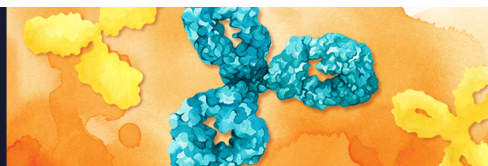


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Ovalbumin-Induced Airway Inflammation Is Ameliorated in Dectin-1–Deficient Mice, in Which Pulmonary Regulatory T Cells Are Expanded through Modification of Intestinal Commensal Bacteria

Wei Han, Ce Tang,¹ Seiya Baba,² Tomofumi Hamada, Tomoyuki Shimazu,³ and Yoichiro Iwakura

Asthma is an allergic chronic respiratory disease that affects more than 300 million people around the world. Dysbiosis of intestinal commensal microbiota influences the development of asthma. Dectin-1 (gene symbol: *Clec7a*), a C-type lectin receptor, plays an important role in the intestinal immune homeostasis by controlling regulatory T (Treg) cell differentiation through regulation of intestinal microbiota. However, it is not clear whether intestinal immune conditions affect immune responses in other organs. In this study, we examined the effects of Dectin-1 deficiency on allergic airway inflammation (AAI). OVA-induced AAI was attenuated in *Clec7a*^{−/−} mice. Treg cells were more abundant in colonic lamina propria, mesenteric lymph nodes, and bronchoalveolar lavage fluid of *Clec7a*^{−/−} mice after AAI induction. Treatment with antibiotics, but not an antifungal agent, decreased the abundance of intestinal Treg cells and aggravated the symptoms of AAI in *Clec7a*^{−/−} mice. Transplantation of gut microbiota from *Clec7a*^{−/−} mice into antibiotic-treated hosts increased the abundance of intestinal Treg cells and ameliorated AAI. Overcolonization by *Lactobacillus murinus*, a Dectin-1 signaling-regulated commensal bacterium, also promoted expansion of Treg cells in the colon and suppressed lung inflammation. Depletion of Treg cells with anti-CD25 Ab eliminated the phenotypic differences between wild-type and *Clec7a*^{−/−} mice in OVA-induced AAI. These observations suggest that inhibition of Dectin-1 signaling ameliorates AAI by increasing the abundance of Treg cells in lungs through modification of intestinal commensal bacteria, suggesting a role for commensal microbiota in regulating inflammation in organs other than the intestine. *The Journal of Immunology*, 2021, 206: 1–10.

Dectin-1 (gene symbol: *Clec7a*), a C-type lectin receptor, is a transmembrane protein expressed on dendritic cells (DCs) and macrophages (1, 2). Dectin-1 contains a carbohydrate recognition domain in its extracellular region and an ITAM in its cytoplasmic domain. Dectin-1 plays an important role in the host defense against fungal infection by facilitating Th17 cell differentiation through recognition of β -(1,3)-D-glucans on pathogens (3–5). Furthermore, we showed recently that Dectin-1 signaling induces IL-17F production from intestinal monocytes and IL-17F promotes antimicrobial protein production, suppressing the growth of specific bacteria, such as *Lactobacillus murinus* (*L. murinus*) and *Clostridium* cluster XIVa in the intestine (6–8). Because these bacteria can induce differentiation of immune-suppressive regulatory T (Treg) cells by inducing production of TGF- β and IL-10 (7) and secreting short-chain fatty acids (SCFAs) (9), inhibition of Dectin-1 signaling increases the proportion of Treg cells in the intestine

through expansion of these bacterial populations. Thus, blocking Dectin-1 signaling can ameliorate development of dextran sodium sulfate (DSS)–induced and naive T cell–induced colitis (6, 7). However, it is not known whether these Treg cells in the intestine also suppress inflammation in other organs.

Asthma, one of the most common chronic respiratory diseases, is characterized by airway hyperresponsiveness (AHR), airway inflammation, airflow obstruction, and airway remodeling (10). Asthma is caused by many factors, including viral and fungal infection to upper respiratory tract, exposure to aeroallergens such as house dust mites (HDMs), and air pollution (11, 12). Asthma can be categorized as Th2 high and corticosteroid sensitive or Th2 low and corticosteroid resistant. In Th2-high asthma, type 2 immune response–associated cytokines, including IL-4, IL-5, IL-13, IL-25, and IL-33, induce IgE synthesis and activate allergy-related effector cells, such as eosinophils, mast cells, ILC2 cells, NH cells, and basophils

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W.H. performed the majority of experimental work and wrote the manuscript, and S.B., T.H. and T.S. performed a part of experiments and analyzed the data. Y.I. and C.T. organized and supervised the project and edited the manuscript. All authors read and approved the final manuscript.

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Abbreviations used in this article: AAI, allergic airway inflammation; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; cLP, colonic lamina propria; DC, dendritic cell; DSS, dextran sodium sulfate; HDM, house dust mite; mLN, mesenteric lymph node; qPCR, quantitative PCR; SCFA, secreting short-chain fatty acid; Treg, regulatory T; WT, wild-type.

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(11, 13–15). Previous studies showed that genetic deficiency of IL-4, IL-5, or IL-13 ameliorates the major symptoms of asthma, indicating that they play indispensable roles in the pathogenesis of Th2-high AHR (16–19). In contrast, Th2-low asthma is induced through activation of CLRs, including Dectin-1 and Dectin-2, upon infection with fungi such as *Aspergillus fumigatus* or exposure to HDMs (11, 20–24). In this type of asthma, IL-23 and IL-1 β induced downstream of Dectin-1 or Dectin-2 promote differentiation of Th17, IL-17, IL-17F, and IL-22. These cytokines recruit neutrophils by inducing chemokines such as CXCL1 and CXCL8 and also promote mucus production and epithelial proliferation, causing development of corticosteroid-resistant asthma (11). We showed previously that OVA/alum-induced AHR is not dependent on IL-17A, whereas OVA-induced AHR without alum in DO11.10 mice is partially IL-17A dependent (25).

Treg cells have been implicated in the regulation of asthma (26, 27), and the proportion of Treg cells in the sputum is significantly lower in asthma patients than in normal subjects (28). Commensal *Clostridium* cluster IV and XIVa induce Treg cell differentiation in the colon and suppress DSS-induced colitis and OVA-induced production of IgE and IL-4 (29). Clostridia produce butyrate, an SCFA, and induce Treg cell differentiation by inducing Foxp3 expression in naive T cells, thereby ameliorating development of colitis (9). In addition, another microbial-derived SCFA propionate potentiates Treg cell differentiation by inhibiting histone deacetylase (30). We showed that commensal *L. murinus* and *Clostridium* cluster XIVa can induce Treg cells in the intestine to suppress colitis. Other bacteria, such as *Bifidobacterium* and *Faecalibacterium*, also facilitate Treg cell differentiation in the intestine (31, 32).

In contrast, involvement of intestinal commensal microbiota in asthma is suggested in many studies. The relative abundances of genera *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* are significantly reduced in the feces of Canadian children with high risk of asthma, as are levels of fecal acetate (33). Inoculation of germ-free mice with these four bacterial taxa ameliorates airway inflammation in their adult progeny (33). Neonates with the lowest relative abundance of the bacterial genera *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* and higher relative abundance of the fungal genera *Candida* and *Rhodotorula* have the greatest risk of developing atopy and asthma (34). However, the mechanisms by which these bacteria suppress development of AHR remain to be elucidated.

