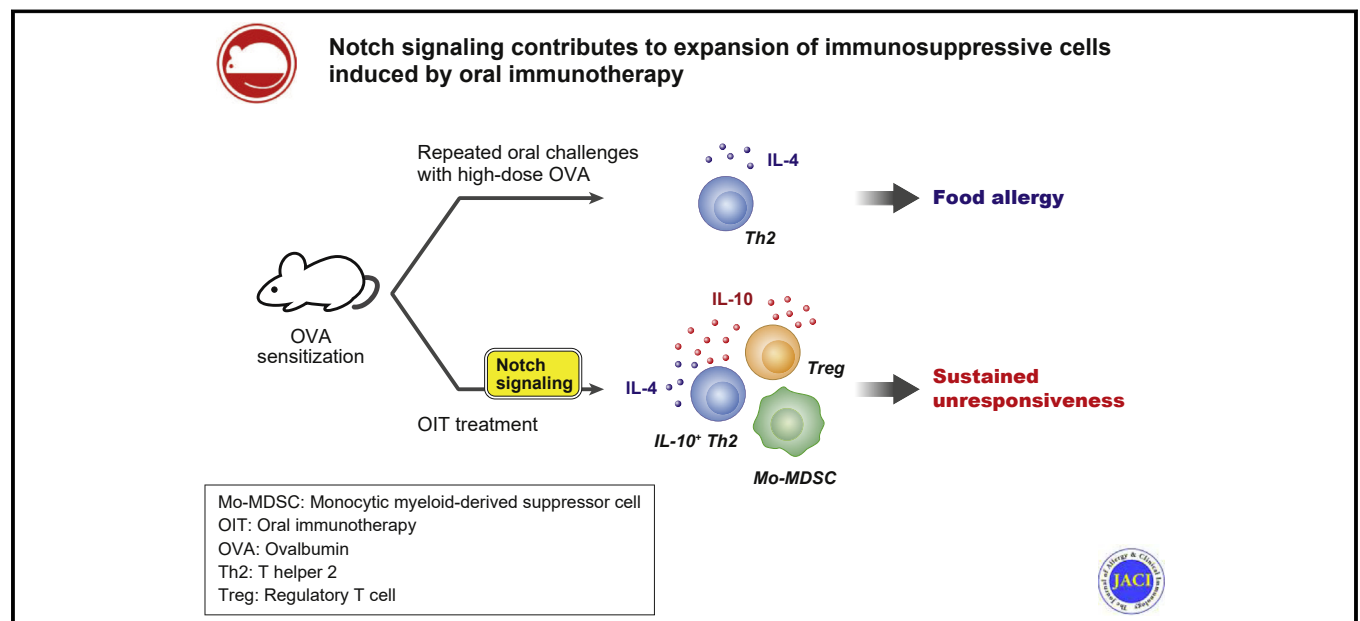


Notch signaling contributes to the establishment of sustained unresponsiveness to food allergens by oral immunotherapy

Toshiyuki Yoneyama, MD,^a Nobuhiro Nakano, PhD,^b Mutsuko Hara, PhD,^b Hiromichi Yamada, MD,^a Kumi Izawa, MD, PhD,^b Koichiro Uchida, MD, PhD,^c Ayako Kaitani, PhD,^b Tomoaki Ando, MD, PhD,^b Jiro Kitaura, MD, PhD,^b Yoshikazu Ohtsuka, MD, PhD,^a Hideoki Ogawa, MD, PhD,^b Ko Okumura, MD, PhD,^b and Toshiaki Shimizu, MD, PhD^a Tokyo, Japan

GRAPHICAL ABSTRACT



Background: Oral immunotherapy (OIT) aims to establish desensitization and sustained unresponsiveness (SU) in patients with food allergy by ingestion of gradually increasing doses of specific food allergens. However, little is known about the mechanisms by which OIT induces SU to specific allergens. **Objectives:** We investigated the role of Notch signaling, which controls cell fate decisions in many types of immune cells in the induction of SU by OIT treatment.

Methods: Two types of mouse models, ovalbumin-induced food allergy and OIT, were generated. To elucidate the role of Notch signaling in OIT-induced SU, mice were intraperitoneally injected with the Notch signaling inhibitor *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester during the OIT treatment period. **Results:** Ovalbumin-sensitized mice were desensitized and also had SU induced by OIT treatment, whereas repeated challenges with ovalbumin caused the development of severe allergic reactions in ovalbumin-sensitized mice. Administration of *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester to mice during the OIT treatment period inhibited the establishment of SU to ovalbumin but did not affect the induction of desensitization. OIT induced a systemic expansion of IL-10-producing CD4⁺ T cells, including Th2 cells, and myeloid-derived suppressor cells (MDSCs), particularly the monocytic MDSC subpopulation. Inhibition of Notch signaling prevented the OIT-induced expansion of those cells. *In vitro* cultures of bone marrow cells showed that Notch signaling directly promoted the generation of monocytic MDSCs. In addition, the contribution of MDSCs to OIT-induced SU was confirmed by MDSC depletion with the anti-Gr1 antibody.

From ^athe Department of Pediatrics and Adolescent Medicine, and ^bthe Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine, Tokyo, and ^cthe Juntendo University Advanced Research Institute for Health Science, Tokyo.

Supported in part by the Ministry of Education, Culture, Sports, Science and Technology Grants-in-Aid for Scientific Research program (grant 18K08416) and Research Grant 2018 from the Foundation for Dietary Scientific Research.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication August 28, 2019; revised June 2, 2020; accepted for publication July 1, 2020.

Corresponding author: Nobuhiro Nakano, PhD, Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan. E-mail: nbnakano@juntendo.ac.jp.

0091-6749/\$36.00

© 2020 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2020.07.011>

Conclusion: Notch signaling contributes to the establishment of SU induced by OIT through systemic expansion of immunosuppressive cells, such as IL-10-producing CD4⁺ T cells and MDSCs. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: Food allergy, oral immunotherapy, sustained unresponsiveness, Notch signaling, myeloid-derived suppressor cell

Food allergies are a growing public health concern worldwide. The prevalence of food allergies in preschool children is now 10% in developed countries and around 7% in the rapidly developing economies of Asia.¹ Currently, there are no therapeutic agents for food allergies, so it is therefore extremely important to develop effective therapeutic methods for treatment. Oral immunotherapy (OIT) has been explored as a viable treatment option for food allergies in the past. OIT is a promising treatment for patients with food allergies to induce desensitization and sustained unresponsiveness (SU) for specific food allergens. Desensitization is defined as a lack of clinical reactivity to allergens and is maintained by regular allergen exposure. Patients achieve SU, which is distinct from desensitization, by exhibiting a long-term loss of reactivity to allergens independent of continued allergen exposure. In general, the process of OIT comprises an initial build-up and maintenance phase, and after avoidance of allergens for several weeks to months, the patients are retested by oral food challenge (OFC) to determine whether SU is present. Over the past decade, the efficacy of OIT for egg, milk, and peanuts has been demonstrated by many randomized placebo-controlled trials.² However, OIT has several problems, including differences in effectiveness among allergens,³ risk of anaphylaxis, adverse reactions such as eosinophilic gastrointestinal disorders,⁴ and a lack of biomarkers to predict SU or immunologic tolerance.⁵ In addition, current OIT therapy has not been able to efficiently induce SU for specific food allergens in patients with allergy. Burks et al⁶ reported that OIT for children with egg allergies transiently desensitized 75% of subjects, but only 28% of subjects had SU. Importantly, the mechanisms by which OIT induces desensitization and SU for specific allergens are poorly understood.

Induced regulatory T (iTreg) cells, which are characterized by the expression of CD4, CD25, and the transcription factor Foxp3, are essential for establishing oral tolerance.⁷ During the induction of healthy tolerance to food antigens, CD103⁺ dendritic cells are known to play a crucial role through the development of antigen-specific iTregs.⁸ However, inducing immune tolerance in an individual who has already been sensitized to food allergens is no easy task. To achieve oral tolerance or SU against the pressure of antigen-specific effector cells, an immunosuppressive environment may be necessary. Importantly, in addition to Treg cells, some lymphoid and myeloid cells that produce immunosuppressive cytokines, such as IL-10 and TGF- β , are known. Myeloid-derived suppressor cells (MDSCs) have the ability to suppress effector T-cell responses and activate Treg cells by producing immune suppressive factors such as IL-10, TGF- β , arginase 1, and nitric oxide.⁹ MDSCs are a heterogeneous population of cells that consist of myeloid progenitor cells and immature myeloid cells. In mice, MDSCs are identified by the coexpression of the CD11b and Gr1 markers and include 2 distinct subsets, Ly6G⁺Ly6C^{int} polymorphonuclear MDSCs (PMN-MDSCs) and Ly6G⁺Ly6C^{hi} monocytic MDSCs (Mo-MDSCs).¹⁰ Various cytokines, such as GM-CSF and IL-6, which are released during

Abbreviations used

DAPT:	N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester
DLL1:	Delta-like 1
iNOS:	Inducible nitric oxide synthase
LP:	Lamina propria
MDSC:	Myeloid-derived suppressor cell
Mo-MDSC:	Monocytic myeloid-derived suppressor cell
OFC:	Oral food challenge
OIT:	Oral immunotherapy
PMN-MDSC:	Polymorphonuclear myeloid-derived suppressor cell
SU:	Sustained unresponsiveness
Treg:	Regulatory T

pathologic conditions (including cancer, infection, autoimmunity, and allergic airway inflammation), cause the expansion and accumulation of MDSCs.¹¹⁻¹³

In this study, to explore the factors involved in establishing SU by OIT, we generated a mouse model of OIT and administered a Notch signaling inhibitor to the mouse model. Notch signaling influences multiple lineage decisions of developing lymphoid and myeloid cells, such as thymic T cells,^{14,15} marginal zone B cells,^{14,16} splenic CD8⁺ dendritic cells,¹⁷ and intestinal mucosal mast cells.¹⁸ Notch signaling is initiated by the binding of Notch receptors to their ligands, leading to 2 successive proteolytic cleavages catalyzed by metalloprotease and γ -secretase. The liberated intracellular domain of the Notch receptor induces transcriptional expression of target genes.¹⁵ Although Notch signaling is a key regulator of immune functions, little is known about the role of Notch signaling in the induction of SU by OIT.

