several allergic diseases as an innate type 2 cytokine producer.[38] However, the frequency of ILC2s among the noses from PBS-, Ec-OVA–, and Ef-OVA–challenged mice was not significantly different (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting that ILC2s were not activated in this model.

To investigate whether Ec-OVA–elicited rhinitis in OVA-T_H2–transferred mice was IgE-independent, as for the OVA immunization model (Fig 1), we transferred OVA-T_H2 cells into *Fcer1a*$^{-/-}$ and T- and B-cell–deficient *Rag2*$^{-/-}$ mice. Both *Fcer1a*$^{-/-}$ (Fig 2, *H*) and *Rag2*$^{-/-}$ mice (Fig 2, *I*) had a comparable frequency of sneezing to that of WT mice in response to Ec-OVA. Therefore, neither IgE nor other antibody-mediated signals were involved in Ec-OVA–elicited rhinitis.


A
Th2 transfer
Sacrifice
-1 0 1 2 3 4 5
Day
Challenge (i.n.)
PBS, Ec-OVA, or Ef-OVA
B
Transfer Challenge
+ PBS (n=7)
- PBS (n=7)
+ Ec-OVA (n=11)
- Ec-OVA (n=11)
Sneezing (/10 minutes)
Days
E
Transfer Challenge
+ PBS (n=8)
+ Ec-OVA (n=10)
+ Ef-OVA (n=11)
Sneezing (/10 minutes)
Days
C
CD4+DO11.10TCR+ cells (% in CD45+ cells)
Transfer
Challenge
PBS (n=7)
Ec-OVA (n=11)
F
CD4+DO11.10TCR+ cells (% in CD45+ cells)
N.S.
PBS (n=8)
Ec-OVA (n=10)
Ef-OVA (n=11)
D
CCR3+Siglec-F+ cells (% in CD45+ cells)
G
CCR3+Siglec-F+ cells (% in CD45+ cells)
H
Genotype Transfer Challenge
Fcer1a+/+ + PBS (n=4)
Fcer1a-/- + PBS (n=4)
Fcer1a+/+ + Ec-OVA (n=8)
Fcer1a-/- + Ec-OVA (n=8)
Sneezing (/10 minutes)
Days
I
Genotype Transfer Challenge
Rag2+/+ + PBS (n=4)
Rag2-/- + PBS (n=4)
Rag2+/+ + Ec-OVA (n=8)
Rag2-/- + Ec-OVA (n=8)
Sneezing (/10 minutes)
Days

Local CD4$^+$ T-cell activation followed by LPS stimulation triggers rhinitis

Next, we investigated whether endotoxin-mediated sneezing responses are induced through the conventional TLR4 signaling pathway. Ec-OVA challenge failed to elicit sneezing in OVA-T_H2–transferred *Tlr4*$^{-/-}$ (Fig 3, *A*) and *Myd88*$^{-/-}$ mice (Fig 3, *B*). Nevertheless, nasal OVA-T_H2 accumulation in *Tlr4*$^{-/-}$ (Fig 3, *C*) and *Myd88*$^{-/-}$ (Fig 3, *D*) mice was comparable to that in WT mice. In addition, *Tlr4*$^{-/-}$ (Fig 3, *E*) and *Myd88*$^{-/-}$ (Fig 3, *F*) mice showed substantial, although partially reduced, nasal eosinophilia in response to Ec-OVA challenge. These results indicated that although TLR4-MyD88 signaling contributed to local T_H2 activation, antigen stimulation alone could induce substantial nasal accumulation and activation of T_H2 cells. In addition to activating T_H2 cells, activation of the TLR4-MyD88 pathway in cells of recipient mice was critical for triggering sneezing responses.

Because TLR4-MyD88 signaling played a critical role in eliciting sneezing in our model, we investigated whether LPS could directly trigger sneezing. OVA-T_H2–transferred mice were challenged with Ec-OVA for 4 days, or Ef-OVA for 2 days (days 1 and 2), followed by LPS (50 ng/mL) challenge on days 3 and 4 (Fig 4, *A*). As shown in Fig 4, *B*, Ef-OVA followed by LPS challenge elicited sneezing comparable to that with Ec-OVA challenge on day 3. However, at day 4, although Ec-OVA–challenged mice maintained high sneezing levels, LPS challenge alone failed to elicit sneezing (Fig 4, *B*). Mice challenged with Ef-OVA (days 1-2) and left on day 3 also did not respond to LPS challenge at day 4 (see Fig E5 in this article's Online Repository at www.jacionline.org), suggesting that the time after nasal CD4$^+$ T-cell activation was important to elicit sneezing by LPS challenge. To determine the LPS concentration required to elicit sneezing, Ef-OVA–primed mice were challenged with several doses of LPS (Fig 4, *A*, Ef-OVA → LPS). Doses of 0.5 and 5 ng/mL LPS had some effect, while 50 ng/mL LPS elicited substantial levels of sneezing (Fig 4, *C*).

Taken together, these observations clearly demonstrate that LPS directly triggers sneezing responses, but antigen-dependent local activation of CD4$^+$ T cells is a prerequisite and is also essential for maintaining the response.

Mast cell/basophil-independent histamine mediates Ec-OVA–elicited rhinitis

Histamine release is central to sneezing responses in AR,[8] and antihistamines are also effective in patients with non–IgE-mediated rhinitis.[11] Thus, to investigate whether histamine mediates Ec-OVA–elicited rhinitis, mice were i.p. injected with antihistamines, diphenhydramine or fexofenadine, 90 minutes before Ec-OVA challenge. Diphenhydramine (Fig 5; see Fig E6, *A*, in this article's Online Repository at www.jacionline.org) and fexofenadine (Fig E6, *B*) completely suppressed Ec-OVA–elicited sneezing, demonstrating that histamine release mediated the Ec-OVA–elicited rhinitis similar to that in classical IgE-mediated type 1 hypersensitivity reactions.

Mast cells and basophils are major sources of histamine in classical allergic responses,[2,8] and their degranulation can also be induced by non–IgE-mediated mechanisms.[4] Therefore, we investigated the involvement of mast cells and basophils in Ec-OVA–elicited rhinitis. Mas-TRECK mice, a transgenic mouse strain expressing the human DT receptor gene under the control of a mouse mast cell–specific *Il4* intronic enhancer,[39] were i.p. injected with DT (Fig 6, *A*). As previously demonstrated in the peritoneal cavity or skin,[39] both mast cells and basophils were completely depleted from the noses of DT-injected Mas-TRECK mice, as assessed by nasal mRNA levels of *Mcpt1*, *Mcpt2* (markers for mucosal mast cells), and *Mcpt8* (a marker for basophils) (see Fig E7, *A*, in this article's Online Repository at www.jacionline.org). Although IgE-mediated sneezing responses (induced by OVA sensitization followed by Ef-OVA challenge) were completely abrogated in DT-treated Mas-TRECK mice (Fig E7, *B* and *C*), OVA-T_H2–transferred and DT-injected Mas-TRECK mice showed increased sneezing in response to Ec-OVA challenge comparable to that of OVA-T_H2–transferred and DT-injected WT mice (Fig 6, *B*). In addition, mast cell– and basophil-depleted noses still had substantial histidine decarboxylase mRNA expression, an enzyme that catalyzes the reaction for histamine synthesis (Fig E7, *A*). Therefore, although histamine was required for Ec-OVA–elicited rhinitis, the cellular source(s) was not mast cells or basophils.

Monocytes/macrophages are involved in Ec-OVA–elicited rhinitis

We next investigated whether other cell types might mediate Ec-OVA–elicited rhinitis. Monocytes/macrophages were depleted from OVA-T_H2–transferred and Ec-OVA–challenged mice by intraperitoneal injection of clodronate-containing liposomes on day 3, 6 hours after the third OVA challenge (Fig 7, *A*). Clodronate treatment depleted Ly-6C^{high}F4/80^{int}CD11b^{high} monocytes and F4/80^{high}CD11b^{int} macrophages without affecting CD11c$^+$MHC-II^{high} dendritic cells and Ly-6G$^+$CD11b$^+$ neutrophils as demonstrated in mouse spleens (see Fig E8 in this article's Online Repository at www.jacionline.org). As shown in Fig 7, *B*, clodronate treatment completely abrogated Ec-OVA–induced sneezing responses at day 4 in the OVA-T_H2 transfer model. Clodronate treatment did not affect OVA-challenge–induced T_H2 and eosinophil recruitment into the nose (see Fig E9 in this article's Online Repository at www.jacionline.org), indicating the minimal effect of the treatment on nasal T_H2 activation. To

FIG 2. Nasal T-cell activation is required for Ec-OVA-induced sneezing. **A,** Experimental schema. **B-D,** WT mice were adoptively transferred with or without DO11.10-derived T_H2 cells. Mice were then i.n. challenged with PBS or Ec-OVA. **E-G,** T_H2-transferred mice were i.n. challenged with PBS, Ec-OVA, or Ef-OVA. **B** and **E,** Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. **C, D, F,** and **G,** Frequency of OVA-T_H2 cells (CD4$^+$DO11.10TCR$^+$ cells in CD45$^+$ cells) (**C** and **F**) and eosinophils (CCR3$^+$Siglec-F$^+$ cells in CD45$^+$ cells) (**D** and **G**) in nasal mucosa 24 hours after the final challenge was examined by FACS. **H** and **I,** *Fcer1a*$^{+/+}$ or *Fcer1a*$^{-/-}$ (Fig 2, *H*), or *Rag2*$^{+/+}$ or *Rag2*$^{-/-}$ mice (Fig 2, *I*) were adoptively transferred with DO11.10-derived T_H2 cells and i.n. challenged with PBS or Ec-OVA. Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. Pooled data from 3 (Fig 2, *B-G*) or 2 (Fig 2, *H* and *I*) independent experiments are shown (mean, SEM, n = indicated in figure). *N.S.*, Not significant. $^{**}P < .01$; $^{***}P < .001$.