In this study, we investigated whether Treg-inducing microbiota in the colon can suppress inflammation in organs other than the intestine. We found that OVA-induced AHR development was suppressed in *Clec7a*^{-/-} mice through modification of intestinal commensal microbiota. Gut bacteria, but not fungi, were important for this suppression. Moreover, we found that intestinal colonization of *L. murinus* attenuates allergic airway inflammation (AAI) associated with elevated abundance of Treg cells in the intestine. Thus, we demonstrated that increased colonization of *L. murinus* in the intestine ameliorates OVA-induced AAI in *Clec7a*^{-/-} mice by promoting accumulation of Treg cells in colon, suggesting that intestinal microbiota can regulate inflammation in organs other than the intestine.

Materials and Methods

Mice

Clec7a^{-/-} mice were generated by homologous recombination using E14.1 ES cells (4) and were used for experiments after backcrossing for 10 generations to BALB/cA mice. BALB/cA wild-type (WT) control mice were bred in the same facility as *Clec7a*^{-/-} mice, and age- and sex-matched mice were used. WT and genetically modified mice were first cohoused after weaning

for several days and then separately housed for at least 4 wk before use. All mice were kept under specific pathogen-free conditions with γ -ray-sterilized normal diet (F1; Funahashi Farm, Chiba, Japan), acidified (0.002 N HCl, pH 2.5) tap water, and an autoclaved wooden chip bed in environmentally controlled clean rooms at the Research Institute for Biomedical Sciences, Tokyo University of Science. The experiments were carried out according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments and were approved by the relevant institutional committees.

AAI induction and sneeze counting

One hundred microliters of OVA/saline (0.5 mg/ml; Sigma-Aldrich, Germany) mixed with 100 μ l of aluminum hydroxide gel (20 mg/ml; FUJIFILM, Japan) was i.p. injected into BALB/c WT and *Clec7a*^{-/-} mice on days 0, 7, and 14 for sensitization. One week after the last sensitization, the mice were treated with 20 μ l of OVA/saline (60 mg/ml) as nasal drops every day for 7 d. Five minutes after the last nasal immunization, mice were placed individually in a transparent chamber, and sneezes during 15 min were counted using the recorded footage taken by a charge-coupled device camera (Supplemental Video 1).

Antibiotic and antifungal treatments

Mice were pretreated for 3 wk with an antibiotic mixture containing 0.5 mg/ml vancomycin (Wako, Tokyo, Japan), 1 mg/ml ampicillin (Nacalai Tesque, Tokyo, Japan), 1 mg/ml metronidazole (Nacalai Tesque), and 1 mg/ml neomycin (Nacalai Tesque) or an antifungal agent (fluconazole, 0.5 mg/ml; Wako); allowed to rest for 1–2 d; and then subjected to transfer of fecal microflora or *Lactobacillus*.

Bronchoalveolar lavage fluid preparation

After mice were sacrificed, the skin and muscle around the neck were gently removed to expose the trachea. A surgical suture line ~15 cm long was placed under the trachea using forceps, and a 22-G syringe needle was shallowly inserted into the exposed trachea for 1 cm and then tied with the prepared suture line. One milliliter of PBS was injected and removed slowly with a 1-ml syringe, and the recovered lavage fluid was collected in a 15-ml tube. This process was repeated five times.

Cell preparation

Colonic lamina propria (cLP) cells and lung-infiltrating cells were isolated as previously described with slight modifications (30). Briefly, colons or lungs were washed with PBS, cut into 1-mm slices, and then shaken in HBSS containing 3 mM EDTA at 37°C for 30 min. Organ slices were washed with HBSS and then shaken at 37°C for another 120 min in RPMI-1640 medium containing 10% FBS and 1% streptomycin + penicillin, 400 U/ml collagenase (C2139; Sigma-Aldrich), and 5 U/ml DNase I (Sigma-Aldrich). After incubation, samples were vortexed for 30 s, and single-cell suspensions were harvested after sterile gauze filtration. In some experiments, cLP or lung cells were further purified to exclude epithelial cells on a 45 and 66.6% discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden) at 2200 rpm for 20 min.

Flow cytometry

After preparation of single-cell suspension, cells were washed twice with FACS HBSS (containing 2% FCS and 0.1% sodium azide) and then treated with 2.4G2 to block Fc γ binding. Cells were then surface stained with the anti-mouse Abs described in Supplemental Table I. All Abs were used at 1:250 dilution. After 25–30 min, cells were washed twice and resuspended in FACS buffer, followed by flow cytometry with Canto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo 10.0 FACS software. Treg cell was identified as CD25⁺ Foxp3⁺ subset after 7AAD⁻ CD45⁺ CD4⁺ cell gating.

RNA extraction and real-time PCR

Total RNA was extracted with Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and cDNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, San Francisco, CA). Real-time PCR was performed with a C1000 Thermal Cycler System (Bio-Rad, Hercules, CA) using SYBR Green Master Mix (Applied Biosystems) with the sets of primers described in Supplemental Table II. Relative levels of cytokine-encoding mRNAs were calculated with the comparative cycle threshold (Ct value) method and normalized against the corresponding level of *Gapdh* mRNA. For quantitative PCR (qPCR) analysis of bacterial 16S rRNA genes, genomic DNA of mouse fecal microflora was purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), and qPCR was carried out using the same system as above. The relative quantity of

each bacteria was calculated by the ΔCt method and normalized to the total level of bacterial 16S rRNA genes.

Treg depletion by anti-CD25 Ab

WT and *Clec7a*^{-/-} mice were given i.p. injections of anti-mouse CD25 (clone no. PC-61.5.3; BioXCell) or control IgG1 K isotype-matched Ab (clone no. HRPN; BioXCell) at a dosage of 100 μg per mouse every 3 d for 4 wk during AAI induction (Fig. 6A). The absence of CD25⁺Foxp3⁺ Treg cells from mesenteric lymph nodes (mLNs) and bronchoalveolar lavage fluid (BALF) was confirmed by flow cytometry after the final injection.

Measurement of cytokine concentrations

The BALF supernatant was collected after AAI induction and assayed using the ELISA Development Kit for mouse IL-4 (R&D Systems, Minneapolis, MN) following reagent instructions. IL-4 concentration was measured on an MTP-300 microplate reader (Corona Electric, Hitachinaka, Japan).

