# Short-chain fatty acids ameliorate allergic airway inflammation via sequential induction of PMN-MDSCs and Treg cells

Min-Ting Huang, MSc,[a] Chiao-Juno Chiu, PhD,[b] Ching-Yi Tsai, MSc,[a] Yue-Ru Lee, PhD,[a] Wei-Liang Liu, PhD,[c] Hsiao-Li Chuang, PhD,[d] and Miao-Tzu Huang, MD, PhD[a,b,e] *Taipei and Miaoli, Taiwan*

**Background: Reinforcement of the immune-regulatory pathway is a feasible strategy for prevention and therapy of allergic asthma. The short-chain fatty acids (SCFAs) acetate, propionate, and butyrate are pleiotropic microbial fermentation products known to induce regulatory T (Treg) cells and exert an immune-regulatory effect. The cellular mechanism underlying SCFA immune regulation in asthma is not fully understood. Objective: We investigated the role of myeloid-derived suppressor cells (MDSCs) and Treg cells, the immune-regulatory cells of innate and adaptive origin, respectively, in SCFA-elicited protection against allergic airway inflammation. Methods: BALB/c mice were given SCFA-containing drinking water before being rendered asthmatic in response to ovalbumen. When indicated, mice were given a GR1-depleting antibody to investigate the function of MDSCs in allergic inflammation of the airways. MDSCs were sorted to examine their immunosuppressive function and interaction with T cells. Results: The mice receiving SCFAs developed less severe asthma that was accompanied by expansion of PMN-MDSCs and Treg cells. Mice depleted of PMN-MDSCs exhibited aggravated asthma, and the protective effect of SCFAs was abrogated after PMN-MDSC depletion. SCFAs were able to directly induce T-cell differentiation toward Treg cells. Additionally, we found that PMN-MDSCs enhanced Treg cell expansion in a cell contact–dependent manner. Whilst membrane-bound TGF-β has been shown to induce Treg cell differentiation, we found that MDSCs upregulated surface expression of TGF-β after coculture with T-cells and that MDSC-induced Treg cell differentiation was partially inhibited by TGF-β blockage. Conclusions: Although previous studies revealed Treg cells as the effector mechanism of SCFA immune regulation, we found that SCFAs ameliorate allergic airway inflammation by relaying immune regulation, with sequential induction of PMN-MDSCs and Treg cells. (J Allergy Clin Immunol Global 2023;2:100163.)**

***Key words:*** *Asthma, short-chain fatty acids, SCFAs, microbiota, myeloid-derived suppressor cells, MDSCs, Treg cells*

Mammals harbor 10 to 100 trillion commensal microbes in the gut that have collectively been termed the *commensal microbiota* and have symbiotically coevolved with the host not only for better utilization of food but also, as is now known, to shape the development of the immune system and participate in disease pathogenesis. Gut microbiomes are broadly similar between humans and rodents. In both hosts, more than 500 to 1000 different species from around 10 different microbial divisions colonize the gastrointestinal tract.[1,2] The gut microbiome is driven primarily by the diet of the host and is constantly being shaped by host physiopathologic conditions in a reciprocal manner. Although a crucial role of commensal flora in the expansion and maintenance of specific T-cell subsets has been found,[3,4] the molecular mechanisms whereby the commensal microbiota contributes to health or diseases are not fully understood.

Recent advances have brought to light the fact that microbial functions are, to a great extent, executed by the postbiotics, especially at distant organs. Among them, the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate are microbial metabolites that are produced by microbial fermentation of indigestible polysaccharides and dietary fibers. SCFAs are known for their pleiotropic functions in crucial physiologic processes, including lipid and glucose metabolism through the production of the satiety hormones GLP-1, PYY, and leptin, and for enhancing insulin sensitivity as well as maintaining epithelial barrier function and homeostasis.[5,6] From the standpoint of immune regulation, exogenous administration of SCFAs inhibits LPS-induced nuclear falctor-κB activation in neutrophils and macrophages[7] and promotes development of regulatory T (Treg) cells by binding to the receptors GPR41, GPR43, and GPR109A and inhibition of histone deacetylase.[8-10] Previous studies using SCFA supplementation[11] or high-fiber diets[12,13] have shown a protective effect of SCFAs against asthma through the induction of Treg cells or regulation at the bone marrow (BM) and dendritic cell (DC) precursor levels, respectively.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells characterized by the expression of $CD11b^{+}GR1^{+}$ and a remarkable capacity to dampen T-cell immune activation in cancers, organ transplantation, and inflammation.[14-16] It is currently believed that MDSCs originate from an arrest of the myeloid differentiation process in various pathologic conditions such as cancer, infection, and autoimmune diseases, wherein the factors released into the

From [a]the Department of Medical Research and [c]the Department of Pediatrics, National Taiwan University Hospital; [b]the Graduate Institute of Clinical Medicine, School of Medicine, National Taiwan University, Taipei; [c]the National Mosquito-Borne Diseases Control Research Center, National Health Research Institutes, Miaoli; and [d]the National Applied Research Laboratories, National Laboratory Animal Center, Taipei.

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Corresponding author: Miao-Tzu Huang, MD, PhD, Department of Medical Research National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, Taiwan 100. E-mail: mthuang@ntu.edu.tw.



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*Abbreviations used*

BALF: Bronchoalveolar lavage fluid
BM: Bone marrow
DC: Dendritic cell
LAP: Latency-associated peptide
MDSC: Myeloid-derived suppressor cell
Mo-MDSC: Monocytic myeloid-derived suppressor cell
OVA: Ovalbumin
pDC: Plasmacytoid dendritic cell
PMN-MDSC: Polymorphonuclear myeloid-derived suppressor cell
SCFA: Short-chain fatty acid
Treg: Regulatory T

microenvironment hinder differentiation of myeloid progenitors into mature leukocyte lineages, such as DCs, granulocytes, and macrophages. There are currently 2 major subsets of MDSCs, namely, MDSCs (PMN-MDSCs) and the monocytic MDSCs (Mo-MDSCs). In mice, PMN-MDSCs and Mo-MDSCs are identified by their surface expression of $CD11b^+$ $GR1^+$ $Ly6G^+$ $Ly6C^{lo}$ and $CD11b^+$ $GR1^+$ $Ly6G^-$ $Ly6C^{hi}$, respectively. MDSC subsets regulate immune responses by producing distinct immunosuppressive factors. For example, PMN-MDSCs preferentially produce reactive oxygen species, arginase-1, and prostaglandin $E_2$ to execute immune suppression, whereas Mo-MDSCs exploit nitric oxide and immunoregulatory cytokines and molecules such as IL-10, TGF-β, and PD-L1.[16,17] In addition to playing a role in tumor progression, infections, and transplant tolerance, MDSCs mitigate the progression of autoimmunity, including experimental autoimmune encephalomyelitis, inflammatory bowel disease, type I diabetes, and autoimmune uveitis.[18-20] MDSCs, in parallel with Treg cells, can be an important target in harnessing immune-mediated inflammatory diseases.

MDSCs and Treg cells represent the main immune-regulatory cells of innate and adaptive immune origin, respectively. Although it is conceivable that the 2 regulatory cells collaborate to dampen inflammatory processes, knowledge regarding whether and how they interact is scant. Similarly, although SCFAs exploit Treg cells as the effector of immune suppression, the role of MDSCs in SCFA immune regulation has not been investigated. In our current studies, we found that the microbial SCFA metabolites acetate, propionate, and butyrate promoted PMN-MDSC and Treg cell development and alleviated allergen-induced airway inflammation. In addition, the protective effect of SCFAs in ameliorating allergic inflammation of the airways was abrogated by depletion of PMN-MDSCs. We found that MDSCs induced Treg cell differentiation at least in part by surface TGF-β signaling irrespective of antigen engagement or specificity. These findings demonstrate a feedforward regulatory loop involving sequential induction of the PMN-MDSCs and Treg cells in mediating SCFAs' protection against allergic airway inflammation.