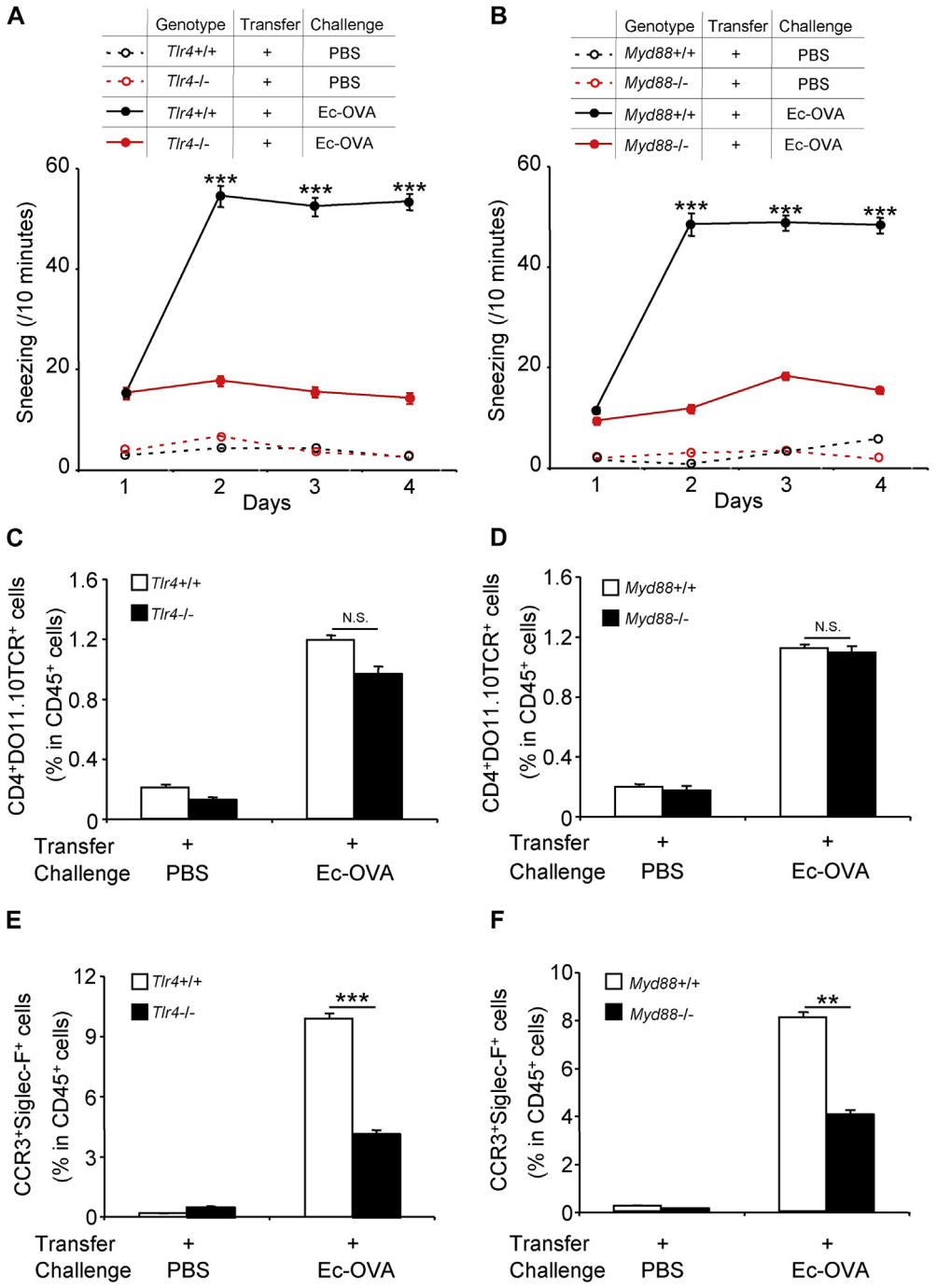


FIG 3. TLR4-MyD88–signaling pathway is essential for Ec-OVA–induced sneezing. $Tlr4^{+/+}$ or $Tlr4^{-/-}$ (**A**, **C**, and **E**), or $Myd88^{+/+}$ or $Myd88^{-/-}$ (**B**, **D**, and **F**), mice were adoptively transferred with DO11.10-derived T_H2 cells. Mice were then i.n. challenged with PBS or Ec-OVA. **A** and **B,** Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. **C-F,** Frequency of OVA-T_H2 cells ($CD4^+DO11.10TCR^+$ cells in $CD45^+$ cells) (Fig 3, *C* and *D*) and eosinophils ($CCR3^+Siglec\text{-}F^+$ cells in $CD45^+$ cells) (Fig 3, *E* and *F*) in nasal mucosa 24 hours after the final challenge was examined by FACS. Pooled data from 3 independent experiments are shown (mean, SEM, n = 5 [PBS groups], n = 12-13 (OVA groups in **A**, **C**, and **E**), n = 13-14 [OVA groups in *B, D, F*]). *N.S.*, Not significant. $^{**}P < .01$; $^{***}P < .001$.

investigate the specificity of clodronate treatment on sneezing responses, we investigated the effect of clodronate in the OVA immunization model (Fig 7, *C*). Clodronate treatment completely abrogated sneezing responses in OVA-immunized and Ec-OVA–challenged $Fcer1a^{-/-}$ mice at day 4 in which IgE signaling did not mediate the sneezing (Fig 7, *D*). Clodronate partially, but significantly, reduced the sneezing responses in Ec-OVA–challenged WT mice in which both IgE and endotoxin mediated the sneezing (Fig 7, *E*), although it did not affect the sneezing responses in Ef-OVA–challenged WT mice in which only IgE