Statistical analysis

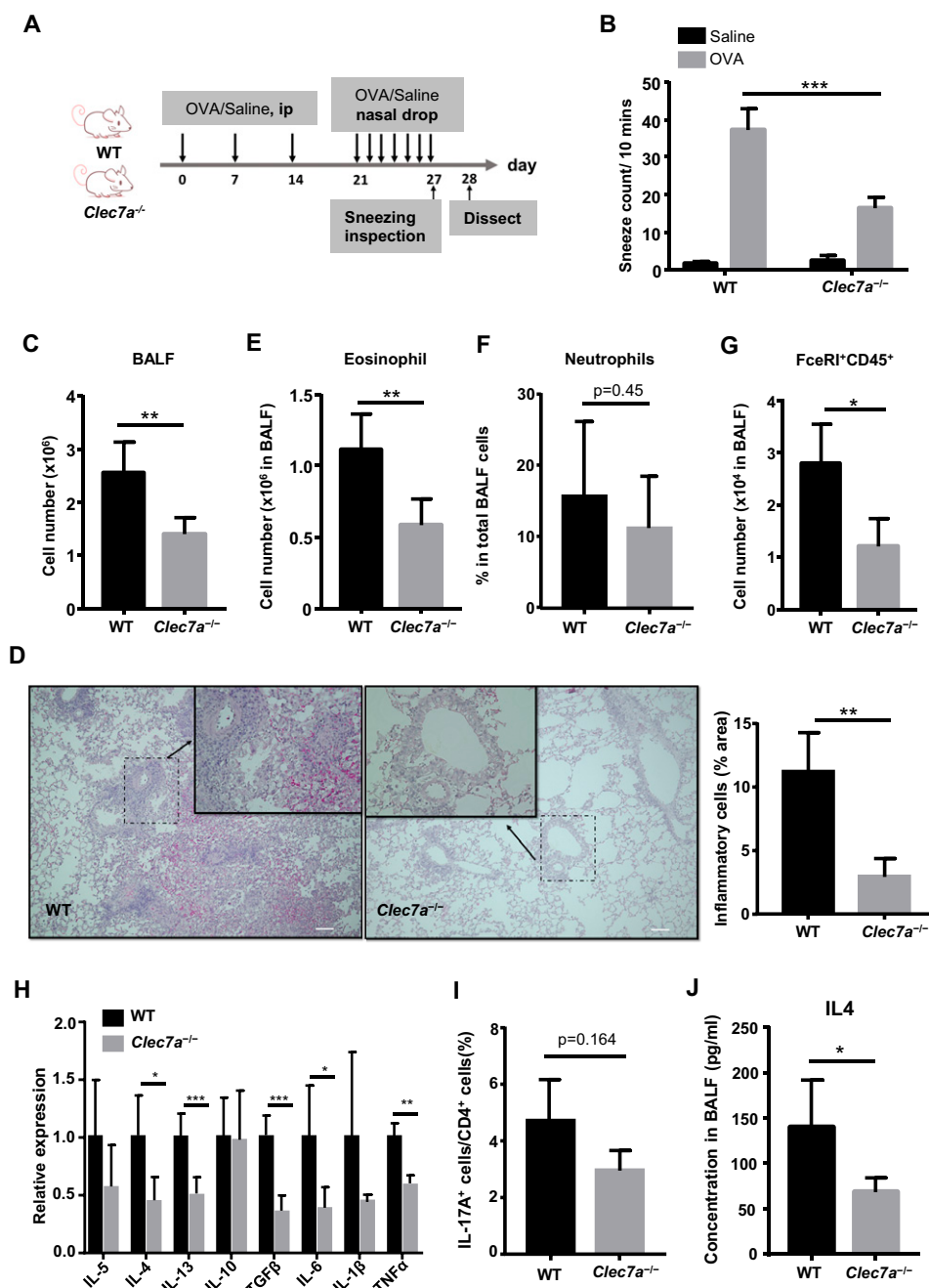
Differences in parametric data were evaluated by unpaired two-tailed Student *t* test. For experiments comparing more than two groups, one-way ANOVA was performed followed by Dunnett test or Tukey multiple comparisons test. Statistics were computed using the Prism 7 software (GraphPad Software). A *p* value < 0.05 was considered statistically significant.

Results

OVA-induced AAI is attenuated in *Clec7a*^{-/-} mice

To investigate the involvement of Dectin-1 in airway inflammation, we examined an OVA-induced AAI model in WT and *Clec7a*^{-/-} mice (Fig. 1A). *Clec7a*^{-/-} mice exhibited significantly milder AAI than WT mice, as evaluated by sneeze count after the last nasal OVA administration (Fig. 1B). Consistent with the reduction in sneezing, the total number of cells in BALF was reduced in *Clec7a*^{-/-} mice (Fig. 1C). Infiltration of inflammatory cells around

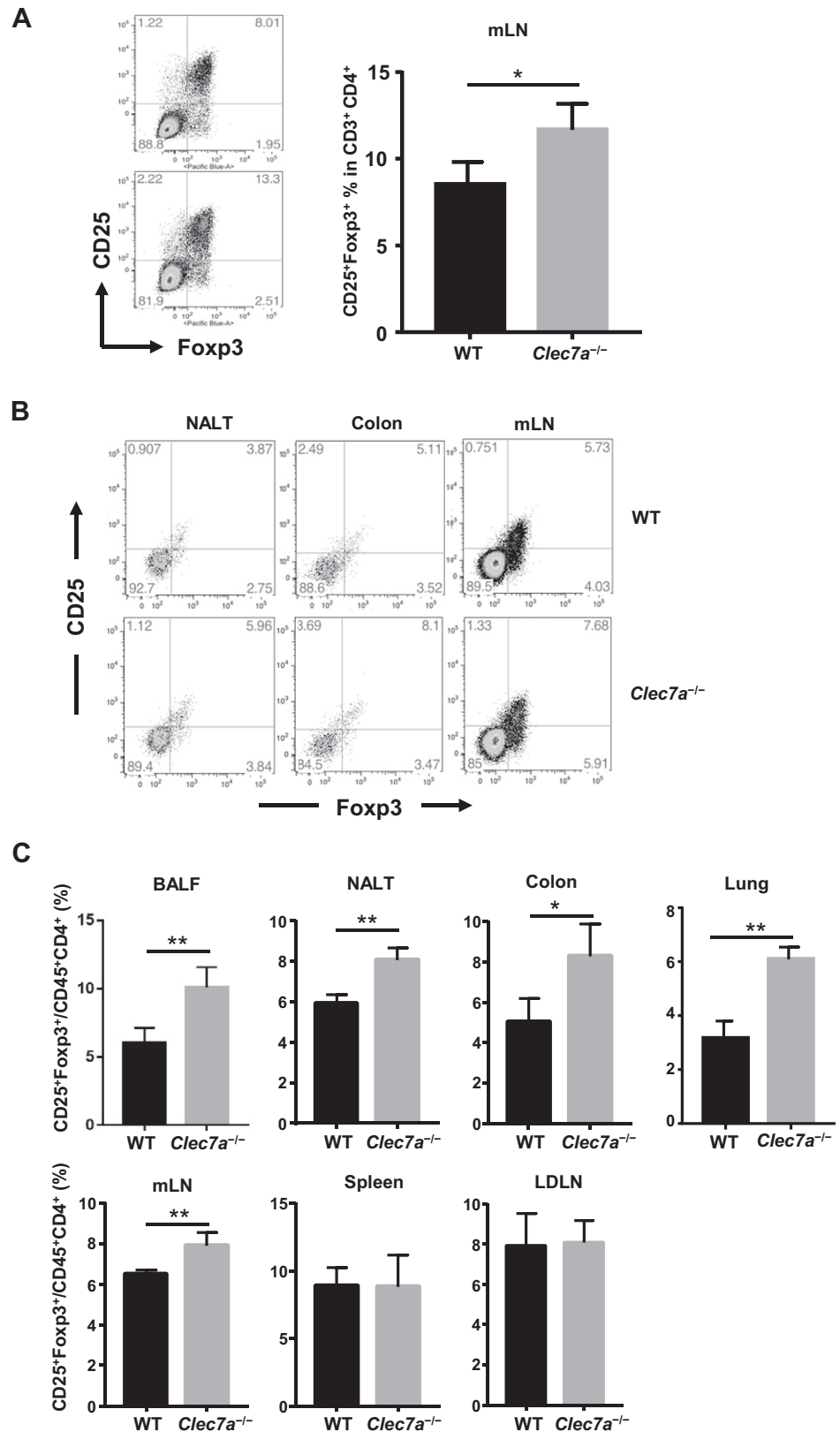
FIGURE 1. *Clec7a*^{-/-} mice are refractory to OVA-induced AAI. Separately housed WT and *Clec7a*^{-/-} mice were i.p. immunized with OVA once a week for 3 wk and then nasally boosted with OVA every day for 1 wk. (A) Protocol for AAI induction (10 wk old, *n* = 5 per group, female). (B) Five minutes after the last nasal administration of OVA, sneezes were counted over 15 min, and we present the count per 10 min. Data are representative of seven experiments. (C) After the final OVA administration, mice were sacrificed, and the numbers of total cells in BALF were evaluated by flow cytometry. Data are representative of four experiments. (D) Representative lung histology (H&E stain). Scale bar, 100 μm . Inflammatory cell infiltration around the airway and airway muscle layer (square indicated by arrows) was examined after AAI induction (right panel). Percentage of inflammatory cells in the interstitial area was calculated. (E–G) After the final OVA administration, mice were sacrificed, and the SiglecF⁺ eosinophils (E), Ly6G⁺ neutrophils (F), and FcεRI⁺ mast cells (G) in BALF were evaluated by flow cytometry. (H) Expression of mRNAs encoding indicated cytokines in the lungs of the indicated mice was analyzed by qPCR after the final OVA administration. (I) IL-17⁺ CD4 T cells in BALF were measured by flow cytometry. (J) IL-4 concentration in BALF was examined by ELISA. (E–J) Data are representative of at least two independent experiments. Data are expressed as mean $\pm$ SD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.