Here, using a mouse model of OIT for the treatment of food allergies, we found that the systemic expansion of IL-10-producing CD4⁺ T cells and MDSCs may contribute to the induction of SU by OIT. The inhibition of Notch signaling interferes with the establishment of SU by OIT, probably by suppressing the expansion of those immunosuppressive cells. These findings provide insight into how patients with food allergies can achieve SU by OIT.

METHODS

A more detailed description of our methods can be found in the Methods section of this article's Online Repository (at www.jacionline.org).

Experimental food allergy and the OIT model

Female BALB/c mice (5 to 8 weeks old) were intraperitoneally sensitized with 50 μ g of ovalbumin (albumin from chicken egg white) (Merck, Darmstadt, Germany) plus 2 mg of aluminum hydroxide and magnesium hydroxide (Alum [Thermo Fisher Scientific, Waltham, Mass]) on days 0 and 14. In the experimental food allergy model, mice were challenged intragastrically with 20 mg of ovalbumin 7 times every other day from day 28 to day 40. In the OIT model, ovalbumin-sensitized mice were intragastrically administered gradually increasing doses of heated ovalbumin (which was heat-treated at 65°C for 1 minute) daily from day 28 according to the following schedule: 0.25 mg on day 28, 0.5 mg on day 29, 1 mg on day 30, 2 mg on day 31, 4 mg on day 32, 8 mg on day 33, 12 mg on day 34, 16 mg on day 35, and 6 daily oral administrations of 20 mg of heated ovalbumin from day 36 to day 41. The establishment of desensitization was assessed by OFC (oral administration of 20 mg of unheated ovalbumin) on day 42. To confirm SU, mice underwent a second OFC (on day 56) after 2 weeks of complete elimination of ovalbumin.

Allergic diarrhea was monitored visually for 60 minutes after OFC. The severity of diarrhea was evaluated in terms of a clinical score depending on stool condition as follows: solid (score 0), funicular (score 1), slurry (score 2), and watery (score 3). Anaphylaxis was assessed by measuring changes in rectal temperature by using a digital thermometer (Natsume Seisakusho, Tokyo, Japan) 25 minutes after OFC.

Administration of a Notch signaling inhibitor to mice

N-(3,5-Difluorophenyl)acetyl-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) (ChemScene, Monmouth Junction, NJ), a γ -secretase inhibitor, was used as a Notch signaling inhibitor. In the OIT model, mice were injected intraperitoneally with 4 mg of DAPT in dimethyl sulfoxide or vehicle every other day from day 27 to day 41 during the OIT treatment period.

RESULTS

OIT induces desensitization and SU to mice sensitized to ovalbumin allergen

To investigate the role of Notch signaling in the induction of desensitization and SU to individuals sensitized with food allergens, we first generated 2 mouse models: an experimental food allergy model (Fig 1, A) and an OIT model (Fig 1, B). As shown in Fig 1, C, the clinical score of allergic diarrhea induced by OFC immediately after OIT treatment (on day 42) was significantly lower in the OIT-treated mice than in the mice with a developed food allergy. This unresponsiveness was maintained in OIT-treated mice after 2 weeks of discontinuation of OIT treatment (on day 56). OFC also induced marked hypothermia in mice with food allergy, but not in OIT-treated mice, on both day 42 and day 56 (Fig 1, D). Although serum total IgE levels were increased equally in both mice with food allergy and OIT-treated mice (Fig 1, E), the elevation of serum ovalbumin-specific IgE levels peaked on day 42 in OIT-treated mice, but not in mice with food allergy (Fig 1, F). Serum ovalbumin-specific IgG1 levels were significantly higher in the OIT-treated mice than in mice with food allergy on both day 42 and day 56 (Fig 1, G). From these results, we concluded that ovalbumin-sensitized mice were desensitized by OIT on day 42 and then achieved SU on day 56.

Establishment of SU by OIT is prevented by inhibition of Notch signaling

To elucidate the role of Notch signaling in OIT, we next administered DAPT (a γ -secretase inhibitor that functions as a Notch signaling inhibitor) intraperitoneally to mice during the OIT treatment period (Fig 2, A). OFC-induced allergic diarrhea was prevented in both groups on day 42 (Fig 2, B). Interestingly, on day 56, the clinical scores of OFC-induced allergic diarrhea were markedly increased in the OIT-treated mice administered DAPT compared with in the normal OIT-treated mice (Fig 2, B). OFC-induced hypothermia was also observed in OIT-treated mice administered DAPT on day 56, but not in normal OIT-treated mice (Fig 2, C). These results indicate that the administration of the Notch signaling inhibitor during the OIT treatment period leads to disappearance of the induction of SU, but not to desensitization. As shown in Fig 2, D-F, there was no significant difference in the levels of serum total IgE or ovalbumin-specific IgE and IgG1 between the 2 groups. Administration of DAPT for 2 weeks did not affect antibody production in the

OIT-treated mice. Therefore, the disappearance of SU induction cannot be explained by the difference in the levels of ovalbumin-specific antibodies.

OIT suppresses mast cell expansion and promotes Treg cell and T_H2 cell expansion in the intestines, and the T_H2 cell expansion is suppressed by inhibition of the Notch signaling

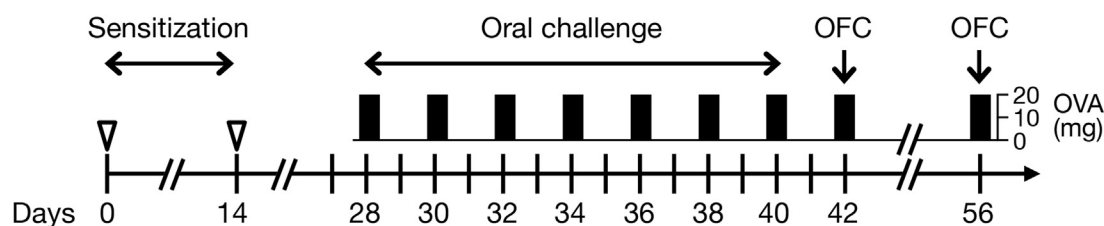
Food allergies result in a dramatic increase in the number of intestinal mast cells and mast cell-dependent intestinal inflammation.¹⁹ In this mouse model, OIT treatment prevented the increase in the number of intestinal mast cells (Fig 3, A). The serum levels of mast cell protease mMCP-1 after the second OFC (on day 56) were significantly lower in normal OIT-treated mice, but not in OIT/DAPT-treated mice, than in mice with food allergy (Fig 3, B).

Foxp3⁺ regulatory Treg cells are potent immunosuppressive cells. Immunosuppressive cytokines, including IL-10, secreted by Treg cells directly inhibit cytokine production by effector T cells and IgE-dependent mast cell activation.²⁰ An increase in the number of Treg cells in the intestinal lamina propria (LP) is a crucial step induced by OIT.²¹⁻²³ As shown in Fig 3, C, on day 57, the proportion of Treg cells was clearly increased in the intestinal LP by OIT, and the administration of DAPT did not affect the increase in Treg cell population by OIT. *Ex vivo* antigen restimulation assays revealed that the IL-10-producing CD4⁺CD25⁺ activated T-cell population, including Treg cells, was increased in the intestinal LP of the OIT-treated mice (Fig 3, D and E). In addition, the populations of IL-4-producing T_H2 cells and, interestingly, T_H2 cells producing both IL-4 and IL-10 were also significantly larger in OIT-treated mice than in mice with food allergy (Fig 3, D and E and see Fig E1, A in this article's Online Repository at www.jacionline.org). These effects of OIT were abolished by the administration of DAPT (Fig 3, D and E). These results in peripheral tissues show that OIT succeeded in inducing SU in this mouse model and that administration of DAPT to this model prevented an OIT-induced increase in intestinal IL-10-producing CD4⁺ T cells, including Treg cells and T_H2 cells.