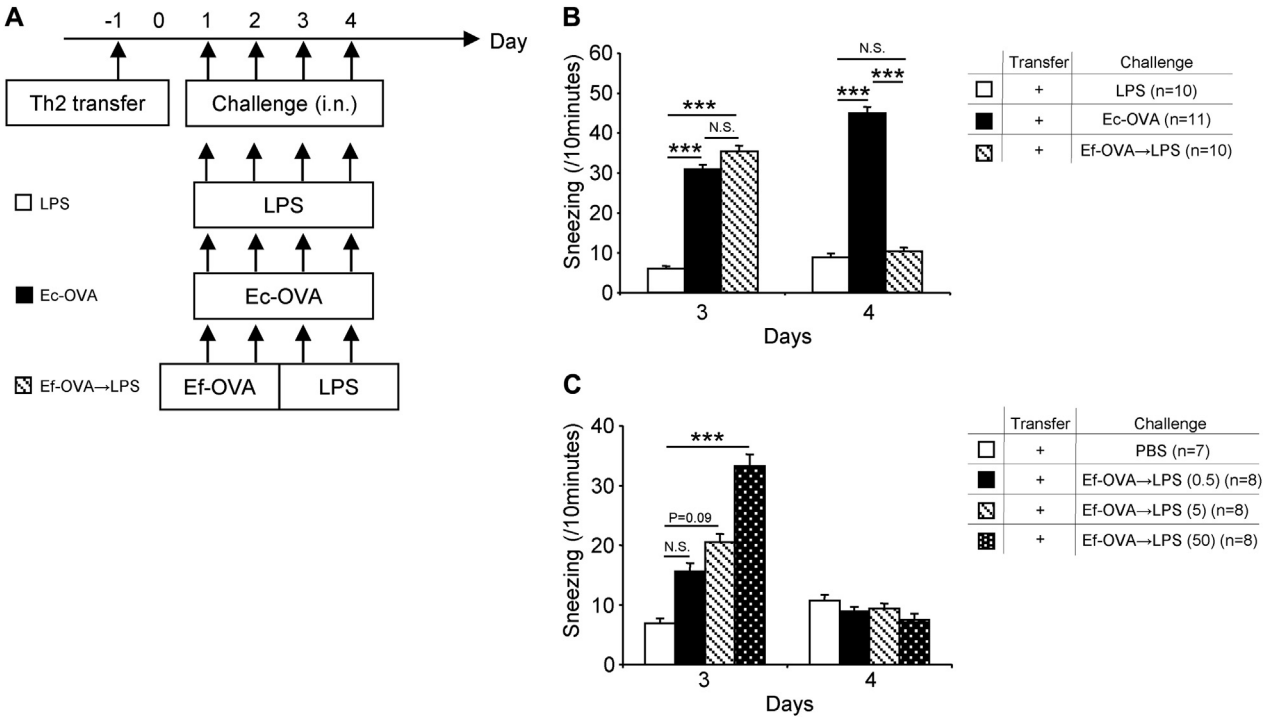


FIG 4. LPS triggers sneezing in T-cell–activated noses. **A,** Experimental schema. **B,** WT mice were adoptively transferred with DO11.10-derived T_H2. Mice were then i.n. challenged with LPS (days 1-4), Ec-OVA (days 1-4), or Ef-OVA (days 1-2) followed by LPS (days 3-4). **C,** Mice i.n. challenged with Ef-OVA (days 1-2) were then i.n. challenged with variable concentrations of LPS (days 3-4). **B** and **C,** Number of sneezes at days 3 and 4 was counted for 10 minutes immediately after i.n. challenge. Pooled data from 3 (Fig 4, *B*) or 2 (Fig 4, *C*) independent experiments are shown (mean, SEM, n = indicated in figure). *N.S.*, Not significant. ***$P < .001$.

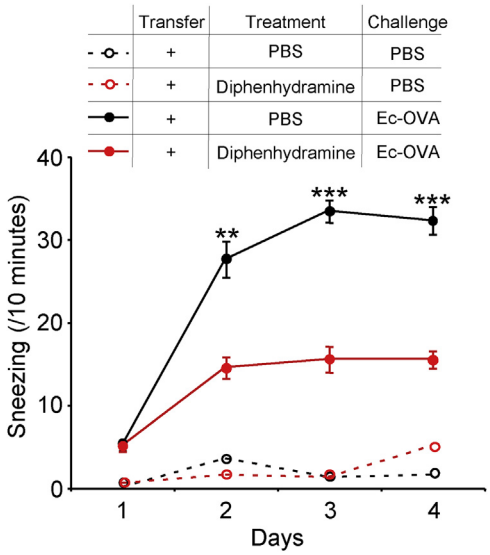


FIG 5. Histamine mediates Ec-OVA–induced sneezing. WT mice adoptively transferred with DO11.10-derived T_H2 cells were i.n. challenged with PBS or Ec-OVA in the presence or absence of diphenhydramine treatment (1 mg/dose), 90 minutes before each intranasal challenge. Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. Pooled data from 2 independent experiments are shown (mean, SEM, n = 4 [PBS groups], n = 8 [OVA groups]). **$P < .01$; ***$P < .001$.

mediated the sneezing (Fig 7, *F*). These results demonstrated that clodronate treatment abrogated endotoxin-mediated, but not IgE-mediated, sneezing responses. Therefore, monocytes and macrophages are specifically involved in endotoxin-elicited rhinitis.

DISCUSSION

In this study, we report a previously unknown nasal type-1-hypersensitivity-like reaction in mice. Nasal activation of antigen-specific $CD4^+$ T cells followed by LPS challenge induced rhinitis symptoms. The sneezing response was mediated by histamine, but the cellular source(s) was not mast cells or basophils. This is the first report to demonstrate that LPS elicits histamine-mediated hypersensitivity-like reactions in the nose.

Although allergen-mediated IgE-cross-linking is central to AR pathogenesis, it may not be the sole trigger of AR. Nasal provocation tests with an allergen plus LPS enhanced nasal responses and increased total nasal resistance and clinical scores in children with AR compared with allergen alone.[40] In addition, high-dose nasal LPS challenge (~500 μg/mL) induced mild nasal symptoms such as itching or nasal secretion, even in nonallergic individuals.[41,42] In this study, we showed that even low-dose LPS (50 ng/mL) directly triggered sneezing responses in nasally T_H2-activated mice. Thus, the previously demonstrated LPS-mediated augmentation of nasal responses in children with AR[40] might be caused by direct LPS-triggered responses in addition to classical antigen-IgE–mediated responses. Because LPS concentration that elicited sneezing in this study was within the range of endotoxin levels found in extracts from household dust,[25-27] endotoxin-elicited reactions might mediate rhinitis symptoms in

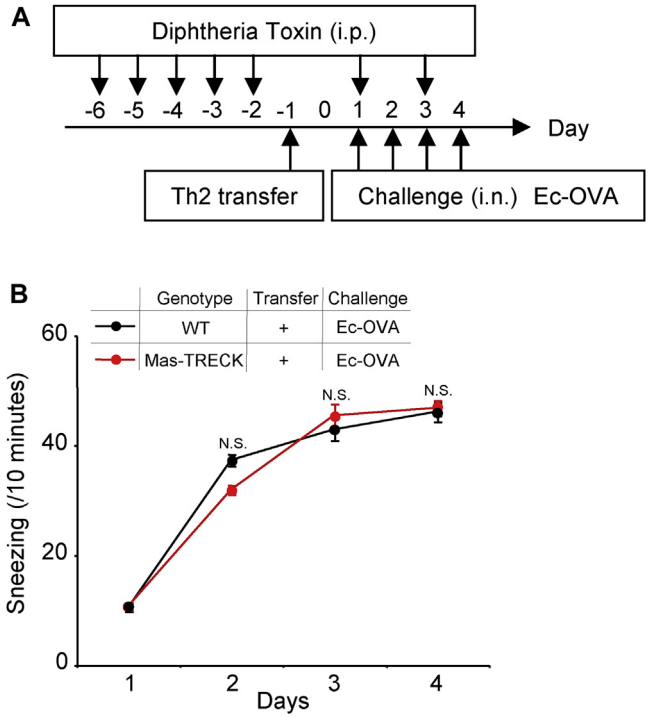


FIG 6. Mast cells/basophils are dispensable for Ec-OVA–induced sneezing. **A,** Experimental schema. WT or Mas-TRECK mice were adoptively transferred with DO11.10-derived T_H2 cells, i.p. injected with DT, and i.n. challenged with Ec-OVA. **B,** Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. Pooled data from 2 independent experiments are shown (mean, SEM, n = 10 [WT], n = 11 [Mas-TRECK]). *N.S.*, Not significant.

actual disease settings of AR. In addition, LPS or microbial products are also found in ambient air particles that are considered important exacerbation factors for respiratory allergy.[28] Although treatment with an anti-IgE mAb, omalizumab, significantly improved AR symptoms, there were a substantial number of patients with AR who did not respond to therapy.[7] Omalizumab-unresponsive rhinitis symptoms might be mediated by allergens contaminated with endotoxin or environmental pollutants.

In addition to classical AR, endotoxin-elicited rhinitis might mediate some disease settings of NAR. NAR is diagnosed by the presence of persistent rhinitis symptoms with negative antigen-specific IgE and/or skin prick test result.[11] Patients with NAR may include patients misdiagnosed with local AR whose symptoms are indeed mediated by locally (nasally) detectable allergen-specific IgE.[10] Some patients with authentic NAR, who really do not have allergen-specific IgE, even show nasal infiltration of inflammatory cells.[12-15] This NAR with inflammation can be further classified into differential subtypes, such as NAR with eosinophilia syndrome or NAR with neutrophils, on the basis of infiltrating cell types.[12-15] Among them, our mouse model best resembled NAR with eosinophilia syndrome: nasal eosinophilic inflammation with IgE-independent rhinitis symptoms. Furthermore, rhinitis symptoms of NAR can often be controlled by antihistamines,[11] which were also effective in our mouse model. Whether T cells are activated in the noses of patients with NAR with inflammation is currently unknown. However, because the classification of "nonallergic" is based on the absence of antigen-specific IgE and/or a negative skin prick test result, it might be useful to examine the presence of activated T cells in the noses of patients with NAR with inflammation, especially patients with NAR with eosinophilia syndrome. Another potential consideration is ILC2-mediated inflammation. Even though we did not find potential involvement of ILC2s in our model, nasal inflammation of some patients with NAR might be induced by ILC2, but not T-cell, activation. And the presence of local inflammation might be sufficient to induce endotoxin-elicited rhinitis.