the airway was also reduced in *Clec7a*^{-/-} mice (Fig. 1D). Because eosinophils play crucial roles in tissue damage and asthma development by secreting granule proteins, such as major basic protein (35), we analyzed the composition of BALF cells. Flow cytometry analysis revealed that the number of SiglecF⁺ eosinophils, but not neutrophils, in the BALF was significantly lower in *Clec7a*^{-/-} mice

than in WT mice (Fig. 1E, 1F). FcεRI is expressed on eosinophils, mast cells, DC, and macrophages (36), and asthma severity is associated with the abundance of FcεRI⁺ cells (37). Relative to WT mice, the population of FcεRI⁺CD45⁺ cells in BALF was lower in *Clec7a*^{-/-} mice (Fig. 1G). Expression of type 2 response-associated cytokines, such as IL-4, IL-6, and IL-13, was reduced in lungs of

FIGURE 2. Proportion of Treg cells is elevated in *Clec7a*^{-/-} mice after AAI induction. **(A)** FACS panels and histogram showing the proportion of CD25⁺ Foxp3⁺ Treg cells in total CD4⁺ T cells of WT (upper) or *Clec7a*^{-/-} (lower) mouse mLN under the physiological conditions (*n* = 5 per group). Data are reproduced in another experiment. **(B and C)** AAI was induced as described in Fig. 1. Representative FACS dot panels for CD25⁺ Foxp3⁺ Treg cells **(B)** and FACS-based counting of Treg cells in the indicated organs **(C)** are shown (10 wk old, *n* = 5 per group, female). Data are representative of three independent experiments and are expressed as mean ± SD. **p* < 0.05, ***p* < 0.01. LDNL, lung-associated draining lymph nodes; NALT, nasal-associated lymphoid tissues.



Clec7a^{-/-} mice relative to WT lungs (Fig. 1H). We could not detect IL-17A in these mice, and Th17 cell content was similar between WT and *Clec7a*^{-/-} mice (Fig. 1I), consistent with the notion that Th2 cytokines, but not IL-17A, are important in OVA/alum-induced airway hypersensitivity response (25). Concentration of IL-4 in BALF was significantly reduced in *Clec7a*^{-/-} mice (Fig. 1J), consistent with the reduction of BALF cell numbers in these mice (Fig. 1C). Together, these results show that suppression of Dectin-1 signaling ameliorates development of AAI.

The Treg cell population is overexpanded in BALF, colon, and mLN of Clec7a^{-/-} mice after AAI induction

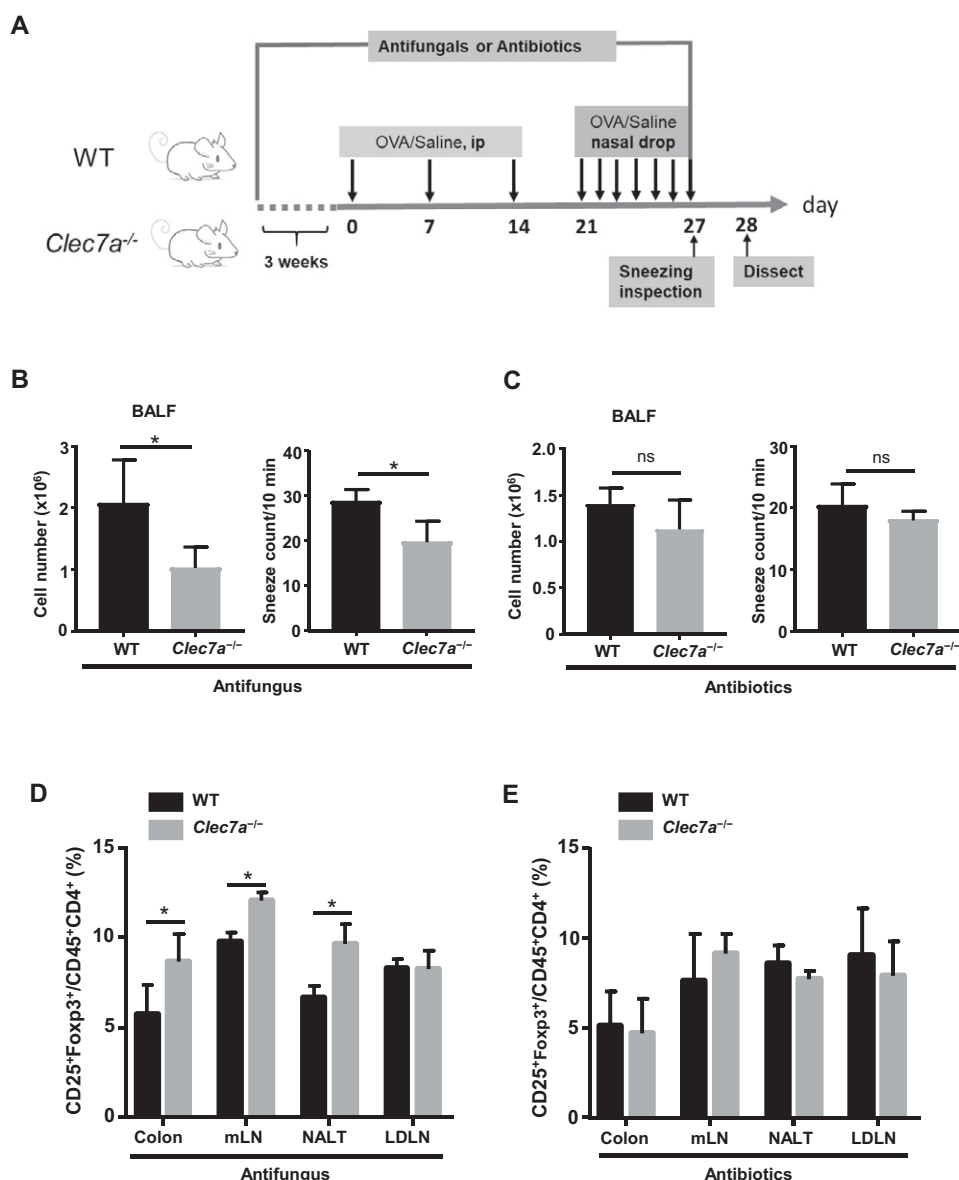
Treg cells play indispensable roles in the suppression of chronic inflammatory diseases, including OVA-induced AHR (38, 39). Remarkably, the proportion of Treg cells in mLN was higher in *Clec7a*^{-/-} mice than in WT mice at steady state (Fig. 2A). To clarify the mechanism by which Dectin-1 deficiency inhibits AAI development, we harvested cells from different organs of WT and *Clec7a*^{-/-} mice after AHR induction and evaluated Treg population by FACS. We found that Treg cell population in BALF, nasal-associated lymphoid tissue, cLP, mLN, and lungs was higher in *Clec7a*^{-/-} mice than in WT mice (Fig. 2B, 2C). The overexpansion