OIT-induced expansion of IL-10-producing splenic CD4⁺ T cells is suppressed by inhibition of Notch signaling

SU is a state in which systemic clinical tolerance is maintained. Thus, to explore why the administration of the Notch signaling inhibitor inhibited SU establishment, we next evaluated the systemic T-cell response in these mice on day 57. In the spleen, unlike in the intestine, no OIT-induced increase in Treg cells was observed (Fig 4, A). However, as in the intestine, the frequencies of splenic IL-10-producing CD4⁺CD25⁺ activated T cells, including T_H2 cells, were significantly higher in the OIT-treated mice (but not in the OIT/DAPT-treated mice) than in the mice with food allergy (Fig 4, B and C and see Fig E1, B). Consistent with the increased frequency of IL-10-producing CD4⁺ T cells in OIT-treated mice, the splenocytes from OIT-treated mice produced a higher level of IL-10 in response to *ex vivo* stimulation with ovalbumin than did the splenocytes from mice with food allergy, but this high level of IL-10 production was not observed in OIT/DAPT-treated mice (Fig 4, D). Next, splenic CD4⁺ T cells

A Allergy model



B OIT model

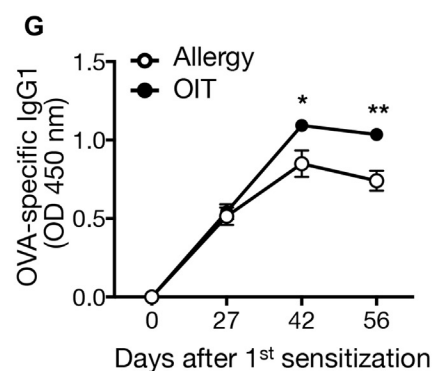
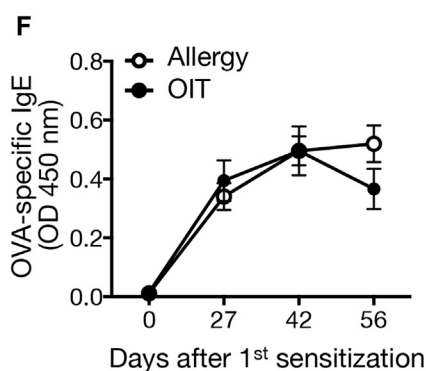
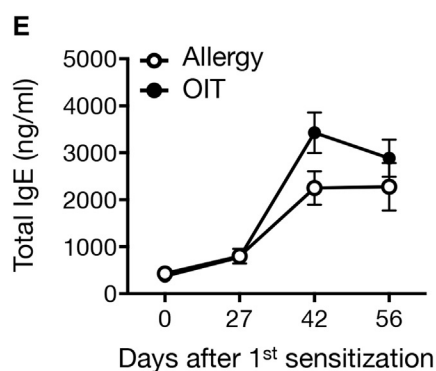
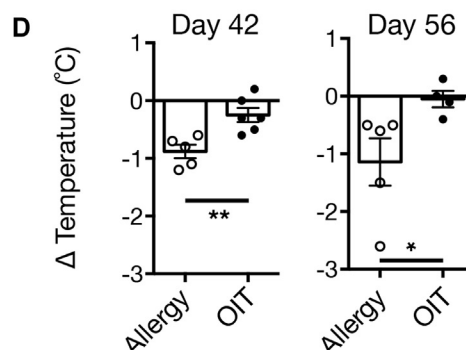
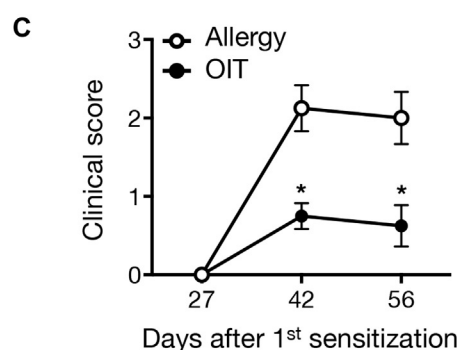
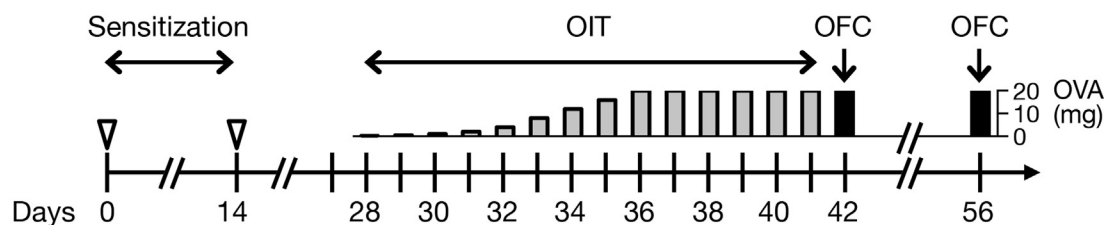


FIG 1. Desensitization and SU are induced in ovalbumin (OVA)-sensitized mice by OIT. Schematic of procedures to establish the experimental food allergy model (A) and the OIT model (B) are shown. Immediate hypersensitivity reactions to OVA were assessed by OFC on days 42 and 56. Black bar indicates unheated OVA; gray bar indicates heated OVA. C, The severity of allergic diarrhea induced by OFC (*n* = 8 or 9). **P* < .05 compared with mice with food allergy. D, Changes in rectal temperature at 25 minutes after OFC. **P* < .05; ***P* < .005. Total IgE (E) and OVA-specific IgE (F) and IgG1 (G) in sera (*n* = 6-9). **P* < .05; ***P* < .005 compared with mice with food allergy. Error bars indicate means ± SEMs. Data are either combined results from 4 (C) or 2 (D) independent experiments or results from an individual experiment that represents 3 independent experiments with similar results (E-G).

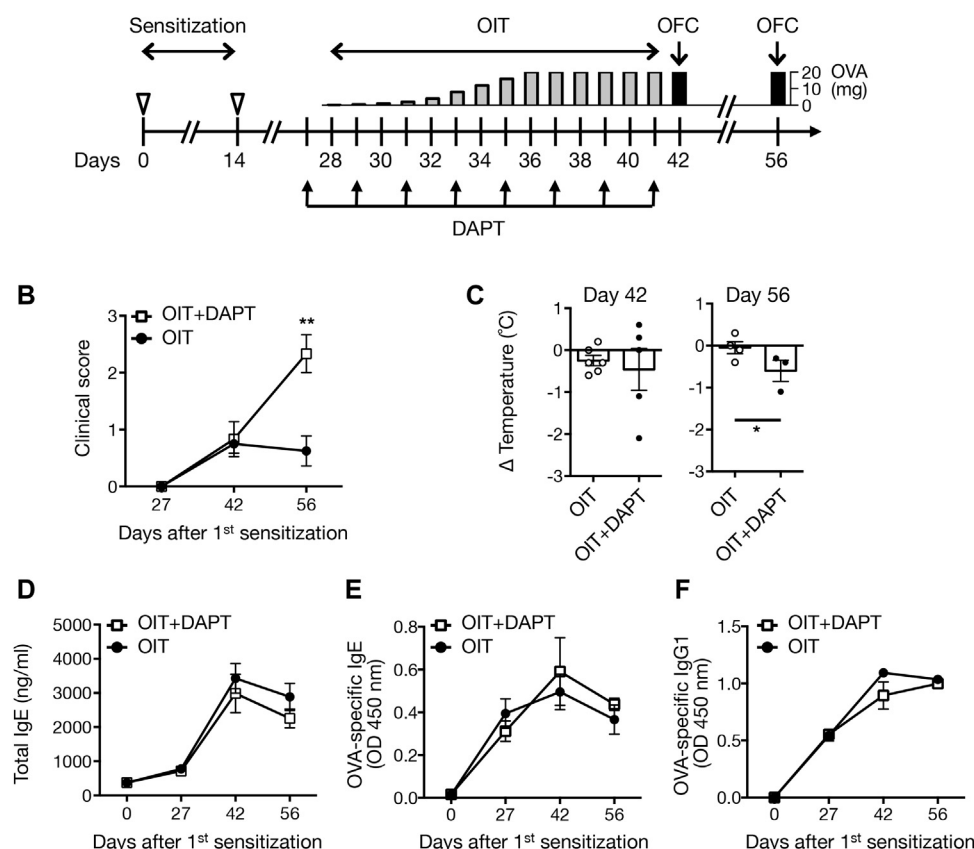
A OIT + Notch signaling inhibitor

FIG 2. Induction of SU by OIT is prevented by the inhibition of Notch signaling. **A**, Schematic procedure administering the Notch signaling inhibitor DAPT to the OIT model. Immediate hypersensitivity reactions to ovalbumin (OVA) were assessed by OFC on days 42 and 56. Black bar indicates unheated OVA; gray bar indicates heated OVA. **B**, The severity of allergic diarrhea induced by OFC ($n = 7$ or 8). $**P < .005$ compared with OIT-treated mice. **C**, Changes in rectal temperature at 25 minutes after OFC ($*P < .05$). Levels of total IgE (**D**) and OVA-specific IgE (**E**) and IgG1 (**F**) in sera ($n = 6$ -9). Error bars indicate means $\pm$ SEMs. Data are either combined results from 4 (**B**) or 2 (**C**) independent experiments or results from an individual experiment that represents 3 independent experiments with similar results (**D**-**F**).

isolated from each group of mice were cocultured with splenic CD11c⁺ dendritic cells in the presence and absence of ovalbumin. Although the proliferation of CD4⁺ T cells in response to ovalbumin was decreased in the OIT-treated mice compared with in the mice with food allergy, a decrease in CD4⁺ T-cell proliferation was hardly observed in the OIT/DAPT-treated mice (see Fig E1, C). These results suggest that the development of ovalbumin-specific IL-10-producing CD4⁺ T cells induced by OIT is prevented by inhibition of Notch signaling.