Whether the endotoxin-elicited reaction demonstrated here could be induced in organs other than nose is currently unclear. Some patients with food allergy, mainly infants or young children, are diagnosed with non–IgE-mediated gastrointestinal food allergies.[3] The patients manifest food allergy symptoms, vomiting and diarrhea after ingestion of offending foods, yet show negative skin prick test results and serum specific IgE against the food antigens.[3] Although T cells are assumed to play a role in the disease, its pathogenesis is poorly understood.[3] Therefore, it would be of interest to investigate the involvement of endotoxin-elicited reactions in several non–IgE-mediated allergic disorders including non–IgE-mediated gastrointestinal food allergies.

Mast cells and basophils are the major sources of histamine in allergic reactions.[43] However, the present study showed that they were not involved in Ec-OVA–elicited rhinitis because DT-treated Mas-TRECK mice had a frequency of sneezing comparable to that of DT-treated WT mice. Other than these cell types, several hematopoietic cell types (macrophages,[17] neutrophils,[18] lymphocytes,[19] and platelets[20]), as well as nonhematopoietic cells (keratinocytes[21] and fibroblasts[22]), can produce histamine. LPS stimulation upregulates histamine production from macrophages,[17] neutrophils,[18] and keratinocytes.[21] In this study, we showed that the LPS-TLR4 pathway triggered the sneezing response, and clodronate treatment specifically abrogated endotoxin-elicited, but not IgE-mediated, rhinitis. Although we do not clearly exclude the potential off-target effects of clodronate, we believe that depletion of monocytes/macrophages mediates amelioration of Ec-OVA–elicited rhinitis by clodronate. Clodronate might affect dendritic cells, neutrophils, and lymphocytes either directly or indirectly. However, dendritic cells and neutrophils were not depleted, and local T_H2 activation was not compromised by clodronate treatment in our model. These results imply that monocytes/macrophages might be a histamine source in our model, although we did not show a direct link between the monocytes/macrophages and nasal histamine release. If these cells do produce histamine, how they release histamine immediately after endotoxin stimulation should be investigated, because macrophages were reported to release histamine gradually.[17,44] Because macrophages have specialized roles and tissue- and disease-specific characteristics,[45,46] nasal and/or T_H2-activated microenvironments might harbor specific macrophage populations that release histamine immediately in response to endotoxin. Further study is required to clarify how monocytes/macrophages contribute to histamine-mediated nasal immediate-hypersensitivity-like reactions.

Another question suggested by this study is how activated T_H2 cells contribute to the induction of Ec-OVA–elicited rhinitis. We showed that LPS directly triggered sneezing in T_H2-activated noses. However, LPS challenge 48 hours after the antigen exposure failed to elicit sneezing responses regardless of the presence of LPS challenge 24 hours after the antigen exposure.

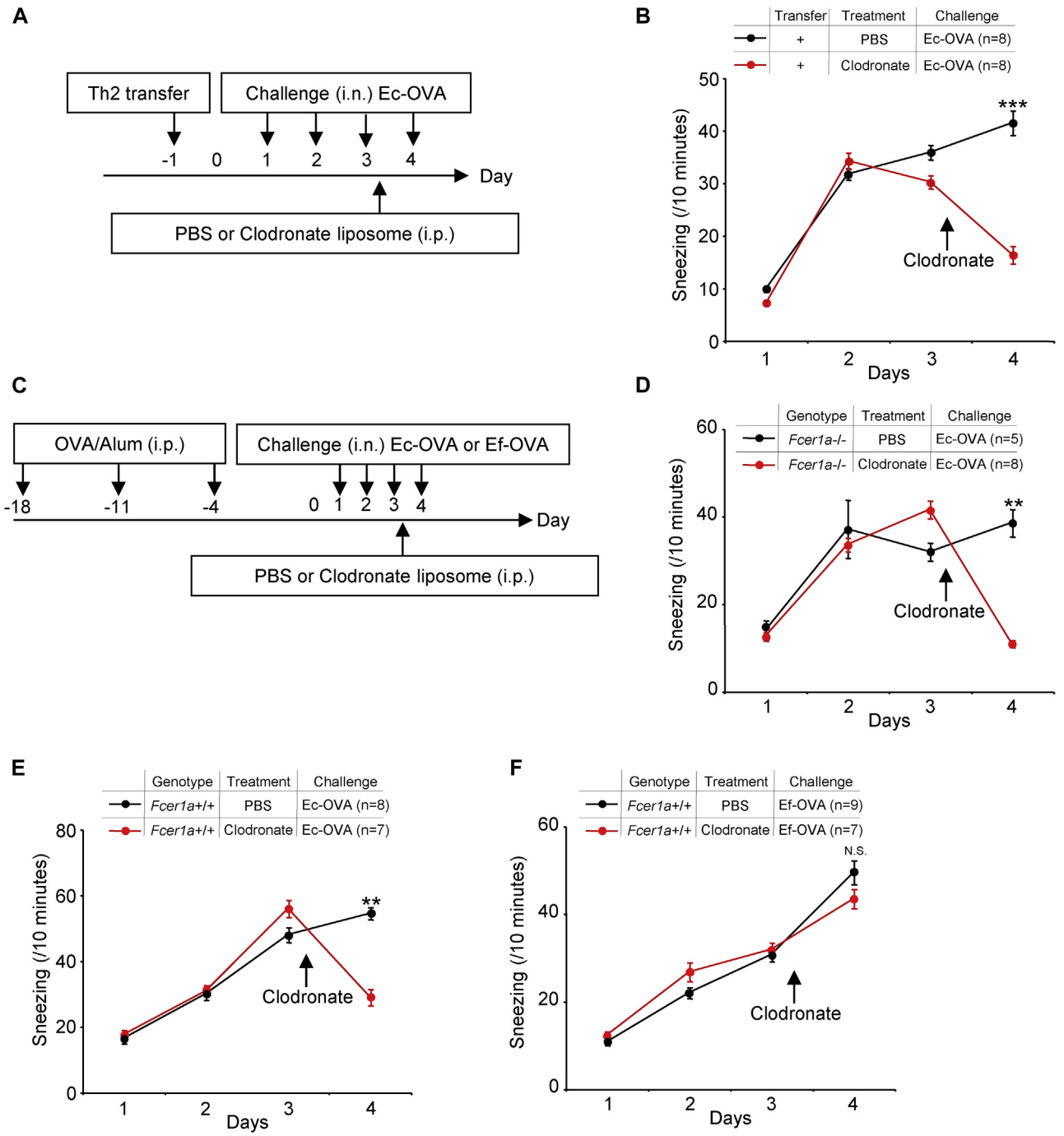


FIG 7. Monocytes/macrophages are essential for Ec-OVA–induced sneezing. **A,** Experimental schema. WT mice adoptively transferred with DO11.10-derived T_H2 cells were i.n. challenged with Ec-OVA. On day 3, 6 hours after the third nasal challenge, mice were i.p. injected with PBS or clodronate-containing liposome. **B,** Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. **C,** Experimental schema. *Fcer1a*$^{-/-}$ (**D**) or WT (**E** and **F**) mice were i.p. immunized with OVA, then i.n. challenged with Ec-OVA (Fig 7, *D* and *E*) or Ef-OVA (Fig 7, *F*). On day 3, 6 hours after the third nasal challenge, mice were i.p. injected with PBS or clodronate-containing liposome. **D-F,** Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. Pooled data from 2 independent experiments are shown (mean, SEM, n = indicated in figure). *N.S.*, Not significant. $^{**}P < .01$; $^{***}P < .001$.

These results suggested that loss of antigen-mediated T-cell activation (eg, by loss of the antigen on dendritic cells in the nose), rather than LPS tolerance, caused the LPS unresponsiveness. Therefore, local T_H2 activation is a prerequisite for inducing endotoxin-elicited rhinitis, and T cells might need to be activated at the time of, or until shortly before, endotoxin exposure. Activated T_H2 cells may activate and/or increase histamine content in nasal cells, such as macrophages, by producing

cytokines. Indeed, IL-4 induces tissue macrophage proliferation during T_H2 inflammation,[47] and granulocyte/macrophage-colony stimulating factor increases histidine decarboxylase activity in macrophages.[17] Such cytokines and endotoxin may cooperatively activate nasal histamine production. Further study is required to uncover the molecular mechanisms of T-cell–mediated preparation of the nasal environment that allow it to respond to endotoxin by evoking rhinitis.

We report a novel T-cell– and histamine-mediated type-1-hypersensitivity-like reaction in mice that might participate in classical and mechanism-unknown non–IgE-mediated allergic disorders. Further studies to increase our understanding of the mechanisms involved and broadening the reaction to different organs might indicate novel therapeutic targets as well as aid the development of better diagnostic and classification methods for less well understood allergic(-like) disorders.