of Treg cells and reduced levels of proinflammatory cells and cytokines in the lungs of *Clec7a*^{-/-} mice (Fig. 1) suggested that Treg cells regulate AAI development.

Commensal microbiota are involved in the suppression of AAI

We previously reported that expansion of commensal *Lactobacillus* in *Clec7a*^{-/-} mice causes the increase in the abundance of intestinal Treg cells, leading to suppression of DSS-induced colitis (7). Because commensal fungi strongly influence the development of colitis (40), we examined the effects of commensal fungi on the development of OVA-induced AAI (Fig. 3A). After treatment of mice with an antifungal drug before and during OVA sensitization, *Clec7a*^{-/-} mice were still less sensitive than WT mice to OVA-induced AAI, as evaluated by total BALF cell number and sneezing frequency (Fig. 3B). In contrast, when mice were treated with antibiotics, BALF cell number and sneezing frequency of WT and *Clec7a*^{-/-} mice were similar (Fig. 3C). After antifungal drug treatment, the proportion of Treg cells in the colon, mLN, and nasal-associated lymphoid tissue was greater in *Clec7a*^{-/-} mice than in WT mice after AAI induction (Fig. 3D). By contrast, Treg cell expansion in *Clec7a*^{-/-} mice was completely abolished after antibiotic treatment (Fig. 3E). These results suggest that commensal bacteria, but not

FIGURE 3. Intestinal commensal bacteria, but not fungi, of *Clec7a*^{-/-} mice are involved in suppression of AAI. WT and *Clec7a*^{-/-} mice (6 wk old, *n* = 4 per group, female) were treated with antifungal agent (500 µg/ml fluconazole) or antibiotics (1 mg/ml ampicillin, 500 µg/ml vancomycin, 1 mg/ml neomycin sulfate, 1 mg/ml metronidazole) in drinking water for 6 wk. After the first 3 wk of antimicrobial treatment, OVA was administered for 4 wk to induce AAI. (A) Protocol for antimicrobial treatment and AAI induction. (B and C) Five minutes after the last nasal administration of OVA, sneeze number (left) and total cell numbers in the BALF (right) of mice treated with antifungal (B) or antibiotics (C) were determined. (D and E) Proportions of CD25⁺ Foxp3⁺ Treg cells in the indicated organs from AAI-induced mice after treatment with antifungal (D) or antibiotics (E) were measured based on FACS. (B–E) Data are representative of two independent experiments and are expressed as mean ± SD. **p* < 0.05.



fungi, are involved in the expansion of Treg cells and suppression of OVA-induced AAI in *Clec7a*^{-/-} mice.

Intestinal microbiota in *Clec7a*^{-/-} mice are responsible for suppression of AAI

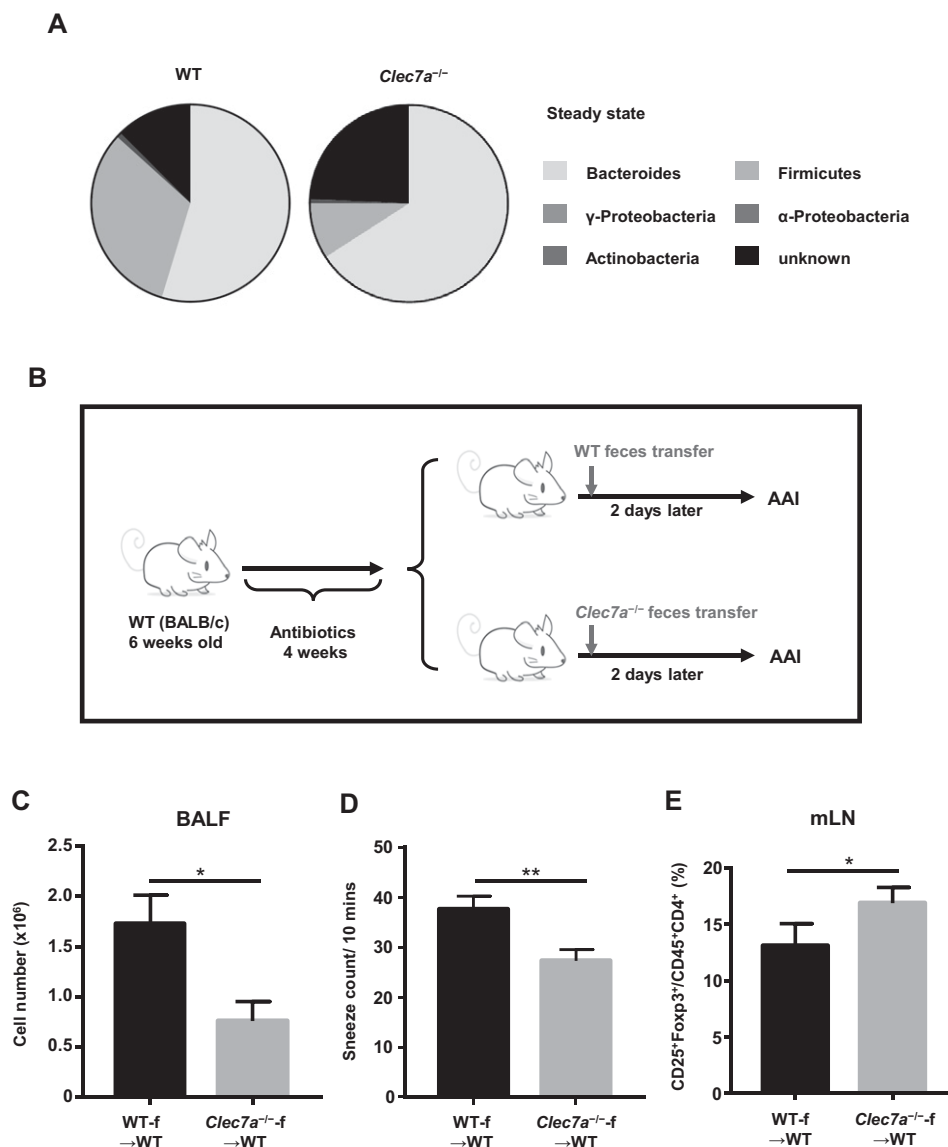
Because the proportion of Treg cells in mLN was higher in *Clec7a*^{-/-} mice than in WT mice even under physiological conditions (Fig. 2A), we analyzed the bacterial composition in the feces of these mice under physiological conditions. Consistent with our previous report, the population of bacteria in phylum Firmicutes, which includes genera *Lactobacilli* and *Clostridia*, was expanded in *Clec7a*^{-/-} mice (Fig. 4A). To directly test the involvement of these commensal bacteria in the regulation of AHR development, we transferred whole fecal bacteria from normal WT or *Clec7a*^{-/-} mice into WT mice pretreated with antibiotics to facilitate colonization of transferred bacteria (Fig. 4B). We found that transfer of fecal bacteria from *Clec7a*^{-/-} mice suppressed the development of AAI more strongly than fecal bacteria from WT mice (Fig. 4C). In mice that received *Clec7a*^{-/-} fecal bacteria, the number of inflammatory cells in BALF was reduced, and the proportion of Treg cells in mLN was elevated (Fig. 4D, 4E). These results indicate that gut microbiota