OIT induces systemic expansion of MDSCs, and the expansion is suppressed by administration of the Notch signaling inhibitor

A systemic increase in IL-10-producing CD4⁺ T cells may be involved in the establishment of SU. However, OIT also increased TH2 cells systemically (Fig 4, B and C and see Fig E1, B). In addition, OIT increased the frequency of Treg cells locally but not systemically (Fig 3, C and Fig 4, A). In spite of the increased frequency of TH2 cells, the allergic responses to ovalbumin were markedly suppressed in the OIT-treated mice compared

with in the mice with food allergy (Fig 1, C and D). From these results, it was inferred that some kind of immunosuppressive cells in addition to IL-10-producing CD4⁺ T cells are involved in the suppression of allergic responses. In fact, besides Treg cells, several cell populations with immunosuppressive function are known. They include M2 macrophages, MDSCs, and regulatory B cells; in particular, MDSCs, which are characterized by the co-expression of CD11b and Gr1 on the cell surface, have a potent immunosuppressive function.^{10,24} As shown in Fig 5, A, flow cytometric analysis on day 57 showed that the frequency of CD11b⁺Gr1⁺ cells was markedly higher in the peripheral blood, spleen, intestinal LP, and mesenteric lymph nodes of the OIT-treated mice than in the same areas of naive mice and mice with food allergy (Fig 5, A and see Fig E2 in this article's Online Repository at www.jacionline.org). In the intestinal epithelial layer, although the frequency of CD11b⁺Gr1⁺ cells was increased in mice with food allergy, very few intraepithelial cells were obtained from the intestines (see Fig E2). Interestingly, an increase in the frequency of CD11b⁺Gr1⁺ cells in the OIT-treated mice was suppressed by the administration of DAPT. On day 43, expansion of the number of CD11b⁺Gr1⁺ cells was

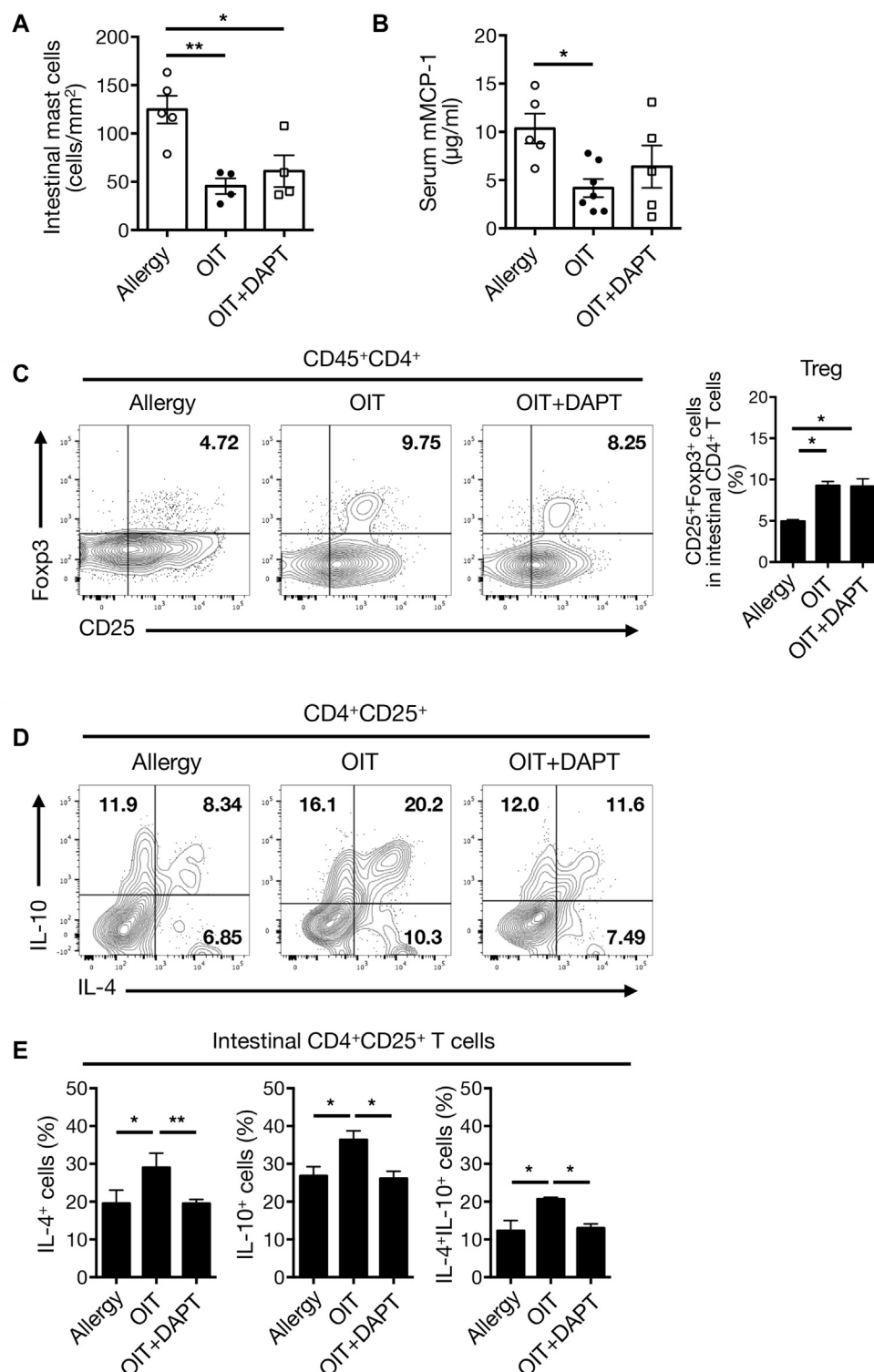


FIG 3. Inhibition of Notch signaling suppresses IL-10 production by intestinal antigen-specific CD4⁺ T cells in OIT-treated mice. **A**, Mast cell numbers in the jejunal mucosa of mice on day 57. *P < .05; **P < .005. **B**, Serum mMCP-1 levels 2 hours after OFC on day 56 (*P < .05). **C**, Flow cytometric analysis (left) and frequencies (right) of Treg cells (gated as CD45⁺CD4⁺CD25⁺Foxp3⁺) in the colonic LP of mice on day 57 (n = 3) (*P < .05). Flow cytometric analysis (**D**) and frequencies (**E**) of intracellular staining in activated CD4⁺ T cells (gated as CD4⁺CD25⁺) for IL-4 and IL-10. Colonic LP cells were isolated from mice on day 57 and cultured with ovalbumin for 6 days for antigen restimulation (n = 3 or 4) (*P < .05; **P < .005). Error bars indicate means ± SEMs. Data are either combined results from 2 independent experiments (**A** and **B**) or results from an individual experiment that represents 3 (**C**) or 2 (**D** and **E**) independent experiments with similar results.

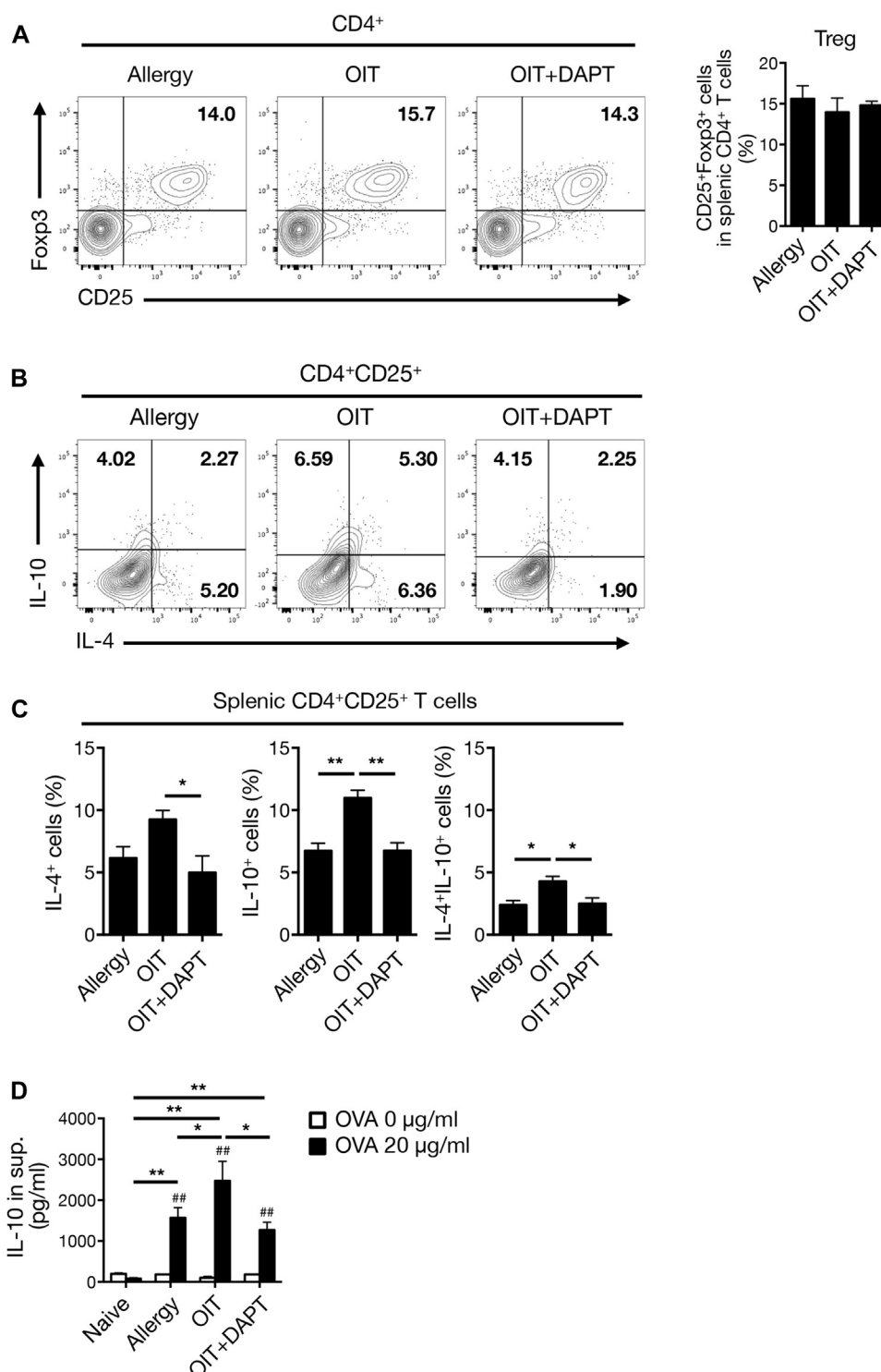


FIG 4. Inhibition of Notch signaling suppresses IL-10 production by splenic antigen-specific CD4⁺ T cells in OIT-treated mice. **A**, Flow cytometric analysis (left) and frequencies (right) of Treg cells (gated as CD4⁺CD25⁺Foxp3⁺) in the splenocytes of mice on day 57 ($n = 3$). Flow cytometric analysis (**B**) and frequencies (**C**) of intracellular staining in activated CD4⁺ T cells (gated as CD4⁺CD25⁺) for IL-4 and IL-10. Splenocytes were isolated from mice on day 57 and cultured with ovalbumin (OVA) for 6 days for antigen restimulation ($n = 3-5$). * $P < .05$; ** $P < .005$. **D**, IL-10 levels in culture supernatant from splenocytes in the presence or absence of OVA ($n = 3-6$). * $P < .05$; ** $P < .005$; and *** $P < .005$, compared with the OVA-unstimulated control. Error bars indicate means $\pm$ SEMs. Data are from an individual experiment that represents 3 (**A**) or 2 (**B-D**) independent experiments with similar results.