## METHODS

### Animals

Specific pathogen–free and germ-free female BALB/c mice, aged 3 or 5 weeks, were provided and maintained by the National Laboratory Animal Center in Taiwan. Germ-free mice were regularly tested for sterility by culturing and PCR analysis of feces amplifying the 16S rRNA genes. All animal procedures were performed according to the regulations approved by the Animal Ethical Committee of National Taiwan University and the National Laboratory of Animal Center.

### OVA-induced allergic airway inflammation model

To establish the allergen-induced murine allergic airway inflammation model, 5-week-old female BALB/c mice were sensitized by 2 intraperitoneal injections of ovalbumin (OVA) (grade V; Sigma-Aldrich, Burlington, Mass) on day 0 and day12, with the 2 injections containing 50 and 20 μg of OVA emulsified in 1% alum (Pierce Biotech, Rockford, Ill), respectively. Acute airway inflammation was induced by challenge consisting of a 30-minute inhalation of 5% OVA for 3 consecutive days (on days 19-21). In the indicated groups, mice were giving drinking water containing a cocktail of SCFAs consisting of 67.5 mM acetate, 25.9 mM propionate, and 40 mM butyrate beginning 2 weeks before allergen sensitization. In addition, where applicable, mice were given a GR1-depleting antibody (clone RB6-8C5; Bio X Cell, Lebanon, NH) at a dose of 10 mg/kg via the tail vein twice a week starting 7 days before intraperitoneal sensitization and inhalation challenge.

### BALF leukocytes

The mice were humanely killed by $CO_2$ asphyxia and their lungs were lavaged with PBS via a tracheal cannula to collect the bronchoalveolar lavage fluid (BALF). The total leukocytes in the BALF were enumerated, and differential leukocyte counts were enumerated by Giemsa- and May-Grünwald–stained cytospins, with a total of 500 cells counted per cytoslide. Cytokines and chemokines in the BALF were analyzed by ELISA or LEGENDplex bead–based immunoassays (BioLegend, San Diego, Calif).

### Measurement of airway hyperresponsiveness

Mice were anesthetized and exposed to escalating concentrations of methacholine (0-60 mg/mL, Sigma-Aldrich) via a tracheal cannula, and airway resistance was recorded by using the Buxco Pulmonary Mechanics System (Buxco Electronics, Wilmington, NC). Airway resistance (cm $H_2O$ × seconds per mL) at each individual methacholine concentration was recorded and compared with that of PBS after subtracting baseline resistance.

### Isolation of pulmonary leukocytes

Lungs were perfused with ice-cold PBS, excised, and gently minced in a strainer, followed by incubation at 37°C for 30 minutes in digestion buffer containing 1 mg/mL of collagenase IV and 0.5 mg/mL of DNase-I (Sigma-Aldrich). After completion of the digestion, leukocytes were suspended in Hanks balanced salt solution and centrifuged over a 60% Percoll gradient (GE Healthcare, Chicago, Ill) at 3200 rpm for 20 minutes.

### Generation of BM-derived conventional DCs

The mouse femurs and tibias were aseptically removed, and the BM was flushed with ice-cold PBS. After lysis of the RBCs, BM cells were cultured in complete 10% Dulbecco modified Eagle

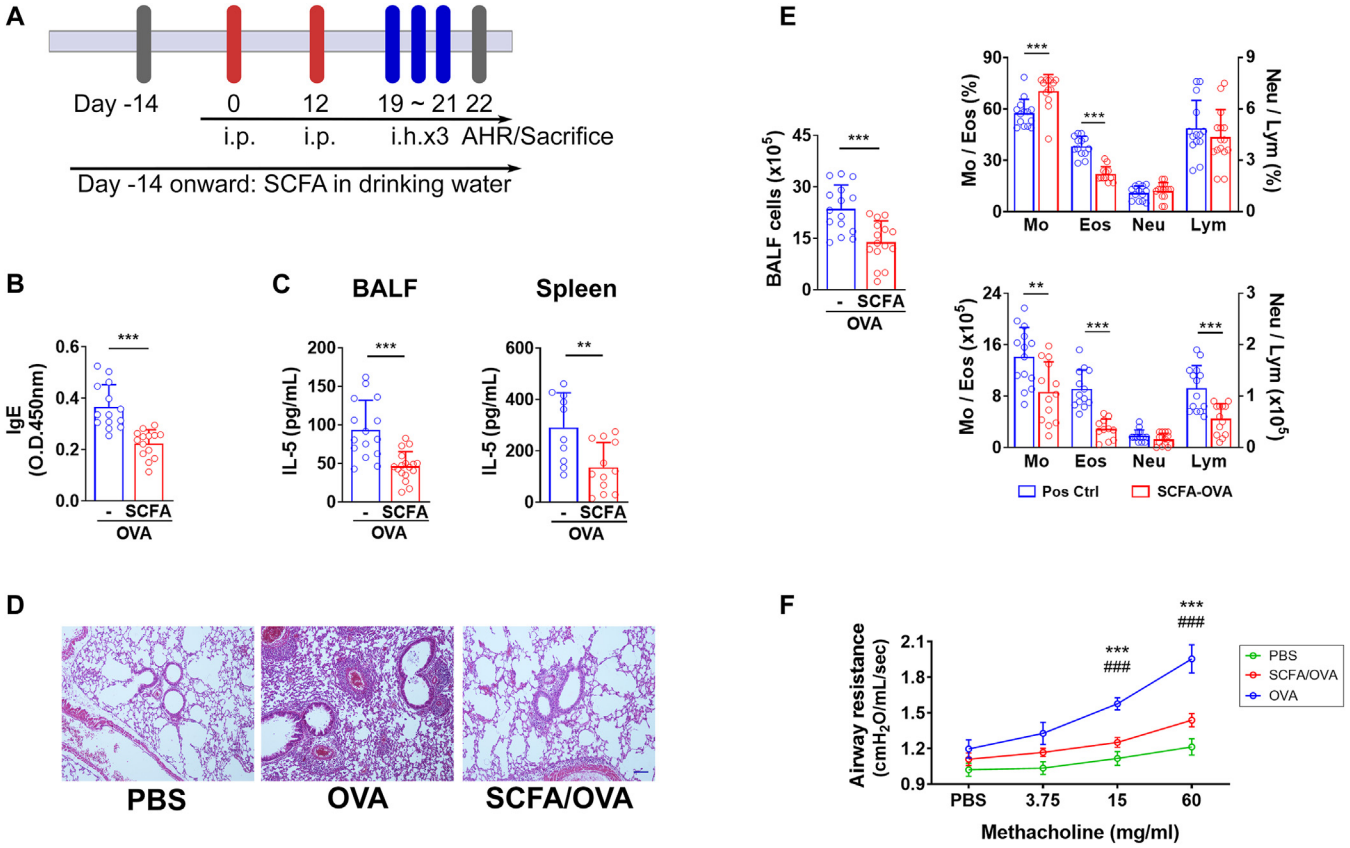


**FIG 1.** Alleviation of allergen-induced acute airway inflammation by SCFAs. **A,** Schematic diagram depicting the experimental protocol for establishing the OVA-induced murine allergic airway inflammation model and SCFA supplementation, as detailed in the Methods section. **B,** Serum levels of OVA-specific IgE antibody examined at 1 week after the second intraperitoneal (i.p.) OVA sensitization. **C,** IL-5 expression in BALF and culture supernatants of OVA-restimulated splenocytes. **D,** Microphotographs showed hematoxylin and eosin–stained lung tissues after aerosol OVA challenge. **E,** Total and differential BALF leukocyte counts enumerated by Giemsa- and May-Grünwald–stained cytospins. **F,** Airway hyperresponsiveness (AHR) presented as airway resistance in response to escalating concentrations of methacholine. n = 7 mice/group. Data shown represent 1 of 4 independent experiments. * and #, OVA-treated group compared with the PBS- and SCFA-treated groups, respectively. **B, C,** and **E,** Each symbol refers to 1 independently treated mouse. *P* values were determined by using an unpaired *t* test. $^{**}P < .01$; $^{***}P < .001$; $^{\#\#\#}P < .001$. *Eos,* Eosinophil; *i.h.,* inhalation; *Lym,* lymphocyte; *Mo,* monocyte; *Neu,* neutrophil; *Pos Ctrl,* positive control.