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Key messages

- **Nasal exposure of endotoxin-containing allergens induces an IgE-independent, yet T-cell– and histamine-dependent, nasal hypersensitivity-like reaction in mice.**
- **Local activation of antigen-specific $CD4^+$ T cells followed by nasal endotoxin exposure triggers sneezing responses in mice.**
- **Monocytes/macrophages, but not mast cells/basophils, are involved in sneezing responses elicited by endotoxin.**

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METHODS

Reagents

Recombinant human IL-2, recombinant mouse IL-4, and fluorescein isothiocyanate (FITC)-antimouse-CCR3 mAbs (83101) were purchased from R&D Systems (Minneapolis, Minn). FITC-anti-mouse-B220 mAb (RA3-6B2), FITC-anti-mouse-CD11b mAb (M1/70), FITC-anti-mouse-CD11c mAb (HL3), FITC-anti-mouse-CD49b mAb (DX5), phycoerythrin-anti-mouse-CD90.2 mAb (53-2.1), and phycoerythrin-anti-mouse-Siglec-F mAb (E50-2440) were purchased from BD Biosciences (San Diego, Calif). FITC-anti-mouse-CD3 mAb (B3B4), FITC-anti-mouse-CD62L mAb (MEL-14), Allophycocyanin (APC)-anti-mouse-DO11.10TCR mAb (KJ1-26), PerCP-Cy5.5-anti-mouse-CD4 mAb (RM4-5), and PacificBlue-anti-mouse-CD45 mAb (30-F11) were purchased from eBiosciences (San Diego, Calif). FITC-anti-mouse-IgE mAb was purchased from SouthernBiotech (Birmingham, Ala). Biotin-anti-mouse-ST2 mAb (DJ8) was purchased from MD Bioproducts (St Paul, Minn). Anti-mouse-IL-12 mAb (C17.8) and anti-mouse-IFN-γ mAb (R4-6A2) were prepared in our laboratory. Antidinitrophenyl IgE (SPE-7), OVA (grade V), LPS from *Salmonella minnesota* 055:B5, aluminum hydroxide hydrate (alum), Triton X-114, and DT from *Corynebacterium diphtheriae* were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Diphenhydramine hydrochloride was purchased from Wako Pure Chemicals (Osaka, Japan). Fexofenadine hydrochloride was purchased from Tokyo Chemical Industry (Tokyo, Japan). $OVA_{323-339}$ peptide was purchased from AnaSpec (Fremont, Calif). Clodronate-containing liposome was purchased from HygieiaBioscience (Osaka, Japan).

Cell preparation for adoptive transfer

For DO11.10$^+$ T_H2 transfer, spleens were dissected from naive DO11.10$^+$ mice and single-cell suspensions were prepared by sieving and gentle pipetting. Naive CD4$^+$ T cells (CD4$^+$CD62L$^+$) were isolated by AutoMACS Separator using the CD4$^+$CD62L$^+$ T-cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). DO11.10$^+$CD4$^+$CD62L$^+$ T cells were cultured in 6-well plates at 3×10^5 cells/3 mL/well with IL-2 (100 pM), IL-4 (20 ng/mL), anti-IL12 mAbs (20 μg/mL), anti–IFN-γ mAb (20 μg/mL), and OVA peptide (323-339) (1 μM) in the presence of 3×10^6 irradiated conventional antigen presenting cells (BALB/c splenocytes) in complete medium—RPMI 1640 supplemented with 10% FBS, 2-mercaptoethanol (50 mM), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 mg/mL). Three days later, cells were subdivided into double the number of wells and cultured with IL-2 (100 pM) in fresh complete medium for another 3 to 5 days. For naive CD4$^+$ T-cell transfer, spleen, cervical lymph nodes, and mesenteric lymph nodes were dissected from naive DO11.10$^+$ mice and single-cell suspensions were prepared. CD4$^+$ T cells were isolated by AutoMACS Separator using antimouse-CD4 MicroBeads (L3T4) (Miltenyi Biotec). Collected CD4$^+$ T cells were incubated with anti-CD4 mAb and anti-CD62L mAb on ice for 30 minutes. Cells were washed twice with PBS, and CD4$^+$CD62L^{high} cells were sorted by FACS Aria III (BD Biosciences). Collected cells were washed with PBS, and used for adoptive transfer.

Mouse sneezing analysis

Awake mice were i.n. administered 20 μL of challenge reagents. Immediately after the challenge, each mouse was put in a new cage, a standard breeding cage but without bedding (1 mouse per cage). An investigator directly monitored the mouse behavior for 10 minutes, and each sneezing behavior accompanied with a sneezing sound (see Video E1) was counted. Usually, 2 mice in the different cages were examined at the same time. Randomization and blinding were not used in the experiments.

FACS analysis

Noses were minced with scissors and digested for 50 minutes at 37°C with collagenase (150 U/mL) and DNase I (10 μg/mL). After filtration with a cell strainer, red blood cells were lysed from the cell suspension. For staining IgE receptor, cells were incubated with anti–DNP-IgE (1 μg/mL) on ice for 30 minutes. Cells were incubated with antibodies against CCR3, CD4, CD45, CD90.2, DO11.10TCR, Siglec-F, ST2, and Lineage markers (CD3, B220, CD11b, CD11c, DX-5, and IgE) on ice for 30 minutes, and then washed twice with PBS. Stained cells were analyzed by FACSCant II flow cytometer (BD Biosciences) and FlowJo software (version 7.6.1, Tree Star Inc, Ashland, Ore). Singlet CD45$^+$ cells were gated as nasal hematopoietic cells (Fig E3, *A*). CD4$^+$DO11.10TCR$^+$ cells (Fig E3, *B*), CD45$^+$CCR3^{high}Siglec-F^{high} cells (Fig E3, *C*), and Lineage$^-$ST2$^+$CD90.2$^+$ cells (Fig E4, *A*) were defined as eosinophils, OVA-specific DO11.10$^+$ T_H2 cells, and ILC2s, respectively. The data were shown as frequency (% in nasal CD45$^+$ cells).

Quantitative PCR analysis of nasal mucosa

mRNA expression levels in nasal mucosa were examined as previously described.[E1,E2] Briefly, total RNAs from nasal cells were isolated using Sepasol (Nakarai, Kyoto, Japan). cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan). Gene expression levels were quantified using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif) and the Thermal cycler dice RT-PCR system (Takara Bio Inc, Otsu, Japan) according to the manufacturer's instructions. The results are shown as the relative expression standardized to eukaryotic 18S rRNA levels. The specific primers and probes used for quantitative RT-PCR were TaqMan probes for *Mcpt1*, *Mcpt2*, *Mcpt8*, *Hdc*, and 18S rRNA (Applied Biosystems).

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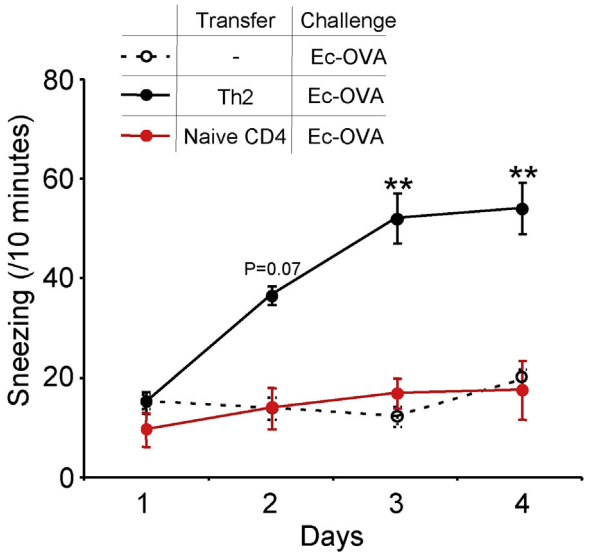

FIG E1. Naive OVA-specific T cells do not mediate Ec-OVA–induced sneezing. WT mice were adoptively transferred with DO11.10-derived naive $CD4^+$ or T_H2 cells, and then i.n. challenged with Ec-OVA. Number of sneezes was counted for 10 minutes immediately after each intranasal challenge (mean, SEM, n = 3). $^{**}P < .01$.