from *Clec7a*^{-/-} mice are responsible for expansion of Treg cells and suppression of AAI.

L. murinus inhibits development of AAI

Previously, we showed that the population of *L. murinus* is expanded in the *Clec7a*^{-/-} mouse intestine and promotes Treg cell differentiation to suppress DSS-induced colitis (7). Next, we investigated whether colonization by *L. murinus* can suppress the development of AAI. To this end, we first treated WT mice with antibiotics and then orally inoculated them with *L. murinus*. Three days after *L. murinus* inoculation, we transferred whole fecal microbiota from WT mice, followed by induction of AHR (Fig. 5A). A single *L. murinus* inoculation was sufficient to maintain high levels of *L. murinus* colonization in the intestine throughout the AHR induction experiment (Fig. 5B). However, *L. murinus* colonization was not detected in lungs. Clearly, *L. murinus* overcolonization in the intestine ameliorated the development of AAI and suppressed cell infiltration into BALF (Fig. 5C, 5D). The proportion of Treg cells was significantly elevated in mLN and also tended to increase in the lungs (Fig. 5E, 5F). Furthermore, expression of proinflammatory cytokines was also reduced following inoculation of *L. murinus* (Fig. 5G). These results suggest that intestinal commensal bacteria

FIGURE 4. Microbiota from *Clec7a*^{-/-} mouse intestine ameliorates AAI. (A) Composition of fecal microbiota of WT and *Clec7a*^{-/-} specific pathogen-free mice ($n = 4$) was analyzed by qPCR of the 16S rRNA gene. (B–E) WT mice (6 wk old, $n = 4$ per group, female) were treated with antibiotics for 4 wk. They then received a transfer of whole fecal bacteria from specific pathogen-free WT or *Clec7a*^{-/-} mice, followed by AAI induction [(B) experiment protocol]. After the final OVA administration, sneeze number (C) and total cell number in BALF (D) of indicated mice were determined, and the proportion of CD25⁺ Foxp3⁺ Treg cells was measured in CD4⁺CD45⁺ gate based on FACS (E) ($n = 5$ per group). WT-f and *Clec7a*^{-/-}-f: feces from WT and *Clec7a*^{-/-} mice, respectively. (C–E) Data are representative of two independent experiments. (A and C–E) Data are expressed as mean $\pm$ SD. * $p < 0.05$.