medium containing differentiation cytokines, IL-4 (1000 U/mL, Peprotech, Cranbury, NJ) and GM-CSF (500 U/mL, Peprotech) for 6 days. The purity of the DCs was examined by the expression of CD11C on the sixth day in culture.

## Isolation of MDSC subsets

Cells were incubated with the fluorescence-conjugated antibodies CD11b, GR-1, Ly6G, and Ly6C at 4°C for 30 minutes. After removal of excess antibodies, the cells were resuspended in ice-cold PBS. PMN-MDSCs and Mo-MDSCs were sorted on a FACSAria III flow cytometer (BD Biosciences, Franklin Lakes, NJ) according to the expression of $CD11b^{+}GR\text{-}1^{+}Ly6C^{lo}Ly6G^{+}$ and $CD11b^{+}GR\text{-}1^{+}Ly6C^{hi}Ly6G^{-}$, respectively.

## *In vitro* cell culture and stimulation

Mouse lung epithelial cells (LA-4, American Type Culture Collection CCL-196) were grown in 10% modified Ham F12 media. After being grown to confluence, the cells were treated with a cocktail of acetate, propionate, and butyrate (each at the same concentration, for a total concentration of 2 mM) for 16 hours, followed by stimulation with LPS (100 ng/mL, LPS-EB Ultrapure, Invivogen, San Diego, Calif). Supernatants were collected 48 hours after LPS stimulation and analyzed by using LEGENDplex bead–based immunoassays (BioLegend). For *in vitro* induction of Treg cells by SCFAs, naive $CD4^{+}$ T cells were negatively selected by using a magnetic bead–conjugated antibody cocktail per the manufacturer's protocol (BioLegend). $CD4^{+}$ T cells were stimulated with plate-bound anti-CD3ε antibody (1 μg/mL) plus anti-CD28 antibody (3 μg/mL) in the presence of various concentrations of acetate, propionate, and butyrate (0.1, 1, and 10 μM) or a mixture of the 3 components. $CD4^{+}$ $FoxP3^{+}$ Treg cells were examined by flow cytometry 72 hours later.

## Proliferation/suppression assay

Spleen cells were cultured in complete 10% Dulbecco modified Eagle medium at a density of $2 \times 10^{5}$ per well in a 96-well plate

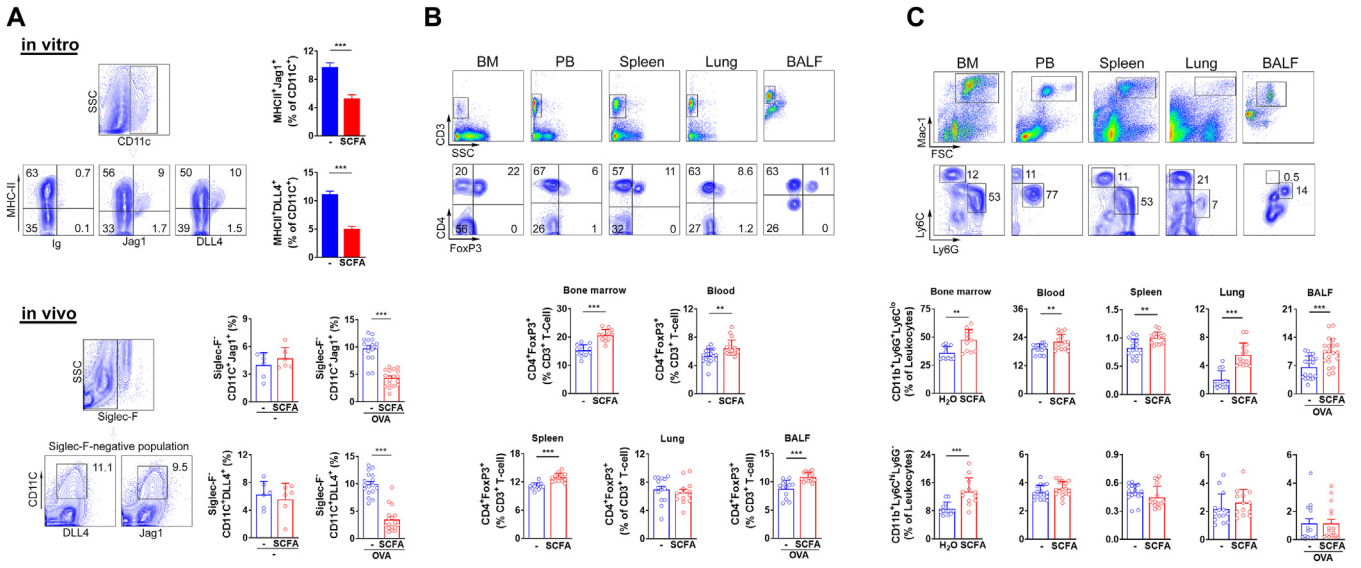


**FIG 2.** SCFAs dampened DC Notch signaling and induced Treg cell and MDSC differentiation. Three-week-old female BALB/c mice were given drinking water containing a cocktail of SCFAs for 2 weeks before initiation of OVA sensitization and challenges as depicted in Fig 1. *A.* **A,** Expression of Notch ligands Jag1 and DLL4 by BM-derived DCs after SCFA treatment (*upper*) or BALF DCs infiltrating into the lung during allergic airway inflammation (*bottom*). Populations of Treg cells (**B**) and MDSCs (**C**) in various tissues, including BM, peripheral blood (PB), spleen, lungs, and BALF, were analyzed by FACS. **A-C**, Each symbol refers to 1 independently treated mouse. **A,** (*top*) Data shown represent 1 of 3 independent *in vitro* culture each performed in triplicate. *P* values were determined by using an unpaired *t* test. $^{**}P < .01$; $^{***}P < .001$.

and stimulated with 200 μg/mL of OVA for 72 hours. Alternatively, for testing MDSC suppression function, DCs were seeded at a density of $5 \times 10^4$ per well and pulsed with 3 μg/mL of $OVA_{323\text{-}339}$ peptide for 1.5 hours before being cocultured with $5 \times 10^4$ $CD4^+$ T cells and $2.5 \times 10^4$ Mo-MDSCs or PMN-MDSCs. Proliferation status was evaluated by pulsing the cells with 1 μCi of [$^3$H]-TdR at 16 hours before harvesting by an automated multisample harvester. [$^3$H]-TdR incorporation was measured in a dry scintillation counter (Packard Instrument Co, Meridan, Conn).