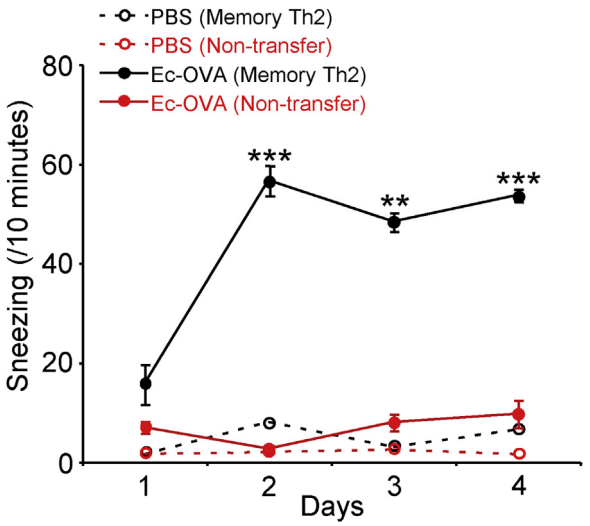

FIG E2. Memory OVA-specific T_H2 cells mediate Ec-OVA–induced sneezing. WT mice were adoptively transferred with or without DO11.10-derived T_H2 cells, and left untreated for 6 weeks. After the generation of memory T_H2 cells, mice were i.n. challenged with PBS or Ec-OVA. Number of sneezes was counted for 10 minutes immediately after each intranasal challenge (mean, SEM, n = 2 [PBS groups], n = 3 [OVA groups]). $^{**}P < .01$; $^{***}P < .001$.

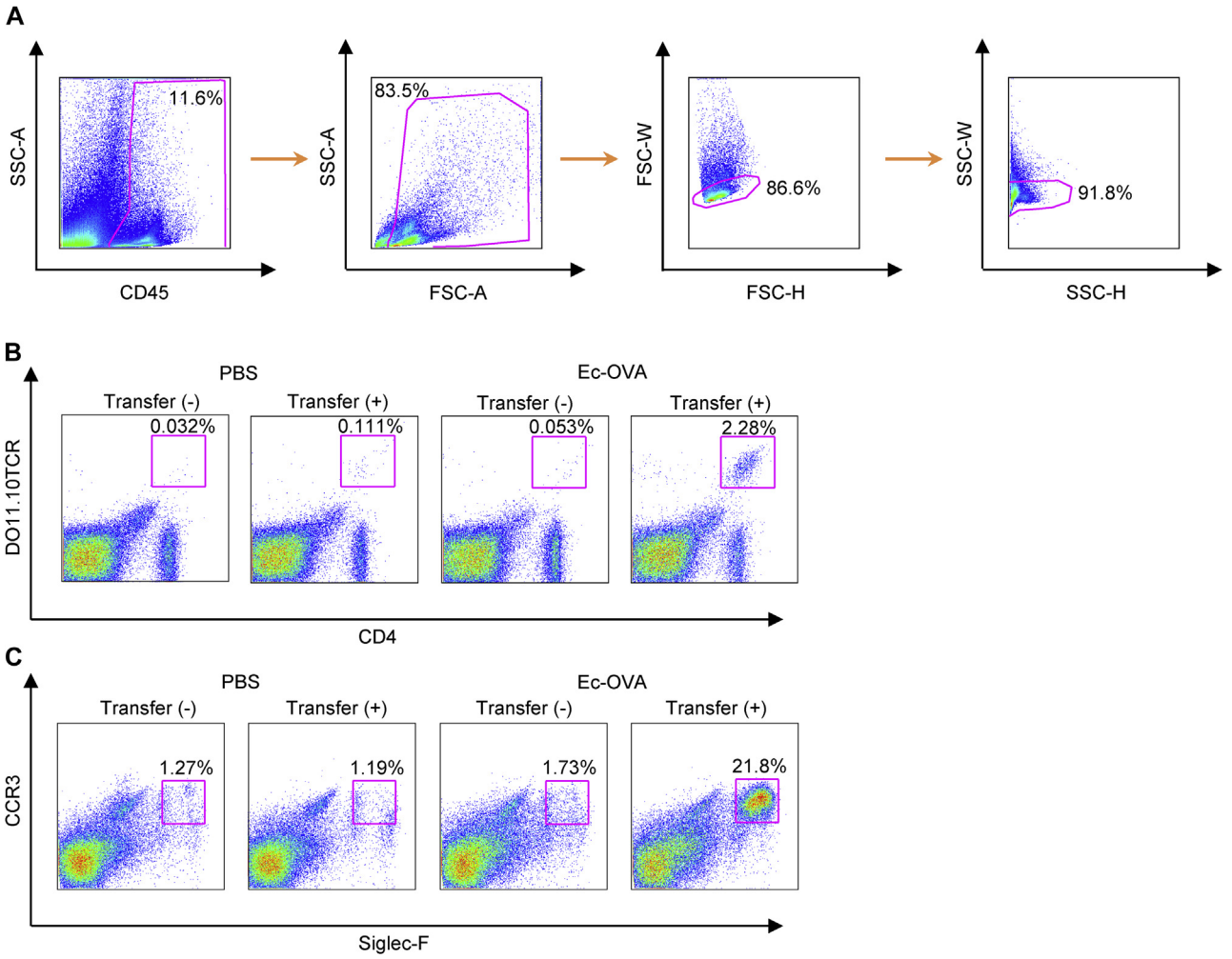

FIG E3. Nasal accumulation of OVA-specific T cells and eosinophils in T_H2-transferred and OVA-challenged mice. WT mice were adoptively transferred with or without DO11.10-derived T_H2 cells. Mice were then i.n. challenged with PBS or Ec-OVA. Frequency of OVA-T_H2 cells ($CD4^+DO11.10TCR^+$ cells in $CD45^+$ cells) and eosinophils ($CCR3^+Siglec\text{-}F^+$ cells in $CD45^+$ cells) in nasal mucosa 24 hours after the final challenge was examined by FACS. **A,** Gating strategy to analyze nasal $CD45^+$ hematopoietic cells. **B** and **C,** Representative flow cytometry plots in Fig 2, *C* (*B*), and Fig 2, *D* (*C*). *FSC-A,* Forward scattered light-area; *FSC-H,* forward scattered light-height; *FSC-W,* forward scattered light-width; *SSC-A,* side scattered light-area; *SSC-H,* side scattered light-height; *SSC-W,* side scattered light-width.