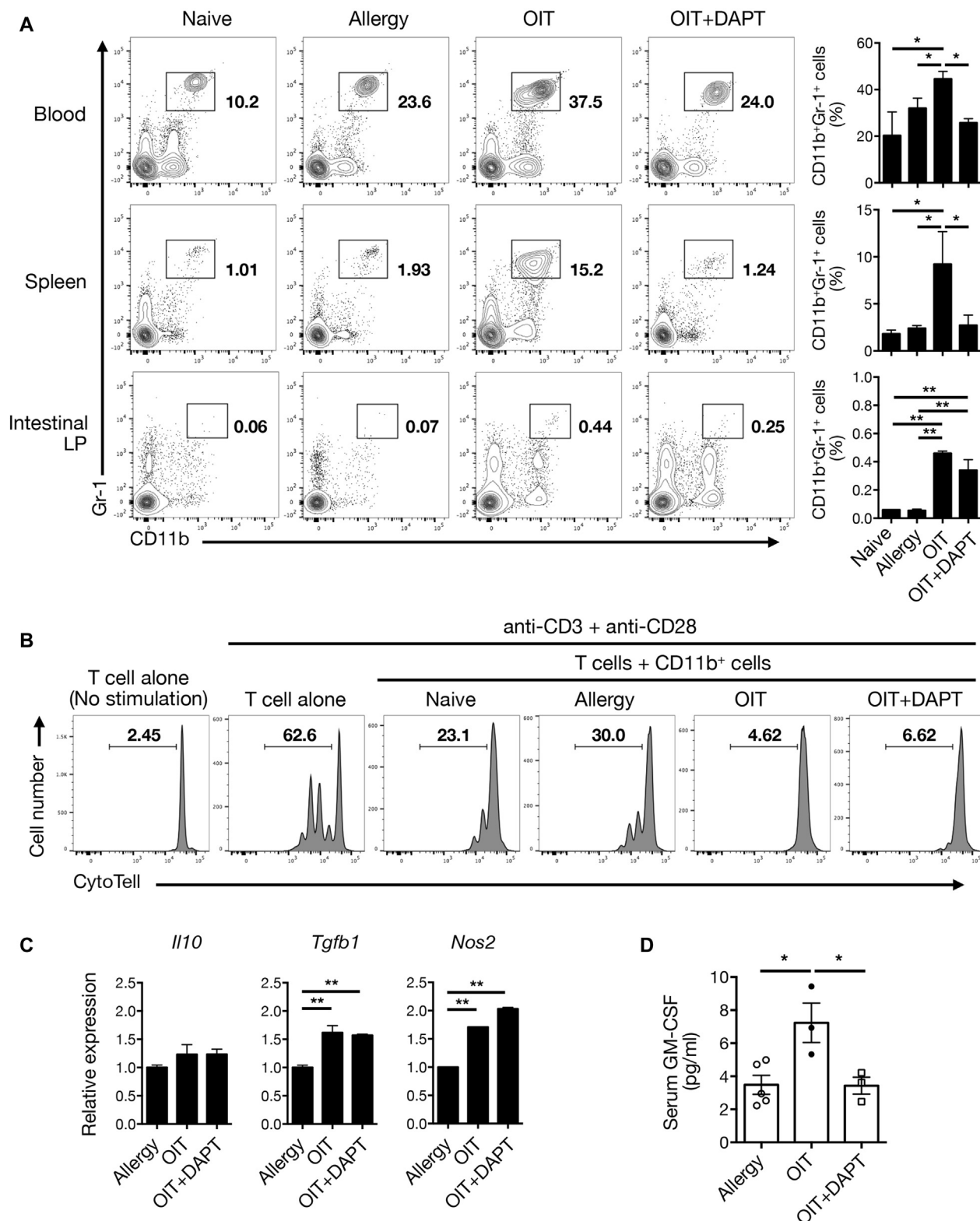


FIG 5. Inhibition of Notch signaling suppresses OIT-induced systemic expansion of MDSCs. **A**, Flow cytometric analysis (left) and frequencies (right) of CD11b⁺Gr1⁺ cells in blood, spleen, and intestinal LP cells of mice on day 57 ($n = 3-5$) ($*P < .05$). **B**, Flow cytometric analysis of proliferation of CytoTell UltraGreen-labeled splenic CD4⁺ T cells cocultured with or without splenic CD11b⁺ cells isolated from each group of mice on day 44 in the presence or absence of anti-CD3/CD28 for 72 hours. **C**, mRNA expression levels in CD11b⁺ cells isolated from each group of mice ($n = 3$) ($**P < .005$). **D**, Serum GM-CSF levels 2 hours after OFC on day 56 ($*P < .05$). Error bars indicate means $\pm$ SEMs. Data are from an individual experiment that represents 4 (**A**) or 2 (**B-D**) independent experiments with similar results.

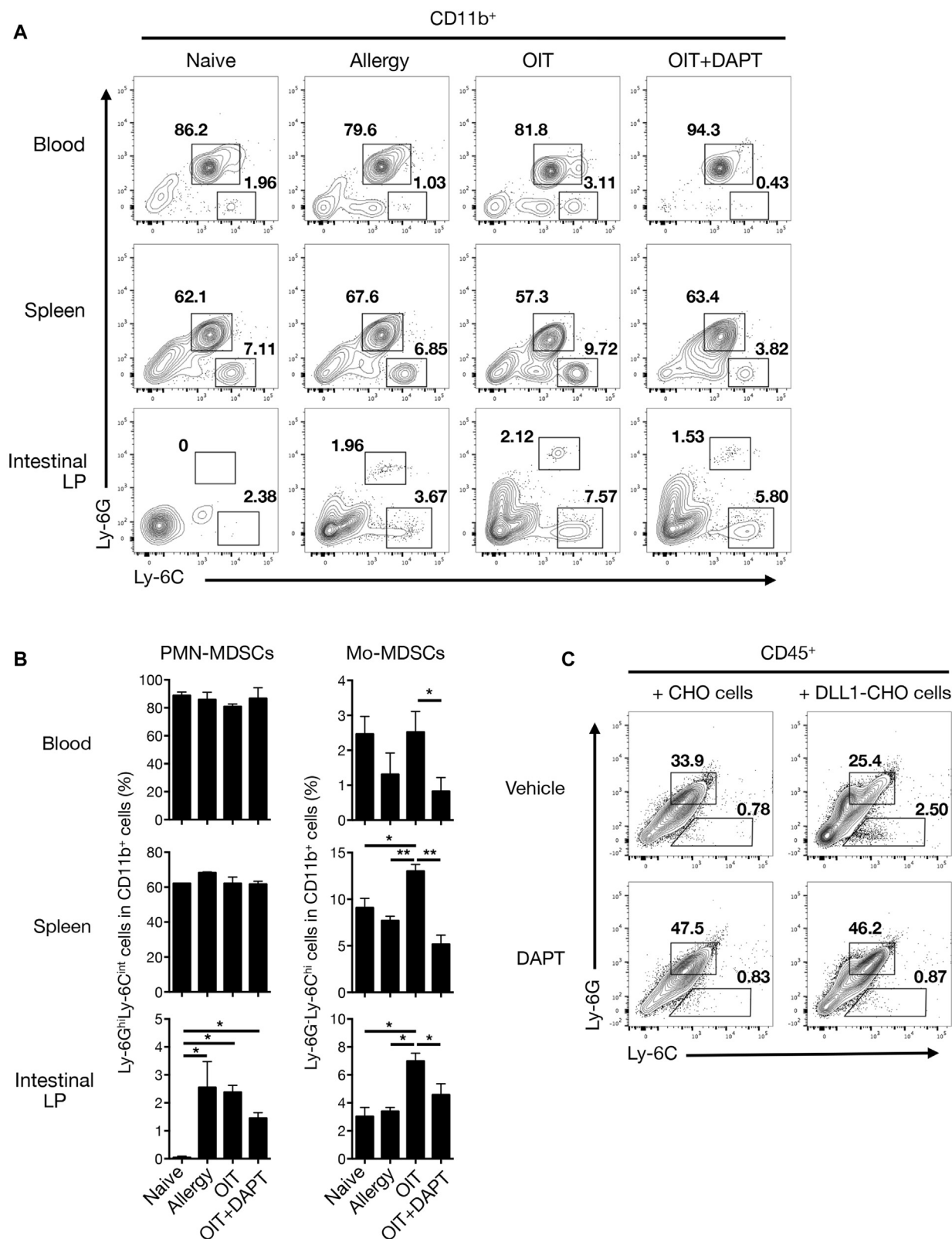


FIG 6. Notch signaling contributes to the generation of Mo-MDSCs. Flow cytometric analysis (**A**) and frequencies (**B**) of CD11b⁺Ly6G⁺Ly6C^{int} cells and CD11b⁺Ly6G⁺Ly6C^{hi} cells in blood, spleen, and intestinal LP cells of mice on day 57 ($n = 3-6$). * $P < .05$; ** $P < .005$. **C**, Flow cytometric analysis of bone marrow-derived MDSCs (gated as Ly6G⁺Ly6C^{int} or Ly6G⁺Ly6C^{hi}). Bone marrow cells isolated from BALB/c mice cocultured with Notch ligand DLL1-expressing CHO cells or control CHO cells for 4 days in the presence of GM-CSF. Error bars indicate means $\pm$ SEMs. Data are from an individual experiment that represents 4 (**A** and **B**) or 3 (**C**) independent experiments with similar results.