## Induction of Treg cells by MDSCs

For the induction of Treg cells by MDSCs, $CD4^+$ T cells were stimulated with plate-bound anti-CD3ε plus anti-CD28 antibody and cocultured with sorted PMN-MDSCs or Mo-MDSCs at an MDSC-to–T-cell ratio of 1:1.5 to 1:10 for 3 days. Alternatively, for the induction of Treg cells by MDSCs under antigen-specific conditions, splenocytes were prepared from OVA-immunized mice and subjected to coculture with MDSC at MDSC-to–T-cell ratio of 1:1.5 to 1:10 in the presence of OVA (200 μg/mL) for 3 days. When indicated, TGF-β blocking antibody (Clone 19D8, BioLegend) was added to the coculture at a concentration of 10 μg/mL.

## Quantitative real-time PCR

For gene expression, cDNA was synthesized by RT-PCR, and gene expression was amplified by using sequence-matched primer pairs and SYBR Supermix (Roche, San Francisco, Calif) in an Applied Biosystems 7900 real-time PCR system (Applied Biosystems, San Francisco, Calif). The data were normalized with expression of glyceraldehyde-3-phosphate dehydrogenase.

## Statistics

Data were expressed as means plus or minus SDs. Statistical comparisons between groups were made by 1-way ANOVA, or unpaired *t* test. A *P* value less than .05 was considered statistically significant.

# RESULTS

## SCFAs alleviate allergen-induced airway inflammation

In recent years, gut microbiota and their particular metabolizing ability have been shown to shape the host immune system and participate in the pathogengesis of allergic airway disease. To investigate the function of SCFAs in regulating allergen-induced airway inflammation, mice were given a cocktail of acetate, propionate, and butyrate before induction of allergic airway inflammation (Fig 1, A and see Fig E1 in the Online Repository at www.jaci-global.org).

In accordance with previous studies supporting an anti-inflammatory role of SCFAs, we found that SCFA-treated mice produced lower levels of OVA-specific IgE antibody (Fig 1, *B*). IL-5 secretion in the BALF after aerosol OVA challenge and in supernatants of OVA-restimulated splenocytes was significantly decreased in SCFA-treated mice versus in untreated controls (Fig 1, *B* and *C*). Leukocytes' infiltration into the lungs after aerosol OVA challenge, in particular, infilatration of eosinophils, lymphocytes, and macrophages, was significantly reduced after SCFA supplementation (Fig 1, *D* and *E*). Airway

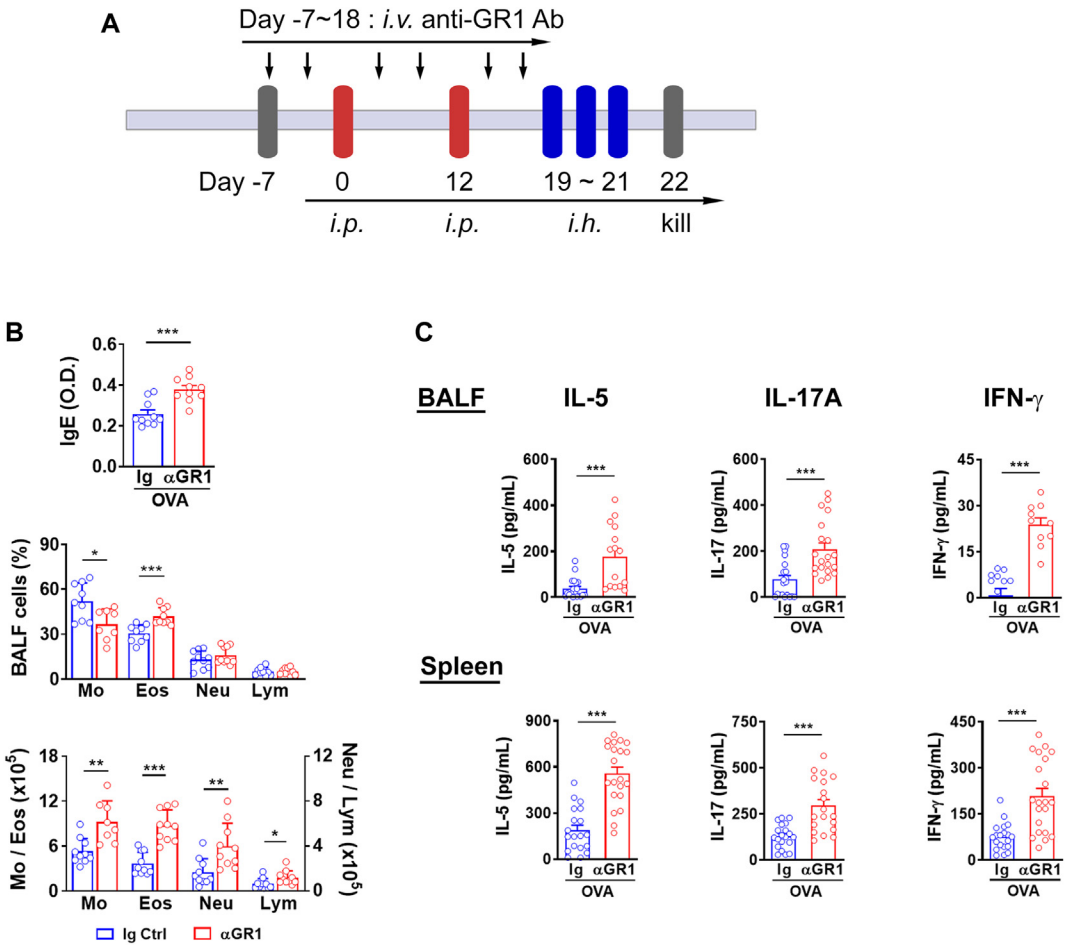


**FIG 3.** MDSC depletion aggravated allergic airway inflammation. **A,** Diagram depicting the experiment protocol of MDSC depletion along the course of OVA-induced airway inflammation. Briefly, the indicated mice were given GR1-depleting antibody twice a week commencing 1 week before OVA sensitization and continued until aerosol OVA challenge. **B,** Serum levels of OVA-specific IgE antibody were measured by ELISA, and leukocyte subpopulations in BALF were enumerated by Giemsa- and May-Grünwald–stained cytospins. **C**, Cytokine expression in BALF and culture supernatants of OVA-restimulated splenocytes were measured by ELISA. Each dot refers to 1 independently treated mouse. *P* values were determined by using an unpaired *t* test. $*P < .05$; $**P < .01$; $***P < .001$. *i.h.,* Inhalation; *i.p.,* intraperitoneal; *i.v.,* intravenous.

hyperrespnsiveness, as provoked by exposing the mice to escalating concentrations of the brchoconstrictor methacholine, showed significantly less airway constriction in SCFA-treated mice than in the asthmatic controls (Fig 1, *F*). Collectively, these data demonstrated a protective role of SCFAs in allergen-induced airway inflammation.

## SCFAs inhibit Notch signaling and induce Treg cell and MDSC differentiation

To investigate the mechanisms underlying the protective function of SCFAs, we first examined the effects of SCFAs on DCs. Notch is a pleiotropic signaling family partipating in various physiologic and pathologic processes. We and others have previously demonstrated the distinct role of DC-derived Notch signaling in allergic airway inflammation.[21-24] In this respect, we found that SCFA treatment downregulated expression of the Notch ligands Jag1 and DLL4 by BM-derived DCs, suggesting inhibition of DC Notch signaling by SCFAs (Fig 2, *A*). Analysis of Notch ligand expression on DCs isolated from the lungs showed that SCFA supplementation alone did not induce a substantial change in DC Notch ligand expresion compared with that in mice given plain drinking water. However, when mice were rendered asthmatic by OVA sensitization and challenge, the lung DCs from mice supplemented with SCFAs expressed significantly lower levels of both Jag1 and DLL4 than did the asthmatic mice given plain drinking water (Fig 2, *A*). Given the role of the Notch signaling family as a signaling hub in the pathogengesis of allergic inflammation,[21-24] our data suggest that SCFAs mitigated allergic airway inflammation via suppression of Notch signaling, which in turn dampened subsequent recruitment of inflammatory leukocytes.