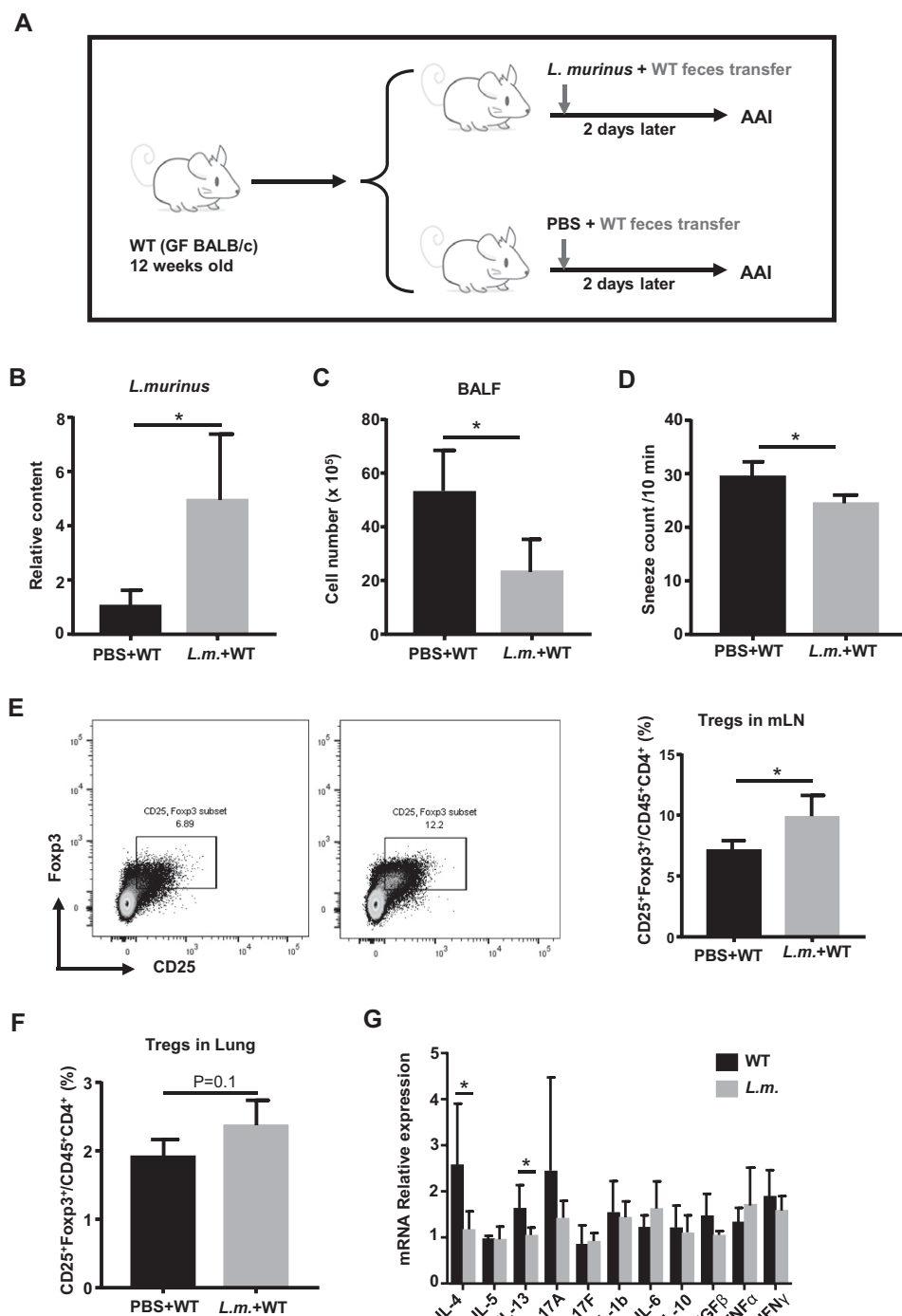


FIGURE 5. Oral transfer of *L. murinus* can improve AAI. WT mice were treated with antibiotics for 4 wk and then orally transferred with *L. murinus*. Three days after the transfer, these mice received whole fecal bacteria from specific pathogen-free WT mice, followed by AAI induction. (**A**) Protocol for microbiota transfer and AAI induction (12 wk old, $n = 5$ per group, female). (**B**) Twenty-eight days after the induction of AAI, feces were collected from *L. murinus*-transferred mice and non-transferred control mice, and the relative content of *L. murinus* was analyzed by qPCR. (**C**) Sneezing frequencies of the indicated mice. (**D**) Total cell number in BALF. (**B–D**) Data are representative of two independent experiments. (**E** and **F**) FACS analysis of mLN cells is shown in left panels (left: WT feces transferred; right: *L. murinus* transferred). Populations of CD25⁺ Foxp3⁺ Treg cells in mLNs (**E**, right panel) and in lungs (**F**) were estimated by FACS. (**G**) Expression of type 2 response-associated cytokines in cells in BALF from indicated mice was evaluated by qPCR. Data are expressed as mean $\pm$ SD. * $p < 0.05$.

such as *L. murinus* have the ability to attenuate chronic inflammation in the respiratory tract.

Treg cells are responsible for the suppression of AAI in *Clec7a*^{-/-} mice

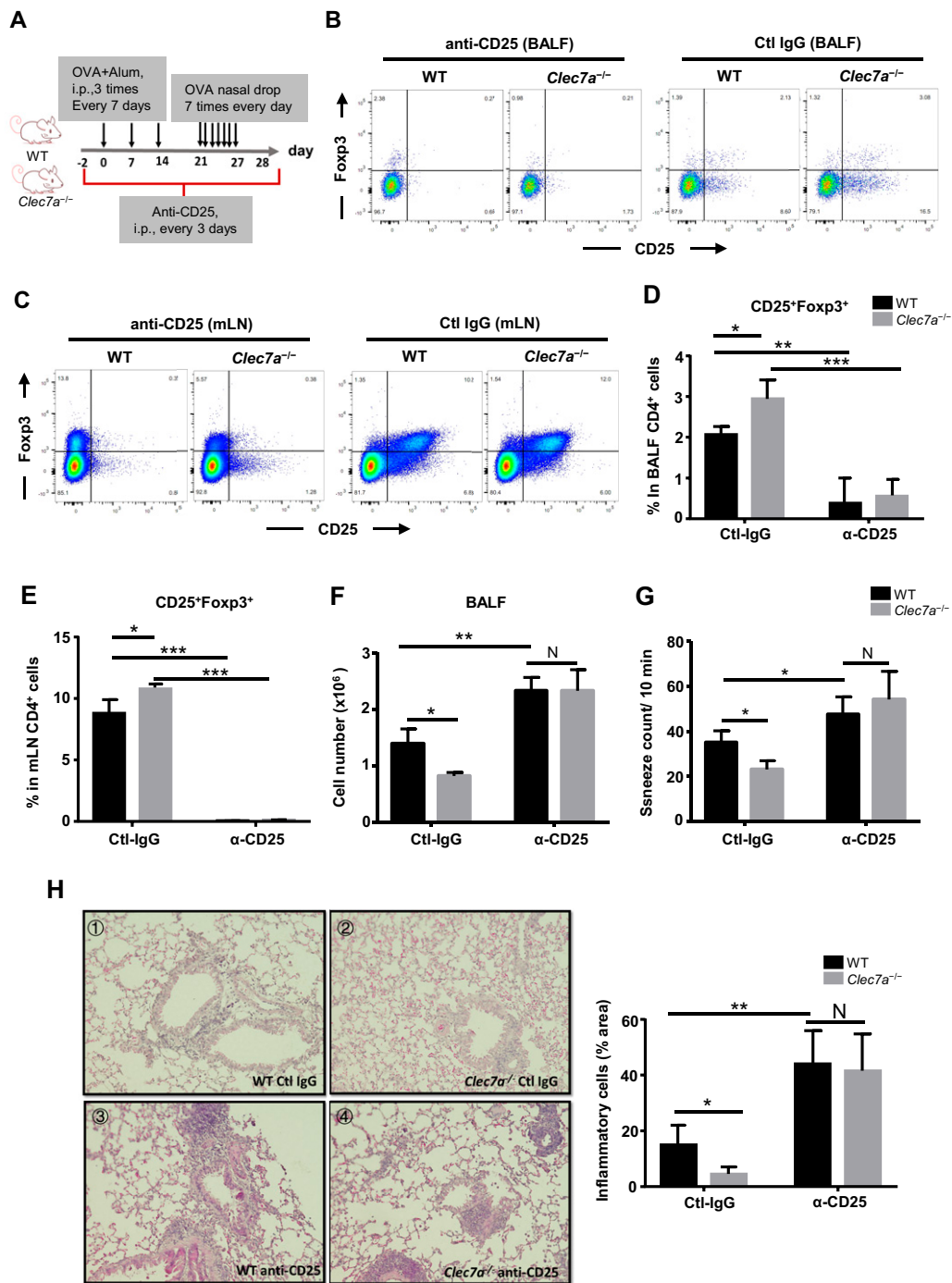
In the experiments described above, we demonstrated that intestinal bacteria from *Clec7a*^{-/-} mice ameliorated OVA-induced AAI, associated with an increase in the Treg cell population. Next, we investigated whether Treg cells are involved in the amelioration of AAI in *Clec7a*^{-/-} mice. For this purpose, we treated mice with either anti-CD25 Ab or isotype control IgG and then monitored development of AAI (Fig. 6A). As expected, Treg cells were greatly reduced in BALF after anti-CD25 Ab treatment relative to control IgG treatment (Fig. 6B, 6D). Anti-CD25 Ab also eliminated Treg cells from mLN (Fig. 6C, 6E). These results confirmed that anti-CD25 Ab could

efficiently deplete Treg cells in our mouse model. Consistent with our previous observations, the Treg cell population was expanded in control IgG-treated *Clec7a*^{-/-} mice (Fig. 6D, 6E), concomitant with reduced abundance of cells in BALF, a reduction in sneezing frequency, and amelioration of inflammatory cell infiltration in the lungs (Fig. 6F–H). By contrast, after anti-CD25 Ab treatment, cell number in BALF and sneezing frequency did not differ between WT and *Clec7a*^{-/-} mice, and no differences were observed in histological examination of the lungs (Fig. 6H). These results suggest that Treg cells are responsible for the suppression of AAI in *Clec7a*^{-/-} mice.