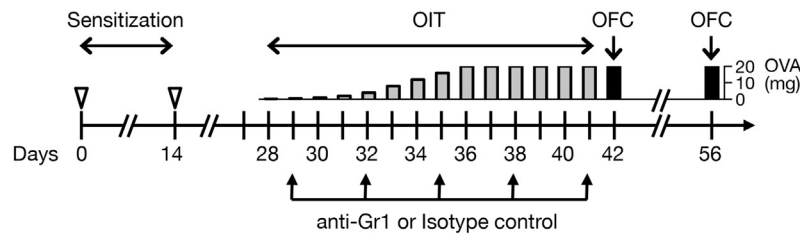
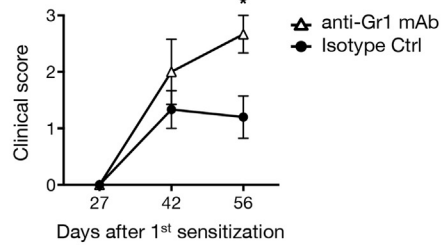
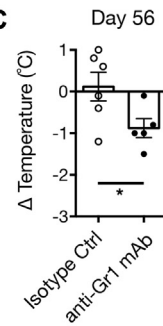
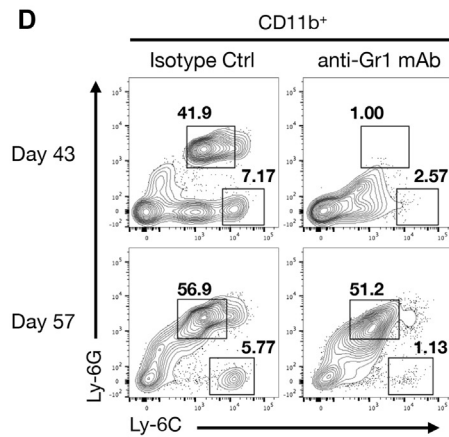
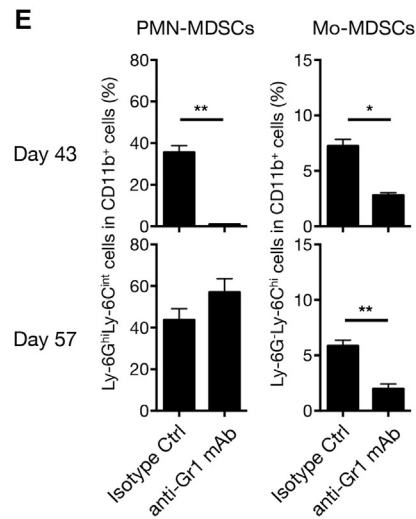
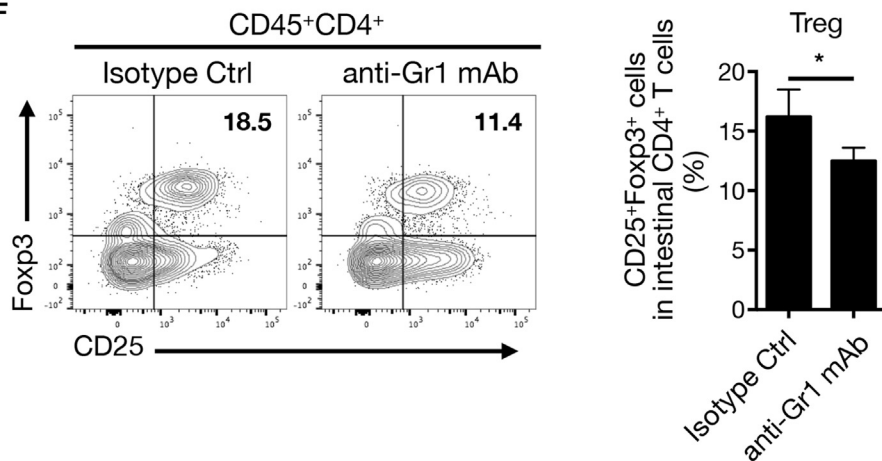
A OIT + Gr1⁺ cell depletion**B****C****D****E****F**

FIG 7. Depletion of MDSCs prevents the establishment of SU by OIT. **A**, Schematic procedure for the OIT model administered anti-Gr1-depleting antibody or isotype control (Ctrl). Immediate hypersensitivity reactions to ovalbumin were assessed by OFC on days 42 and 56. Black bar indicates unheated ovalbumin; gray

observed in the mice that received OIT locally but not in the mice that received OIT systemically (see Fig E3, A in this article's Online Repository at www.jacionline.org). Next, to examine whether they have immunosuppressive activity, we isolated splenic CD11b⁺ cells from each group of mice and cocultured them with splenic CD4⁺ T cells isolated from naive mice in the presence of anti-CD3 and anti-CD28 antibodies. CD11b⁺ cells isolated from OIT-treated or OIT/DAPT-treated mice strongly suppressed the proliferation of CD4⁺ T cells, whereas CD11b⁺ cells isolated from naive or mice with food allergy partially suppressed the proliferation compared with when T cells alone were activated (Fig 5, B). MDSCs are known to suppress T-cell activation through the secretion of immunosuppressive cytokines such as TGF- β and IL-10 and nitric oxide, which is produced by activation of inducible NO synthase (iNOS).⁹ CD11b⁺ cells isolated from OIT-treated or OIT/DAPT-treated mice expressed significantly higher levels of mRNAs for *Tgfb1* (encoding TGF- β 1) and *Nos2* (encoding iNOS), but not for *Il10* (encoding IL-10) (Fig 5, C). Various inducers of MDSCs have been reported to be produced under pathologic conditions, and GM-CSF is a major factor implicated in the expansion and activation of MDSCs.⁹ On day 57, the level of serum GM-CSF was significantly higher in the OIT-treated mice than in the mice with food allergy, and administration of DAPT to the OIT-treated mice significantly reduced the serum GM-CSF level (Fig 5, D). These results indicate that the expansion of CD11b⁺Gr1⁺ MDSCs is induced by OIT and OIT-induced MDSC expansion is suppressed by inhibition of Notch signaling.

Notch signaling is involved in Mo-MDSC generation

Mouse MDSCs have been classified into 2 groups: Ly6G⁺Ly6C^{int} PMN-MDSCs and Ly6G⁺Ly6C^{hi} Mo-MDSCs.¹⁰ In particular, Mo-MDSCs have more potent immunosuppressive activity than PMN-MDSCs do.^{9,12} The antibody RB6-8C5 used to detect Gr1⁺ cells stains not only Ly6G⁺ cells but also cells that express the highest levels of Ly6C.²⁵ Thus, to determine the proportion of the subsets constituting the CD11b⁺Gr1⁺ cell population, CD11b⁺ cells were stained with the antibody 1A8, which specifically binds to Ly6G and Ly6C-specific antibody HK1.4. The proportion of Mo-MDSCs in the spleen was significantly higher in the OIT-treated mice than in the mice with food allergy (Fig 6, A and B). Notably, administration of DAPT to the OIT-treated mice drastically reduced the proportion of Mo-MDSCs in the peripheral blood and spleen (Fig 6, A and B). On day 43, an OIT-induced increase in Mo-MDSC proportion was observed in the intestinal LP, but not in the blood or spleen (see Fig E3, B).

Notch signaling performs multifaceted functions in the differentiation of MDSCs. Several reports have suggested that inhibition of Notch signaling causes an increase in the PMN-MDSC population and/or a decrease in the Mo-MDSC

population.^{26,27} Thus, we examined the effect of DAPT on MDSC differentiation by using *in vitro* culture studies. Bone marrow cells isolated from BALB/c mice were cocultured with Notch ligand Delta-like 1 (DLL1)-expressing CHO cells in the presence of MDSC inducers, GM-CSF, and IL-6, for 4 days. Ly6G⁺Ly6C⁺ Mo-MDSCs were generated by coculture with DLL1-expressing cells, whereas the frequency of Ly6G⁺PMN-MDSCs was decreased in the presence of DLL1. The addition of DAPT prevented generation of Ly6C⁺ cells, indicating that Notch signaling directly promotes the generation of Mo-MDSCs (Fig 6, C).