SCFAs regulate development of the immune system. Notably, SCFAs have been shown to induce *de novo* generation and accumulation of colonic Treg cells.[8-10] Treg cells are well known to dampen inflammation of the airways and alleviate the severity of allergic airway disease. It is conceivable that SCFAs ameliorate allergic airway inflammation by induction of the immunosuppressvie cell populations. We therefore focused on 2 cell populations with crucial immune-regulatory functions,

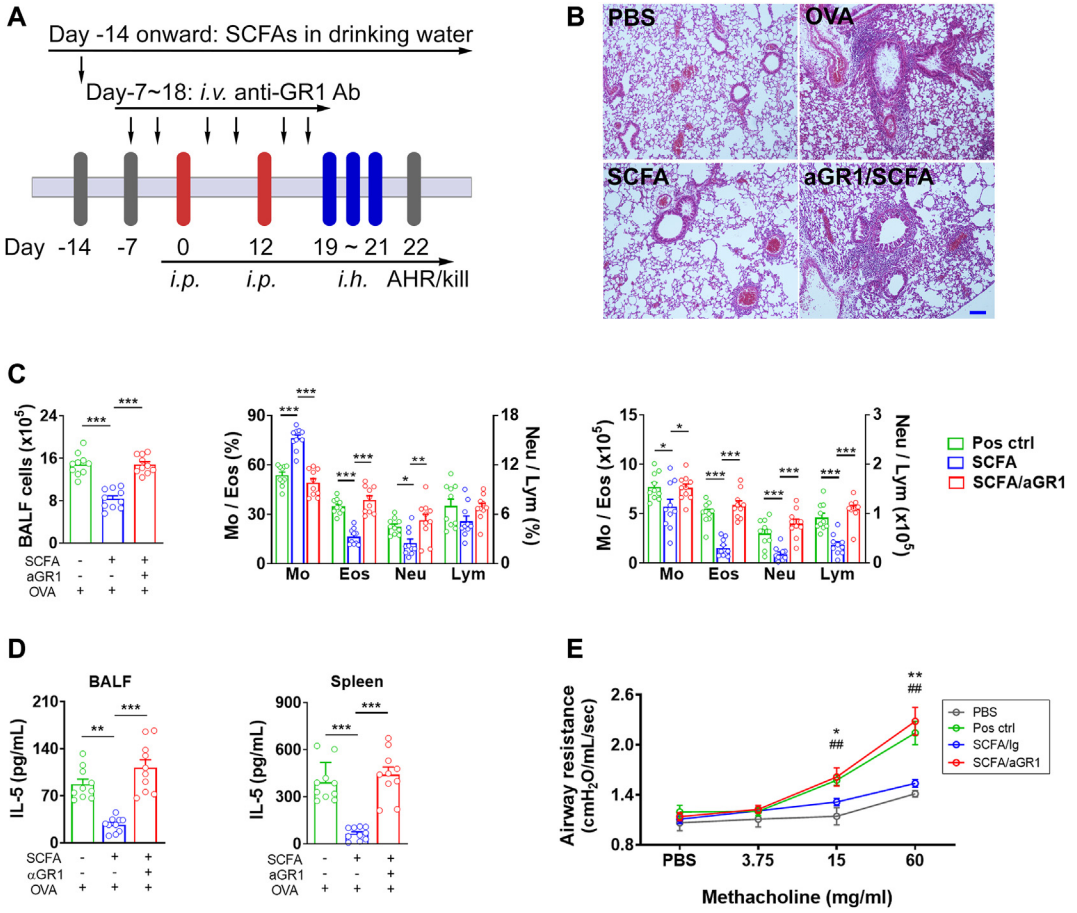


**FIG 4.** Alleviation of airway inflammation by SCFAs was abrogated after MDSC depletion. **A,** Diagram showing the experiment protocol of MDSC depletion and SCFA supplementation in OVA-induced murine allergic airway inflammation model. **B**, Microphotographs showing hematoxylin and eosin–stained lung sections taken at 1 day after OVA challenge. **C,** Total and differential leukocyte counts in the BALF were enumerated by cytospins. **D,** IL-5 expression in BALF and supernatants of OVA-restimulated splenocytes was examined by ELISA. **E,** Airway hyperresponsiveness (AHR) in response to escalating concentrations of methacholine. n = 7 mice/group. Data shown represent 1 of 2 independent experiments. *Positive control and anti-GR1 groups compared with the SCFA group; #Positive control and anti-GR1 groups compared with the PBS group. **C** and **D,** Each symbol refers to 1 independently treated mouse. *P* values were determined by using an unpaired *t* test or 1-way ANOVA. * $P < .05$; # $P < .05$; ** $P < .01$; ## $P < .01$; *** $P < .001$. *Eos,* Eosinophil; *Lym*, lymphocyte; *Mo*, monocyte; *Neu*, neutrophil; *Pos Ctrl*, positive control.

namely, MDSCs of innate origin and Treg cells of the adaptive immune system (Fig 2, *B*). In mice given SCFA-containing drininking water, Treg cells in the BM, peripheral blood, and spleen were significantly enriched versus in mice receiving plain drinking water. In contrast to what was found in the hematopoietic and lymphoid tissues, SCFAs had no effect on Treg cell differentiation of the lungs. However, when mice were renderd asthmatic by OVA sensitization and challenge, an increased Treg cell population was observed in the BALF collected from SCFA-treated mice.

We previously found that MDSCs were underrepresented in the lungs and guts of the germ-free mice compared to mice housed under specific pathogen–free conditions (see Fig E2 in the Online Repository at www.jaci-global.org).

These findings highlighted the requirement of microbiota and/or microbial metabolites in MDSC development. We hence examined 2 MDSC subpopulations, $CD11b^+GR1^+Ly6G^+Ly6C^{lo}$ PMN-MDSCs and $CD11b^+GR1^+Ly6C^{hi}Ly6G^-$ Mo-MDSCs, at various tissue sites after SCFA supplementation (Fig 2, *C*). Although the numbers of PMN-MDSCs significantly expanded in the BM, peripheral blood, spleen, and lungs after SCFA supplementation, the number of Mo-MDSCs showed substantial expansion only in the BM and not in peripheral blood, spleen, or lungs. In mice rendered asthmatic by OVA sensitization and challenge, the numbers of PMN-MDSCs, but not Mo-MDSCs, were also increased in the BALF of SCFA-treated mice. These data demonstrated that SCFAs induced systemic expansion of the immunosuppresive cell populations, the Treg cells and PMN-MDSC subpopulation of MDSCs. It is conceivable that these cells executed the immune suppression effects of SCFAs and ameliorated inflammaiton of the airways.