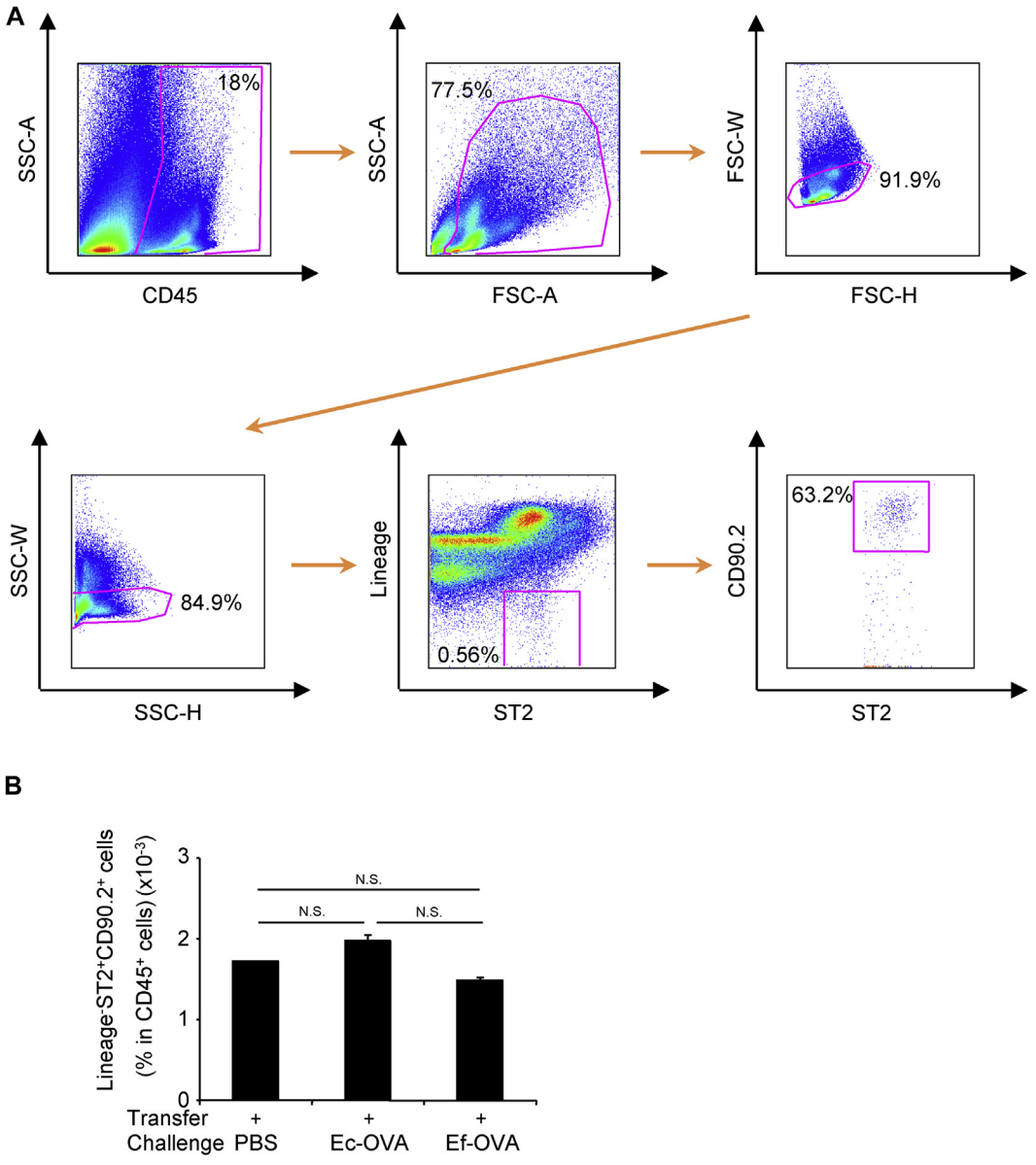

FIG E4. ILC2s do not expand in nasal mucosa in T_H2- transferred and OVA-challenged mice. WT mice adoptively transferred with DO11.10-derived T_H2 cells were i.n. challenged with PBS, Ec-OVA, or Ef-OVA. Frequency of ILC2s ($Lin^-ST2^+CD90.2^+$ cells in $CD45^+$ cells) in nasal mucosa 24 hours after the final challenge was examined by FACS. **A,** Gating strategy to analyze nasal ILC2s. **B,** Quantified graph of ILC2 frequency. Pooled data from 2 independent experiments are shown (mean, SEM, n = 8 [PBS and Ec-OVA groups], n = 7 [Ef-OVA group]). *FSC-A*, Forward scattered light-area; *FSC-H*, forward scattered light-height; *FSC-W*, forward scattered light-width; *SSC-A*, side scattered light-area; *SSC-H*, side scattered light-height; *SSC-W*, side scattered light-width; *N.S.*, not significant.

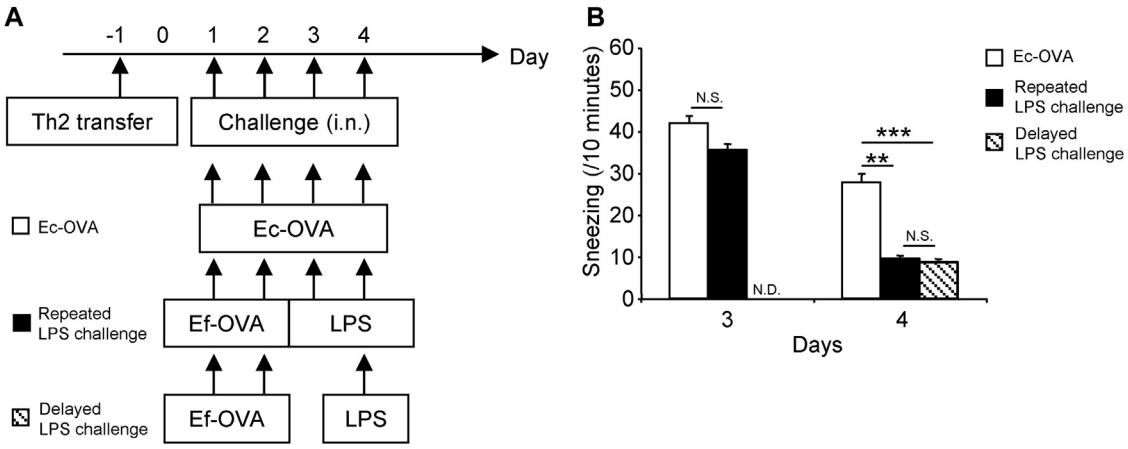

FIG E5. Delayed LPS challenge does not induce sneezing in nasally T_H2-activated mice. **A,** Experimental schema. WT mice were adoptively transferred with DO11.10-derived T_H2 cells. Mice were then i.n. challenged with Ec-OVA (days 1-4), Ef-OVA (days 1-2) followed by LPS (days 3-4), or Ef-OVA (days 1-2) followed by LPS only at day 4. **B,** Number of sneezes at days 3 and 4 was counted for 10 minutes immediately after intranasal challenge. Pooled data from 2 independent experiments are shown (mean, SEM, n = 5 [Ec-OVA group], n = 8 [LPS groups]). *N.D.*, Not determined; *N.S.*, not significant. $^{**}P < .01$; $^{***}P < .001$.

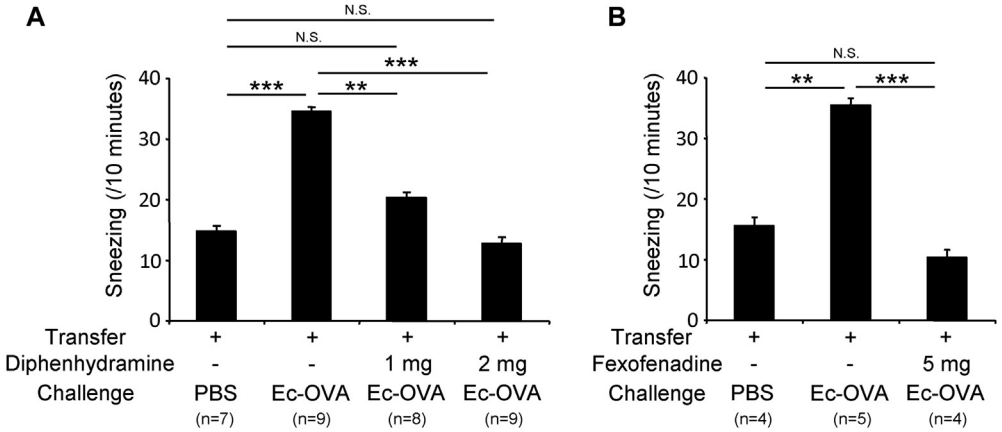

FIG E6. Histamine mediates Ec-OVA–induced sneezing. WT mice adoptively transferred with DO11.10-derived T_H2 cells were i.n. challenged with PBS or Ec-OVA. Mice were i.p. injected with diphenhydramine (1 or 2 mg/dose) **(A)** or fexofenadine (5 mg/dose) **(B)** on day 4, 90 minutes before the final intranasal challenge. Number of sneezes at day 4 was counted for 10 minutes immediately after the intranasal challenge (mean, SEM, n = indicated in figure). *N.S.*, Not significant. $^{**}P < .01$; $^{***}P < .001$.