Repeated oral challenges after OIT treatment prevent the establishment of SU

To determine whether SU is induced even after repeated ovalbumin challenge to OVA-desensitized mice, the phase of repeated ovalbumin challenge was set after OIT treatment period (see Fig E4, A and B in this article's Online Repository at www.jacionline.org). On day 50, the levels of both OFC-induced allergic diarrhea and hypothermia were significantly lower in the OIT-treated mice (OIT(+)/challenge) than in mice with food allergy (OIT(-)/challenge) (see Fig E4, C and D). However, on day 64, there was no difference in the severity of allergic symptoms between the OIT-treated mice and the mice with food allergy. These results indicate that whereas the desensitization to ovalbumin was induced in mice repeatedly challenged with ovalbumin following OIT treatment, the establishment of SU failed in the mice (see Fig E4, C and D). No difference was observed in the frequencies of Mo-MDSCs between OIT(-)/challenge and OIT(+)/challenge (see Fig E4, E).

Depletion of MDSCs prevents the establishment of SU by OIT

To demonstrate that the MDSC is one of the cell types responsible for unresponsiveness to antigens induced by OIT, we depleted Gr1⁺ cells during the OIT treatment period (Fig 7, A). This depletion significantly exacerbated the clinical score of OFC-induced allergic diarrhea and the level of OFC-induced hypothermia on day 56 (Fig 7, B and C). Both PMN-MDSCs and Mo-MDSCs were nearly abolished in OIT-treated mice by administration of Gr1⁺ cell-depleting antibody (on day 43) (Fig 7, D and E). Two weeks after discontinuation of the antibody administration (day 57), the frequency of PMN-MDSCs almost completely recovered, but not the frequency of Mo-MDSCs (Fig 7, D and E). Although it is unclear why the number of Mo-MDSCs did not recover after discontinuation of medication, the frequency of Mo-MDSCs was significantly lower in the Gr1⁺ cell-depleted mice than in the control mice on day 57. In the mice with low Mo-MDSC frequency, the frequency of intestinal Treg cells on day 57 was also significantly lower than in the control mice, whereas the frequency of splenic Treg cells was not affected by

bar indicates heated ovalbumin. **B**, The severity of allergic diarrhea induced by OFC ($n = 3-5$). * $P < .05$ compared with Ctrl mice. **C**, Changes in rectal temperature at 25 minutes after OFC. * $P < .05$. Flow cytometric analysis (**D**) and frequencies (**E**) of CD11b⁺Ly6G⁺Ly6C^{int} cells and CD11b⁺Ly6G⁺Ly6C^{hi} cells in spleen cells of mice on day 43 or 57 ($n = 3$ or 4). * $P < .05$; ** $P < .005$. **F**, Flow cytometric analysis (*left*) and frequencies (*right*) of Treg cells (gated as CD45⁺CD4⁺CD25⁺Foxp3⁺) in the colonic LP of mice on day 57 ($n = 3$) (* $P < .05$). Error bars indicate means $\pm$ SEMs. Data are combined results from 2 independent experiments (**B-E**), from an individual experiment with 4 mice per group, or from an individual experiment that represents 2 independent experiments (**F**) with similar results.

Gr1⁺ cell depletion (Fig 7, *F* and see Fig E5, *A* in this article's Online Repository at www.jacionline.org). More importantly, Gr1⁺ cell depletion reduced the number of IL-10–producing CD4⁺CD25⁺ activated T cells in both the intestine and the spleen on day 57 (see Fig E5, *B* and *C*). In addition, administration of the depleting antibody to a mouse model of food allergy had little effect on the development of food allergy (see Fig E6, *A–C* in this article's Online Repository at www.jacionline.org). These results suggest that Gr1⁺ cells, especially Mo-MDSCs, induced by OIT treatment may contribute to the establishment of SU and the proliferation and activation of food allergen–specific IL-10–producing CD4⁺ T cells, including Treg cells.

DISCUSSION

In patients with food allergy who successfully complete OIT, desensitization has been reported to be associated with increased allergen-specific IgG4 levels.²⁸ Although mice do not have an IgG4 subclass, allergen-specific mouse IgG antibodies act to suppress IgE-mediated mast cell activation through FcγRIIb.²⁹ In addition, the internalization of allergen-specific IgE on mast cells is also involved in OIT-induced desensitization.³⁰ In our mouse models, a significantly higher level of allergen-specific IgG1 was detected in the OIT-treated mice than in the mice with food allergy. This indicates that OVA-specific IgG1 may contribute to the desensitization of OVA-sensitized mice in this OIT model. However, the factors necessary for induction of SU are still poorly understood. In this study, we found that administration of the Notch signaling inhibitor DAPT during the OIT treatment period largely prevented the establishment of SU but did not affect the induction of desensitization. This indicates that Notch signaling plays an important role in the induction of SU by OIT.

It is unclear whether SU induced by OIT and oral tolerance occurring via natural development represent the same state. In the induction of oral tolerance, antigen-specific Foxp3⁺ Treg cells in the intestinal LP play an important role.²³ The development of Treg cells is induced by antigen presentation by CD103⁺ dendritic cells to naive CD4⁺ T cells in the lymph nodes and is promoted by TGF-β and retinoic acid produced by the dendritic cells.³¹ The induced Treg cells produce the immunosuppressive cytokines IL-10 and TGF-β in the LP to inhibit mast cell activation³² and to sustain tolerance.³³ In our mouse model, OIT suppressed the expansion of intestinal mast cells and promoted the proliferation of intestinal Treg cells. Further examinations revealed that OIT also induced the expansion of intestinal IL-4–producing T_H2 cells in addition to Treg cells. Moreover, OIT induced the expansion of splenic IL-10–producing CD4⁺ T cells, including T_H2 cells producing both IL-4 and IL-10, and then enhanced the level of IL-10 produced by splenocytes in response to ovalbumin. These observations suggest that the tolerogenic mechanism differs between OIT-induced SU and oral tolerance by natural development. In this model of OIT, unlike in the induction of healthy tolerance to food antigens, mice are already sensitized to food allergens. To induce unresponsiveness to the allergens in the sensitized mice, effector cells circulating throughout the body must be neutralized. The spleen is involved in systemic regulation of immunity.³⁴ Thus, the altered splenic CD4⁺ T-cell profile probably contributes to the induction of systemic unresponsiveness by OIT. However, the following questions arise. Why is the allergic reaction suppressed in

OIT-treated mice despite the increased frequency of T_H2 cells locally? How does OIT induce a systemic expansion of IL-10–producing CD4⁺ T cells?

Not only Treg cells but also some lymphoid and myeloid cells produce immunosuppressive cytokines such as IL-10 and TGF-β. Interestingly, we found that OIT increased the frequency of T_H2 cells producing both IL-4 and IL-10 locally and systemically. IL-10–producing T_H2 cells have been reported to be involved in tolerance during a successful pregnancy and in allograft.³⁵ Thus, increased frequency of IL-4⁺IL-10⁺ T_H2 cells may contribute to the establishment of SU. Furthermore, we also found that OIT induces a systemic expansion of CD11b⁺Gr1⁺ cells. CD11b⁺ and Gr1⁺ are surface markers common to MDSCs and neutrophils, and thus, it is difficult to distinguish between them by using only the markers. Although MDSCs are well known to have potent immunosuppressive activity, some neutrophils also suppress immune responses by producing IL-10.³⁶ In fact, splenic CD11b⁺Gr1⁺ cells isolated from naive mice or mice with food allergy partially suppressed T-cell activation. On the other hand, splenic CD11b⁺Gr1⁺ cells isolated from OIT-treated mice or OIT/DAPT-treated mice potently suppressed T-cell activation and expression of higher levels of TGF-β and iNOS compared with those from mice with food allergy, indicating that these cells are MDSCs. This suggests that the adverse effects of T_H2 cells increased by OIT may be suppressed by the MDSCs. In addition, as shown in Fig E6, Gr1⁺ cell depletion in mice with food allergy had little effect on the development of food allergy. This observation indicates that Gr1⁺ cells in mice with food allergy have little immunosuppressive effect, which is consistent with the results shown in Fig 5, *B*. Therefore, the systemic expansion of MDSCs, especially Mo-MDSC subset (which has a potent immunosuppressive activity compared with that of the PMN-MDSC subset),^{27,37} may also contribute to the induction of systemic unresponsiveness to food allergens. In fact, the mice that failed to establish SU showed no Mo-MDSC expansion, as shown in Fig E4, *E*, and the OIT-treated mice with an Mo-MDSC subset significantly reduced by administration of Gr1⁺ cell–depleting antibody were not able to induce SU, as shown in Fig 7. Mo-MDSCs are also known to promote Treg cell expansion by changing the surroundings into an immunosuppressive environment.^{9,38} Because an OIT-induced intestinal Treg cell expansion was prevented by Gr1⁺ cell depletion during OIT treatment period, MDSCs also may contribute to the OIT-induced Treg cell expansion.

We next examined the stage at which generation and expansion of Mo-MDSCs occurred in OIT-treated mice. Notably, on day 43, the increased Mo-MDSCs was clearly detected only in the intestinal LP of the OIT-treated mice; an expansion was also observed in the spleen, but it was not significant. Thus, in our mouse models, MDSCs may be generated in the intestines with chronic low-grade inflammation and then expand throughout the body. MDSC expansion is induced by various cytokines, including GM-CSF, that are produced under pathologic conditions.^{9,39} A high level of serum GM-CSF may contribute to the expansion of MDSCs in OIT-treated mice; however, the reason for a low level of serum GM-CSF in OIT-treated mice administered the Notch signaling inhibitor DAPT is unclear. The effect of Notch signaling on MDSC expansion remains controversial,⁴⁰ but at least the treatment of mice with a Notch signaling inhibitor has been reported to inhibit the formation of the Mo-MDSC subset.²⁷ In agreement with these previous reports, the

administration of DAPT during the OIT treatment period markedly reduced the proportion of Mo-MDSCs both locally and systemically. These observations are supported by *in vitro* culture studies in which Notch signaling directly induces the generation of Mo-MDSCs, as shown in Fig 6, C. Thus, both Notch signaling and serum GM-CSF level may contribute to the generation and expansion of Mo-MDSCs. We believe that the suppression of Mo-MDSC generation and expansion by inhibition of Notch signaling is one of the causes of the failure of OIT/DAPT-treated mice to establish SU. On the other hand, Notch signaling is known to directly regulate the differentiation and function of CD4⁺ T cells, such as conventional helper T cells and Treg cells.⁴¹ In this study, the administration of DAPT to mice suppressed OIT-induced expansion of not only MDSCs but also IL-10-producing CD4⁺ T cells, including T_H2 cells and Treg cells, indicating that DAPT also affected these T-cell responses and profiles.