## Depletion of PMN-MDSCs aggravates allergen-induced airway inflammation

The protective effect of Treg cells in allergic asthma is well known. Because SCFAs induced expansion of both PMN-MDSCs and Treg cells in parallel with dampening of allergic

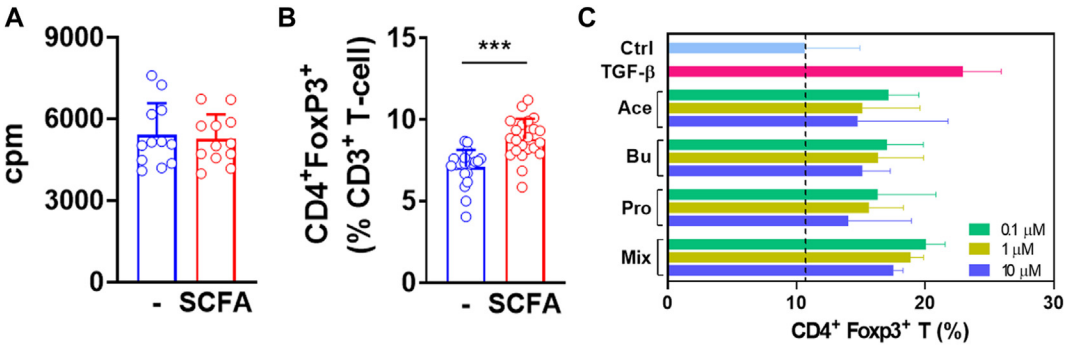


**FIG 5.** SCFAs enhanced Treg cell differentiaion. Female BALB/c mice were given SCFA-supplemented drinking water from the age of 3 weeks, followed by OVA sensitization and challenges as described. **A,** *Ex vivo* proliferation of SCFA-primed splenocytes in response to OVA restimulation. **B,** *Ex vivo* Treg cell differentiation of SCFA-primed splenocytes after OVA restimulation. **C,** Differentiation of $CD4^+$ T cells to Treg cells after SCFA treatment *in vitro*. **A** and **B,** Each symbol represents 1 independently treated mouse. *P* values were determined by using an unpaired *t* test or 1-way ANOVA. $^{**}P < .01$; $^{***}P < .001$. *Ctrl,* Control.

inflammation, we investigated whether the innate immunosuppressive PMN-MDSCs exert beneficial regulation against allergic inflammation of the airways (Fig 3, *A*). The GR1-depleting antibody that we used in this study exhibited a preferential depletion of the PMN-MDSCs owing to their high GR1 expression but not the depletion of the Mo-MDSCs, which express low levels of GR1. It eliminated more than 95% and no more than 50% of the PMN-MDSC and Mo-MDSC subpopulations, respectively (see Fig E3 in the Online Repository at www.jaci-global.org).

With depletion of the PMN-MDSCs, mice developed exaggerated airway inflammation and asthma severity compared to mice receiving a control antibody. Enhanced OVA-specific IgE production and leukocyte infiltration of the lungs (Fig 3, *B*), accompanied by increased production of the inflammatory cytokines IL-5, IL-17A, and IFN-γ in BALF and by OVA-restimulated splenocytes, were found in mice depleted of PMN-MDSCs (Fig 3, *C*). The aggravated inflammatory phenotypes found after depletion of the PMN-MDSCs suggest that PMN-MDSCs function in the same immunosuppressive path as Treg cells during allergic airway inflammation.

## PMN-MDSCs mediate SCFA-elicited immune regulation

After showing that PMN-MDSCs play a regulatory role in allergic airway disease, we investigated whether PMN-MDSCs act as the effector mechanism downstream of SCFA immune-regulatory pathway. To this end, mice were given SCFA-containing drinking water, followed by GR1 antibody–mediated depletion of the PMN-MDSCs along the course of the OVA sensitization and challenge protocol (Fig 4, *A*).

The role of PMN-MDSCs in SCFA immune regulation was revealed by the severity of the asthma phenotype in SCFA-treated and PMN-MDSC–depleted mice. We found that in mice supplemented with SCFAs, PMN-MDSC depletion was capable of abrogating the protective effecets of SCFAs in OVA-induced aiway inflammation compared to their SCFA-fed PMN-MDSC–untouched counterparts. With PMN-MDSC depletion, the lungs were severely inflammed, as exhibited by enhanced leukocyte infiltration (Fig 4, *B* and *C*), increased IL-5 production both in the BALF and by OVA-restimulated splenocytes (Fig 4, *D*), and enhanced airway hyperresponsiveness (Fig 4, *E*). These findings support an indispenable role of PMN-MDSCs in SCFA-elicited regulation of allergic inflammation.

## SCFAs instruct Treg cell differentiaion

Although SCFA treatment elicited immunosuppressive effects, whether the SCFAs caused intrinsic defects in T-cell immune activation is not clear. To address this, splenocytes were isolated from OVA-sensitized mice and restimulated *ex vivo*. The results showed that splenocytes from SCFA-treated mice proliferated undifferentially from spleen cells of the control mice, supporting an immunocompetent state of the T cells after SCFA treatment (Fig 5, *A*).

In addition, we found that a significantly larger population of $CD4^+$ T cells from SCFA-treated mice differentiated into $CD4^+$ $FoxP3^+$ Treg cells following *ex vivo* restimulation (Fig 5, *B*). In accordance with the *ex vivo* findings, naive $CD4^+$ T cells that had been activated in the presence of either acetate, propionate, butyrate, or a mixture of the differentated to Treg cells more prominantly than the untreated controls did (Fig 5, *C*). These data suggest that SCFAs exert a priming effect toward establishment of an immune-tolerant state and which, at lease in part, through independently foster the differentiaion of Treg cells.

## Reciprocal interaction of MDSCs and Treg cells

The immunosuppressive effects of MDSCs have been attributed to the expression of inducible nitric oxide synthase, arginase-1, Cox2, reactive oxygen species, and various immune-regulatory factors under different pathologic conditions. In our study, we found that PMN-MDSCs and Mo-MDSCs isolated from the lungs of the asthmatic mice expressed comparable levels of inducible nitric oxide synthases and arginase-1, whereas PMN-MDSCs expressed significantly higher levels of COX-2 than Mo-MDSCs did (Fig 6, *A* and *B*). The immunosuppressive effect of the MDSC subpopulations was confirmed by examination of the $CD4^+$ T-cell proliferation response in MDSC–$CD4^+$ T-cell coculture, wherein $CD4^+$ T-cell proliferation was significantly inhibited by both PMN-MDSCs and Mo-MDSCs, although the Mo-MDSCs were more potent than the PMN-MDSC subpopulation (Fig 6, *C*). Our findings that depletion of PMN-MDSCs abrogated the protective effects of SCFAs in allergic airway inflammaion hinted at a potential role of MDSCs in Treg cell differentiation. To