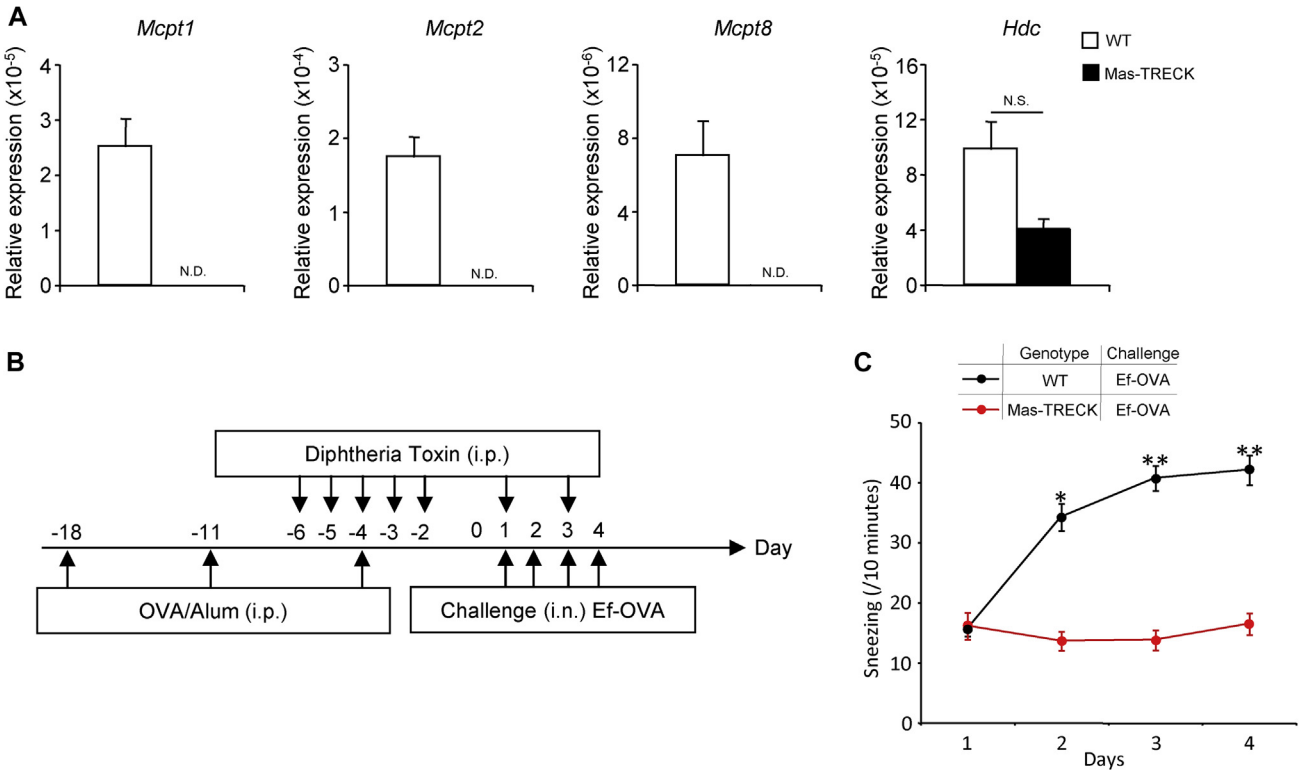

FIG E7. DT treatment in Mas-TRECK mice depletes mast cell/basophil populations. **A,** Mice were treated as in Fig 6, *A*. Total RNAs were extracted from nasal mucosa and subjected to quantitative PCR analysis for the expression of *Mcpt1*, *Mcpt2*, *Mcpt8*, *Hdc*, and *18S*. mRNA expression levels were normalized to 18S rRNA levels. **B** and **C,** WT or Mas-TRECK mice were i.p. immunized with OVA. Mice were then i.p. injected with DT and i.n. challenged with Ef-OVA. **B,** Experimental schema. **C,** Number of sneezes was counted for 10 minutes immediately after each intranasal challenge. Data representative of 2 independent experiments **(A)** and pooled data from 2 independent experiments are shown **(C)** (mean, SEM, n = 3-5 **[A]**, n = 6-8 **[C]**). *N.D.*, Not detected; *N.S.*, not significant. $^{*}P < .05$; $^{**}P < .01$.

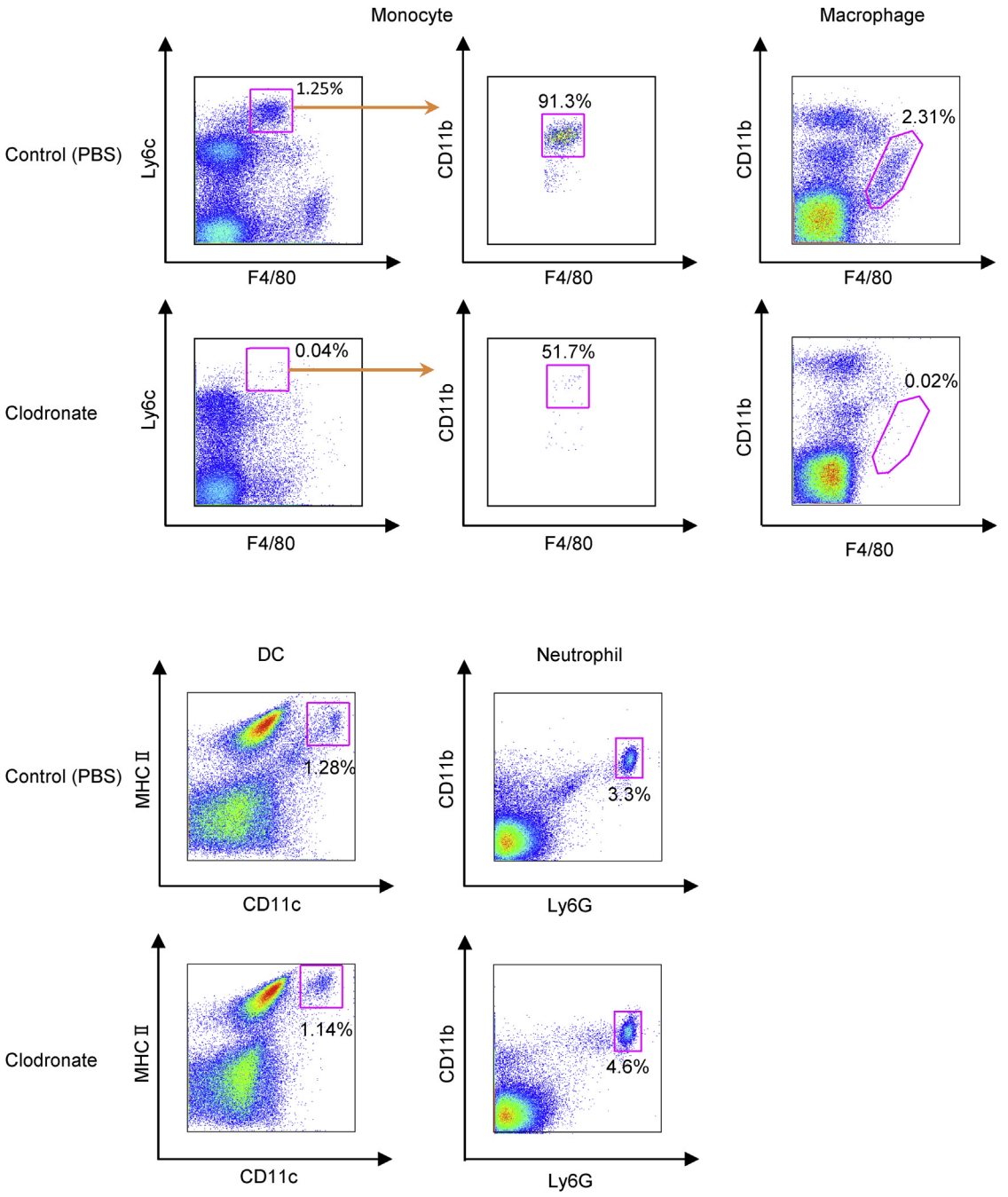

FIG E8. Clodronate treatment depletes monocyte/macrophage populations. WT mice were i.p. injected with clodronate-containing liposome or PBS. Cells in the spleens were isolated 18 hours after the injection. Frequency of Ly-6C^{high}F4/80^{int}CD11b^{high} monocytes, F4/80^{high}CD11b^{int} macrophages, CD11c^{+}MHC-II^{high} dendritic cells, and Ly-6G^{+}CD11b^{+} neutrophils in CD45^{+} cells was examined by FACS. Representative flow cytometry plots from 2 independent experiments are shown. *DC*, Dendritic cells.

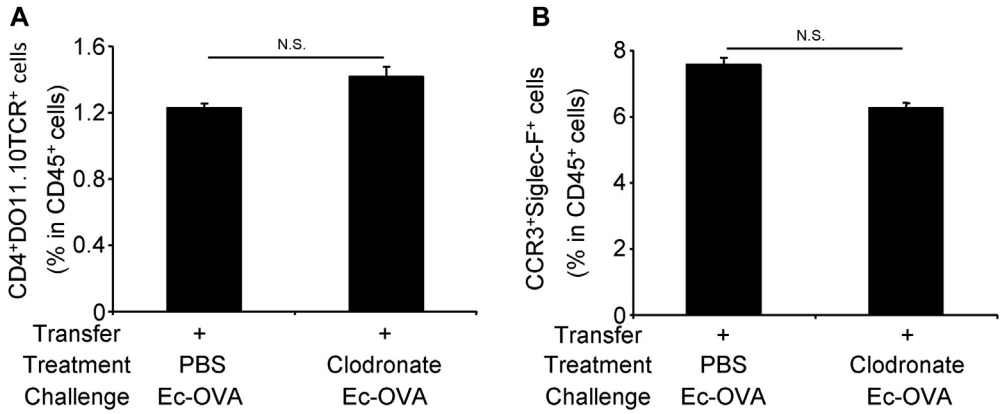

FIG E9. Nasal accumulation of OVA-specific T cells and eosinophils is intact in clodronate-treated mice. WT mice adoptively transferred with DO11.10-derived T_H2 cells were i.n. challenged with Ec-OVA for 4 days. On day 3, 6 hours after the third nasal challenge, mice were i.p. injected with PBS or clodronate-containing liposome. Frequency of OVA-T_H2 cells ($CD4^+DO11.10TCR^+$ cells in $CD45^+$ cells) **(A)** and eosinophils ($CCR3^+Siglec\text{-}F^+$ cells in $CD45^+$ cells) **(B)** in nasal mucosa 60 minutes after the final nasal challenge was examined by FACS. Pooled data from 2 independent experiments are shown (mean, SEM, n = 8). *N.S.,* Not significant.