Discussion

We previously reported that the proportion of intestinal commensal *Lactobacilli*, including *L. murinus* and *L. johnsonii*, is higher in

FIGURE 6. Treg cells are responsible for the amelioration of OVA-induced AAI in *Clec7a*^{-/-} mice through regulation of intestinal bacteria. WT and *Clec7a*^{-/-} mice were induced to develop AAI by injecting OVA as described in Fig. 1. At the same time, mice were injected i.p. with either anti-CD25 Ab or isotype control IgG (Ctl IgG) (100 μ g/mouse, every 3 d). Twenty-eight days later, the mice were sacrificed and subjected to examinations. (A) Protocol for Ab treatment and AAI induction (10 wk old, $n = 4$ per group, female). (B and C) CD25⁺ cells and Foxp3⁺ cells in BALF (B) and mLN (C) were analyzed by FACS. (D and E) Contents of CD25⁺ Foxp3⁺ Treg cells in (B) and (C). (F) Cells in BALF of Ctl IgG- and anti-CD25 Ab-treated mice were counted on day 28 after AAI induction. (G) Frequencies of sneezing are shown. (H) Representative lung histology stained by H&E. Original magnification $\times 20$. Inflammatory cell infiltration around the bronchioles and airway muscle layer was checked after induction of AAI. The percentage of inflammatory cells in the interstitial area was calculated. (E–H) Data are expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Clec7a^{-/-} mice than in WT mice (7). Inoculation of *L. murinus* ameliorates DSS-induced colitis by promoting Treg cell expansion in cLP (6–8, 41). In this report, we showed that OVA-induced AAI is attenuated in BALB/c-*Clec7a*^{-/-} mice. This is because the Treg cell population is expanded in *Clec7a*^{-/-} mice, not only in the colon but also in BALF and airway-associated lymphatic tissues, because of modification of intestinal commensal bacteria. Treg cells regulate immune responses in lungs through several key mechanisms: they can suppress allergic inflammation by interfering with DC-dependent activation of effector Th2 and Th17 cells (42) and they can also directly block the proinflammatory activity of eosinophils, basophils, and mast cells, which are critical for the induction or promotion of asthma (42).

Specific gut microbiota such as *L. murinus*, which overcolonizes in the intestine of *Clec7a*^{-/-} mice, can promote Treg cell differentiation in the intestine by inducing the production of TGF- β and IL-10 from colonic DCs and macrophages (7). A specific group of bacteria, such as *Clostridium* cluster XIVa, also induce differentiation of Treg cells in BALB/c mice by producing SCFAs, such as butyrate and propionate (6, 9, 29, 30). Notably in this regard, children with high levels of butyrate and propionate in the feces at 1 y of age are less likely to have asthma between 3 and 6 y (43), suggesting that SCFAs are also important for regulation of the immune system in humans.

There are two potential explanations for the influence of the intestinal microbiota on lung AAI. First, microbiota-induced Treg cells in the colon migrate into distal organs (including the lungs) to

suppress inflammation. In support of this idea, Treg cells originating from the distal large-intestinal lamina propria can migrate into mLNs and inguinal lymph nodes (44). In this context, Th17 cells induced by segmented filamentous bacteria in the intestine have been shown to aggravate experimental autoimmune encephalomyelitis, suggesting that Th17 cells migrate from the intestine to the CNS (44, 45).

Another possibility is that SCFAs produced by a specific group of bacteria in the intestine disseminate systemically and induce Treg cells in distal organs, resulting in the amelioration of inflammation in these organs. Propionate is recognized by free fatty acid receptor 3 (FFAR3/GPR41) on DCs, leading to differentiation of DCs with an impaired ability to promote Th2 cell effector function and resulting in the suppression of HDM-induced AAI (46). However, in this study, the Treg population was not affected by treatment with propionate (46). In another study, an SCFA mixture containing butyrate, acetate, and propionate ameliorated allergic lung inflammation, but no involvement of Treg cells was observed, and the suppression of Th2 development and DC functions was suggested to prevent lung inflammation (47). Thus, it is not clear whether SCFA-induced Treg cells are involved in amelioration of AAI.

HDM-induced AAI is attenuated in C57BL/6-*Clec7a*^{-/-} mice, associated with a reduction in the levels of inflammatory cytokines and CD11b⁺ DCs in mLN (22). In contrast, however, they did not observe expansion of Treg cells in mLN. This discrepancy may be explained by differences in mouse strains and the immunogens used to induce lung AAI.

Taken together, these observations suggest that intestinal microbiota play important roles in regulating lung AAI, either by inducing Treg cells in the intestine or by producing SCFAs to induce Treg cells in lungs or both. However, Treg-independent regulatory mechanisms involving intestinal microbiota may also be involved in regulating lung inflammation. Further studies are needed to elucidate the mechanism by which intestinal microbiota regulate lung AAI.

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Disclosures

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Supplemental Table 1. Antibodies used for flow cytometry.

Color	Antibody	Clone	Company
Pacific Blue	Foxp3	FJK-16S	eBioscience
Am cyan	CD11c	N418	Biolegend
FITC	CD11b	M1/70	Biolegend
APC	CD3ε	145-2C11	Biolegend
APC	FcεRI	1-Mar	Biolegend
APC	F4/80	BM8	Biolegend
APC-cy7	CD4	RM14-5	Biolegend
PE	CD25	PC61	Biolegend
PE	Dectin-1	RH1	Biolegend
PE	Siglec-F	E50-2440	Biolegend
PE-cy7	CD45	30-F11	Biolegend

Supplemental Table 2. Primers used for real-time PCR.

Target	Forward 5’-3’	Reverse 5’-3’
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
TNF-α	GCCTCCCTCTCATCAGTTCT	CACTTGGTGGTTTGCTACGA
TGF-β	GCAACATGTGGAActCTACCAG A	GACGTCAAAAGACAGTCCACTC A
IFN-γ	GAActGGCAAAAGGATGGTGA	TGTGGGTTGTTGACCTCAAAC
IL-1β	CAACCAACAAGTGATATTCTCC ATG	GATCCACACTCTCCAGCTGCA
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGA T
IL-5	CGTGGGGGTACTGTGGAAAT	AATCCAGGAActGCCTCGTC
IL-6	GAGGATACCACTCCCAACAGAC C	AAGTGCATCATCGTTGTTCATAC A
IL-13	CCTGGCTCTTGCTTGCCCTT	GGTCTTGTTGATGTTGCTCA
IL-17a	TTTAACTCCCTTGCGCAAAA	CTTTCCCTCCGCATTGACAC
IL-17f	TGCTACTGTTGATGTTGGGAC	AATGCCCTGGTTTTGGTTGAA
L.murinus	AGCTAGTTGGTGGGGTAAAG	TAGGATTGTCAAAAGATGTC
16S	AGAGTTTGATCMTGGCTGAG	CTGCTGCCTYCCGTA
Bacteroides	CRAACAGGATTAGATA	GGTAAGGTTCTCTCGCG
Firmicutes	TGAAACTYAAAGGAATTGACG	ACCATGCACCACCTGTG
Actinobacteria	TACGGCCGCAAGGCTA	TCRTCCCCACCTTCCTCCG
gProteobacteria	TCGTCAGCTCGTGTGTGTA	CGTAAGGGCCATGATG
aProteobacteria	CIAGTGTAAGGTGAAATT	CCCCGTCAATTCTTTGAGTT

Supplementary Video 1

Five minutes after the last nasal immunization, each mouse was placed individually in a transparent chamber. Here, this short video gives an example for the sneezing action of mice.