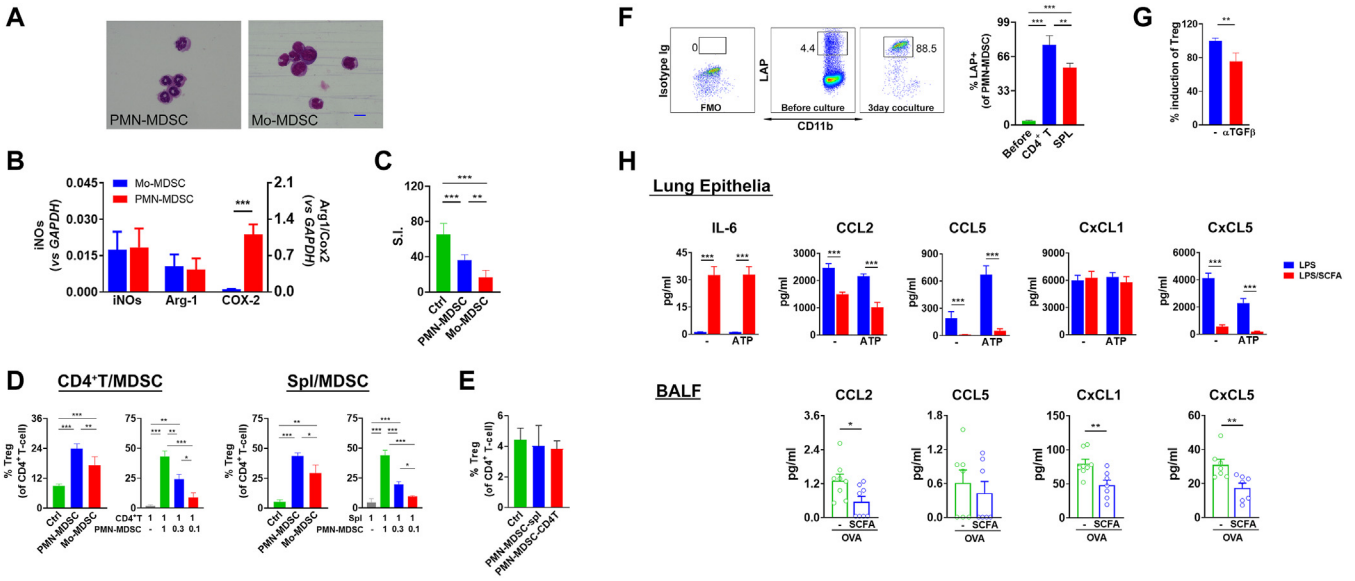


**FIG 6.** MDSCs and Treg cells mediated the immunosuppressive effects of SCFAs. PMN-MDSCs and Mo-MDSCs from the lungs of the asthmatic mice were subjected to fluorescence-activated cell sorting by the expresson of CD11b, Ly6G, and Ly6C after aerosl OVA challenge. **A,** Microphotographs showed the morphology of Liu-stained PMN-MDSCs and Mo-MDSCs. Scale bar = 10 μm. **B,** Gene expressions of the FACS-sorted lung Mo-MDSCs and PMN-MDSCs. **C,** Suppression of $OVA_{323-339}$-elicited $CD4^+$ T-cell proliferation by MDSC subpopulations. Alternatively, to investigate induction of Treg cells by MDSCs, BM PMN-MDSCs and Mo-MDSCs were FACS–sorted and cocultured with $CD4^+$ T cells or spleen cells. **D,** Differentiation of Treg cells from purified $CD4^+$ T cells and splenocytes after MDSC coculture under nonspecific T-cell activation and antigen-specific (OVA) stimulation, respectively **E,** Cell contact–dependent induction of Treg cells by MDSCs was examined in a transwell coculture. **F,** Surface TGF-β expression by PMN-MDSCs after a 3-day coculture with $CD4^+$ T cells and splenocytes was examined by flow cytometry. **G,** Induction of Treg cells by PMN-MDSCs in the presence of an TGF-β–blocking antibody (10 μg/mL). **H,** Chemokine expression by lung epithelial cells and in the BLAF after SCFA treatment. The data shown represent 1 of 3 independent experiments, each performed in triplicate. *P* values were determined by using an unpaired *t* test or 1-way ANOVA. $^{**}P < .01$; $^{***}P < .001$.

address this speculation, PMN-MDSCs and Mo-MDSCs from the BM were sorted and coincubated with either isolated $CD4^+$ T cells or splenocytes under broad stimulation by anti-CD3 antibody or antigen (OVA), respectively. The results showed that differentaion of $CD4^+$ T cells to Treg cells was significantly enhanced by the presence of both Mo-MDSCs and PMN-MDSCs irrespective of antigen recognition or antigen specificity (Fig 6, *D*). In addition, the induction of Treg cells by MDSCs was abolished when T cells and MDSCs were cocultured in a transwell plate, suggesting a cell contact–dependent mechaism in MDSC regulation of Treg cell differention (Fig 6, *E*).

Surface TGF-β associated with latency-associated peptide (LAP) is known to mediate contact-dependent immune suppression and induce Treg cell development. Because we found that MDSCs also required cell contacts to induce Treg cell development, we examined the expression of surface TGF-β-LAP by PMN-MDSCs. The data showed that although resting PMN-MDSCs expressed low levels of LAP, the expression was significantly increased after 3 days of coculture with $CD4^+$ T cells or splenocytes (Fig 6, *F*). Nonetheless, when $CD4^+$ T cells and PMN-MDSCs were coincubated in the presence of an TGF-β blocking antibody, the induction of Treg cells was inhibited by 30%, suggesting the TGF-β pathway as a nonexclusive mechanism in MDSC induction of Treg cells (Fig 6, *G*). Collectively, these results demonstrate that SCFAs induced Treg cell differentiation by both direct effects on the T cells and also through MDSC cross talk with the T cells in a cell contact–dependent manner, which at least in part involved TGF-β.

We next explored the possible mechanism whereby SCFAs stimulated expansion of the MDSC population (Fig 6, *H*). Lung epithelail cells were primed with a cocktail of acetate, propionate, and butyrate, followed by stimulation with LPS. We found that secretion of the proinflammatory chemokines CCL2, CCL5, and CxCL5 in response to LPS were suppressed in the presence of SCFAs, whereas CxCL1 secretion was not affected. On the contrary, the level of IL-6, the cytokine shown in previous publications to promote MDSC differentiation, was significantly increased by SCFA treatment. These data suggest that SCFAs not only dampened epithelial inflammation by suppressing chemokine secretion but also concurrently promoted MDSC differentiation by IL-6 production, which is in accordance with the findings that PMN-MDSCs are also enriched in the BM after SCFA treatment (shown in Fig 2, *C*). We also examined the expression of these chemokines in BALF. Consistent with mitigated airway inflammation after SCFA supplementaion, the levels of expression of CCL2, CxCL1, and CxCL5 were reduced in SCFA-treated mice versus in their asthmatic counterparts.

## DISCUSSION

In pursuing the preventive and therapeutic modalities to harness allergic airway diseases, we found that SCFAs exert a

protective role through reenforcement of the innate and adaptive immune-regulatory pathways. We revealed PMN-MDSCs as the key effector mechainsm mediating the immune-regulatory function of SCFAs by the findings that depletion of PMN-MDSCs abrogated the protective effects of SCFAs in allergic airway inflammaion. SCFA supplementation augmented the differentiation of both PMN-MDSCs and Treg cells. We found that the presence of PMN-MDSCs enhanced Treg cell development during T-cell activation via a cell contact–dependent mechanism and irrespective of antigen recognition or specificity. In parallel, we found that in addition to functioning through reciprocal interaction of MDSCs and T cells to induce Treg cell development, SCFAs also orchestrated Treg cell differentiation by exerting a direct effect on the T cells.

In our current study, we have demonstrated the protective role of PMN-MDSCs in allergic inflammation of the airways by showing an aggravated asthma phenotype after GR1 antibody–mediated PMN-MDSC depletion. In line with our findings, depletion of $GR1^{+}CD11c^{int}$ plasmacytoid DCs (pDCs), also by GR1 antibody, has been shown to cause enhanced allergen sensitization and inflammation of the airways.[25] Although the allergy-ameliorating effect of pDCs was further confirmed by using a pDC-selective depleting antibody and pDC adoptive transfer, the effects of PMN-MDSCs in the setting of GR1 antibody–mediated pDC depletion were not addressed. SCFAs have been shown to regulate DC development and activation, generating DCs with less activating capacity and a tolerizing phenotype.[12,26-28] On the other hand, the effects of SCFAs on pDCs are thus far largely unknown. Although we did not investigate the contribution of $GR1^{+}CD11C^{int}$ pDCs in lessening the allergic airway responses in our studies, our findings that SCFA treatment enhances PMN-MDSC expansion, PMN-MDSCs are necessary to SCFA-exerted immune regulation, and a relationship of reciprocal regulation exists between PMN-MDSCs and Treg cells all support the regulatory effects of PMN-MDSCs downstream of SCFAs in allergic inflammation of the airways.

The gut microbiota is constantly being shaped by the gut microenvironment, and it tends to coevolve with the host; changes in diet led to an altered microbiome in the intestines and, to a lesser extent, in the lungs.[12] An accumulating body of work has linked the gut microbiota with both health and disease[29-31] and indicated microbial metabolites as the crucial executors of microbial effects on the host. Among the microbial metabolites, SCFAs are produced in the gut by microbial fermentation of indigestible carbohydrate and dietary fibers, and they act to provide several health benefits, including amelioration of various inflammatory responses and improved metabolism.[5] SCFAs have been shown by studies using different approaches to play a protective role in allergic airway disease. In a series of these studies, supplementation of SCFAs or a high-fiber diet with high SCFA production elicited ameliorating effects in allergic airway disease.[11-13] Studies have also shown that SCFAs promote extrathymic generation of Treg cells[8-10] and Tr1 cells[26] via histone modification. In line with these findings, SCFAs, as they were produced from dietary fiber, induced differentiation of Treg cells directly by inhibition of HDAC9 and enhanced acetylation of the FoxP3 locus and protein.[13] In addition to modulating T-cell development, SCFAs induced hematopoiesis of DCs, which subsequently adopted an immature phenotype with impaired allergen presentation and activation capacity and swayed T-cell polarization from a $T_H2$ cell fate.[12] In accordance with the findings obtained from using the high-fiber diet strategy, in a study of gut helminth infection, the gut infection caused changes in the commensal microbial composition that resulted in increased SCFA production and amelioration of allergic asthma in a GPR41-dependent manner.[32] These proof-of-principle studies highlighted the interaction between diet, microbiota, and microbial metabolites in shaping the immune niche and disease process, and they showed that it can thus be a therapeutic target for allergic inflammatory disease in the lung and potentially in other organs.

Nonetheless, in contrast to our findings and those reported by others,[11-13] some studies have also shown that the protective effects of SCFAs in allergic airway inflammation are found only in a dysbiotic state and not in the presence of a healthy microbiome.[27] In these studies, oral SCFAs were not able to restore the dysbiotic microbial composition resulting from antibiotic treatment, and similarly in other report, SCFAs failed to effectively influence generation of Treg cells in the presence of a healthy microbiome.[9] These discrepant results can be attributed to the discrete mode of action by the individual SCFAs administered; the routes for supplying SCFAs, which may allow possible uptake of the SCFAs in the gastrointestinal tract; the exact experimental settings, such as the subject's age at which the SCFAs were given and the duration of SCFA supplementation; tissue-specific factors; and the background microbiome, which may vary among the animal facilities housing these animals.[5]

Although the current paradigm for the microbial functions in $T_H2$ cell–mediated inflammation focuses mainly on the induction of Treg cells, our study provides a novel angle for execution of microbial immune regulation in allergic airway inflammation involving microbial metabolites and interactive immune-regulatory cell populations, namely, PMN-MDSCs and Treg cells. Through sequential activation and induction of the innate PMN-MDSC and adaptive Treg cell populations, SCFAs link the gut microbiota to orchestration of the inflammatory process in asthma.

Earlier studies have identified $CD11b^{+}Gr\text{-}1^{hi}$ MDSC-like cells in the lungs of asthmatic mice as the master regulator of airway inflammatory response.[33] Among them, both the $Ly6C^{+}Ly\text{-}6G^{-}$ monocytic and the $Ly6C^{+}Ly\text{-}6G^{+}$ granulocytic MDSC-like cells suppressed T-cell activation and downregulated antigen-induced airway hyperresponsiveness. In this study, $Ly6C^{+}Ly\text{-}6G^{+}$ granulocytic MDSC-like cells suppressed T-cell activation in an arginase-dependent manner; however, their relationship with Treg cells was not examined. On the other hand, $Ly6C^{+}Ly6G^{-}$ monocytic MDSC-like cells induced very moderate expansion of the Treg cells; instead, they recruited Treg cells into the lungs by secretion of chemokine CCL22. In this regard, studies of MDSCs in transplantation tolerance and tumor immunology also demonstrated that MDSCs fulfilled immunosuppressive effects by secretion of chemokine, including CCL3, CCL4, and CCL5, which attracted Treg cell trafficking into the tissues.[34,35]

The first evidence that MDSCs are able to induce the development of Treg cells came from a study in tumor-bearing mice.[36] In this study, the induction of Treg cell development by $Gr\text{-}1^{+}CD115^{+}$ Mo-MDSCs was dependent on the secretion of IL-10 and IFN-γ and independent of the nitric oxide–mediated suppression mechanism.[36] The study further demonstrated that it was the IFN-γ secreted by antigen-activated T cells that stimulated IL-10 and TGF-β production by $Gr\text{-}1^{+}CD115^{+}$ Mo-MDSCs. These findings depict a reciprocal interaction and sequential activation of T cells and MDSCs during the height of

an inflammatory reaction and at the resolution of this process, wherein MDSCs activated by T-cell–derived inflammatory cytokines in return promote the differentiation of Treg cells to resolve the inflammation. The reciprocal interaction between T cells and MDSCs was further demonstrated in a model of murine colitis showing that Treg cell–derived TGF-β is a crucial regulator of MDSC expansion and function and that the populations establish a positive feedback loop under proinflammatory conditions. In this model, whereas PMN-MDSCs supported the expansion of Treg cells, Treg cells in return promoted differentiation and function of PMN-MDSCs by secretion of TGF-β.[37]

Nonetheless, whether and how PMN-MDSCs induced Treg cell development was not thoroughly investigated. TGF-β expressed on the surface of Treg cells in association with LAP has been shown to mediate the immune suppression function of Treg cells[38] and induce Foxp3 expression and Treg cell function in responder cells.[39,40] In addition, the expression of TGF-β–LAP on immature DCs supports Treg cell differentiation and survival.[41] In our present study, we found that PMN-MDSCs significantly promote Treg cell differentiation following either antigen-specific or nonspecific T-cell activation in a cell contact–dependent manner. We further demonstrated that PMN-MDSCs upregulate the expression of membrane-bound TGF-β when cocultured with T cells. In line with our findings, previous studies have shown that human $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs upregulated the expression of membrane-bound TGF-β over time when cocultured with $CD4^{+}$ T cells, whereas blockage of TGF-β drastically reduced the frequency of Treg cells, supporting a TGF-β–dependent induction of Treg cells by MDSCs.[42] In our study however, despite the fact that substantial upregulation of LAP expression by PMN-MDSCs was found after coculture with T cells, blockage of TGF-β prevented Treg cell differentiation by only 30%, suggesting additional mechanisms in MDSCs' induction of Treg cells.

The immune system is constantly being shaped by various microbial encounters in life encompassing not only pathogens but also commensal microbes. In the context of the commensal microbiota, recent advances in metagenomic sequencing, bioinformatics, and multi-omics technologies have brought to light previously underappreciated functions of the microbiota in health and disease and uncovered the gut-lung axis, the gut-brain axis, and so forth. The present study has depicted a prototypical interplay in which metabolic signals originated from specific commensal microbial species promote development of immunosuppressive cells, MDSCs, and Treg cells, which act in concert to execute the immune-regulatory function of the microbial metabolites against inflammatory diseases. Thus, the application of commensal microbiota in mitigating disease pathogenesis is not limited to the utilization of the probiotics *per se* but instead extends to postbiotics, such as the microbial metabolites.

## DISCLOSURE STATEMENT

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