In conclusion, chronic low-grade inflammation induced by OIT probably leads to the expansion of Mo-MDSCs and IL-10-producing CD4⁺ T cells systemically, whereas excessive inflammation induced by allergic reactions leads to the expansion of mast cells locally. The present study suggests that OIT-induced expansion of those immunosuppressive cells may contribute to the induction of unresponsiveness to food allergens and Notch signaling is involved in these processes. Therefore, the frequencies of MDSCs and IL-10-producing CD4⁺ T cells may be markers of whether OIT can be successfully completed. Furthermore, Notch signaling may help to establish unresponsiveness to food allergens.

We thank Prof Shigeru Chiba (University of Tsukuba, Japan) for providing CHO transfectants; Ms Michiyo Matsumoto and Ms Emi Yamaguchi for secretarial assistance; and members of the Laboratory of Morphology and Image Analysis, Research Support Center, Juntendo University Graduate School of Medicine, for technical assistance.

Key messages

- Inhibition of Notch signaling prevents establishment of sustained unresponsiveness to food allergens induced by oral immunotherapy, but it does not affect induction of desensitization.
- Oral immunotherapy induces systemic expansion of IL-10-producing CD4⁺ T cells, including T_H2 cells producing both IL-4 and IL-10, and myeloid-derived suppressor cells in a mouse model of food allergy.
- These immunosuppressive cells contribute to induction of sustained unresponsiveness to food allergens, and Notch signaling is involved in the expansion of these cells.

REFERENCES

- Prescott SL, Pawankar R, Allen KJ, Campbell DE, Sinn J, Fiocchi A, et al. A global survey of changing patterns of food allergy burden in children. *World Allergy Organ J* 2013;6:21.
- Wood RA. Food allergen immunotherapy: current status and prospects for the future. *J Allergy Clin Immunol* 2016;137:973-82.
- Sato S, Yanagida N, Ogura K, Imai T, Utsunomiya T, Iikura K, et al. Clinical studies in oral allergen-specific immunotherapy: differences among allergens. *Int Arch Allergy Immunol* 2014;164:1-9.
- Goldberg MR, Elizur A, Nachshon L, Appel MY, Levy MB, Golobov K, et al. Oral immunotherapy-induced gastrointestinal symptoms and peripheral blood eosinophil responses. *J Allergy Clin Immunol* 2017;139:1388-90.e4.
- Peters RL, Dang TD, Allen KJ. Specific oral tolerance induction in childhood. *Pediatr Allergy Immunol* 2016;27:784-94.
- Burks AW, Jones SM, Wood RA, Fleischer DM, Sicherer SH, Lindblad RW, et al. Oral immunotherapy for treatment of egg allergy in children. *N Engl J Med* 2012;367:233-43.
- Curto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. Adaptive Foxp3⁺ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 2008;29:114-26.
- Scott CL, Aumeunier AM, Mowat AM. Intestinal CD103⁺ dendritic cells: master regulators of tolerance? *Trends Immunol* 2011;32:412-9.
- Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009;9:162-74.
- Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun* 2016;7:12150.
- Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer* 2008;8:618-31.
- Skabytska Y, Wolbing F, Gunther C, Koberle M, Kaesler S, Chen KM, et al. Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. *Immunity* 2014;41:762-75.
- Shi M, Shi G, Tang J, Kong D, Bao Y, Xiao B, et al. Myeloid-derived suppressor cell function is diminished in aspirin-triggered allergic airway hyperresponsiveness in mice. *J Allergy Clin Immunol* 2014;134:1163-74.e16.
- Tanigaki K, Honjo T. Regulation of lymphocyte development by Notch signaling. *Nat Immunol* 2007;8:451-6.
- Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. *Immunity* 2010;32:14-27.
- Hozumi K, Negishi N, Suzuki D, Abe N, Sotomaru Y, Tamaoki N, et al. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells *in vivo*. *Nat Immunol* 2004;5:638-44.
- Caton ML, Smith-Raska MR, Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8⁺ dendritic cells in the spleen. *J Exp Med* 2007;204:1653-64.
- Honjo A, Nakano N, Yamazaki S, Hara M, Uchida K, Kitaura J, et al. Pharmacologic inhibition of Notch signaling suppresses food antigen-induced mucosal mast cell hyperplasia. *J Allergy Clin Immunol* 2017;139:987-96.e10.
- Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;112:1666-77.
- Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014;133:621-31.
- Chinthrajah RS, Hernandez JD, Boyd SD, Galli SJ, Nadeau KC. Molecular and cellular mechanisms of food allergy and food tolerance. *J Allergy Clin Immunol* 2016;137:984-97.
- Wawrzyniak M, O'Mahony L, Akdis M. Role of regulatory cells in oral tolerance. *Allergy Asthma Immunol Res* 2017;9:107-15.
- Freeland DMH, Manohar M, Andorf S, Hobson BD, Zhang W, Nadeau KC. Oral immunotherapy for food allergy. *Semin Immunol* 2017;30:36-44.
- Uttarkar S, Brembilla NC, Boehncke WH. Regulatory cells in the skin: pathophysiologic role and potential targets for anti-inflammatory therapies. *J Allergy Clin Immunol* 2019;143:1302-10.
- Wojtasiak M, Pickett DL, Tate MD, Londrigan SL, Bedoui S, Brooks AG, et al. Depletion of Gr-1⁺, but not Ly6G⁺, immune cells exacerbates virus replication and disease in an intranasal model of herpes simplex virus type 1 infection. *J Gen Virol* 2010;91:2158-66.
- Cheng P, Kumar V, Liu H, Youn JI, Fishman M, Sherman S, et al. Effects of notch signaling on regulation of myeloid cell differentiation in cancer. *Cancer Res* 2014;74:141-52.
- Mao L, Zhao ZL, Yu GT, Wu L, Deng WW, Li YC, et al. gamma-Secretase inhibitor reduces immunosuppressive cells and enhances tumour immunity in head and neck squamous cell carcinoma. *Int J Cancer* 2018;142:999-1009.
- Savilahti EM, Rantanen V, Lin JS, Karinen S, Saarinen KM, Goldis M, et al. Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes. *J Allergy Clin Immunol* 2010;125:1315-21.e9.
- Burton OT, Noval Rivas M, Zhou JS, Logsdon SL, Darling AR, Koleoglou KJ, et al. Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. *Immunity* 2014;41:141-51.

30. Oka T, Rios EJ, Tsai M, Kalesnikoff J, Galli SJ. Rapid desensitization induces internalization of antigen-specific IgE on mouse mast cells. *J Allergy Clin Immunol* 2013;132:922-32.e1-16.
31. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103⁺ DCs induces Foxp3⁺ regulatory T cells via a TGF- β and retinoic acid-dependent mechanism. *J Exp Med* 2007;204:1757-64.
32. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, et al. CD4⁺CD25⁺ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* 2008;29:771-81.
33. Noval Rivas M, Chatila TA. Regulatory T cells in allergic diseases. *J Allergy Clin Immunol* 2016;138:639-52.
34. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. *Immunity* 2013;39:806-18.
35. Fang D, Zhu J. Molecular switches for regulating the differentiation of inflammatory and IL-10-producing anti-inflammatory T-helper cells. *Cell Mol Life Sci* 2020; 77:289-303.
36. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* 2009;31:761-71.
37. Wang SH, Lu QY, Guo YH, Song YY, Liu PJ, Wang YC. The blockage of Notch signalling promoted the generation of polymorphonuclear myeloid-derived suppressor cells with lower immunosuppression. *Eur J Cancer* 2016;68: 90-105.
38. Dorhoi A, Du Plessis N. Monocytic myeloid-derived suppressor cells in chronic infections. *Front Immunol* 2017;8:1895.
39. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* 2004;64:6337-43.
40. Grazioli P, Felli MP, Screpanti I, Campese AF. The mazy case of Notch and immunoregulatory cells. *J Leukoc Biol* 2017;102:361-8.
41. Radtke F, MacDonald HR, Tacchini-Cottier F. Regulation of innate and adaptive immunity by Notch. *Nat Rev Immunol* 2013;13:427-37.

METHODS

Mice

Female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were maintained in specific pathogen-free conditions, and all animal experiments were performed according to the approved manual of the institutional review board of Juntendo University.

Measurement of serum antibodies and mediators

Concentrations of serum total IgE and OVA-specific IgE and IgG1 were quantified by sandwich ELISA as described previously.^{E1} The concentrations of mouse mast cell protease-1 (mMCP-1) and GM-CSF in sera collected 2 hours after OFC on days 42 and 56 were quantified by using an mMCP-1 ELISA kit (Thermo Fisher Scientific) and a Mouse GM-CSF DuoSet ELISA kit (R&D Systems, Minneapolis, Minn), respectively. The concentrations of IL-10 in cell culture supernatants were quantified by using a mouse IL-10 ELISA kit (BioLegend, San Diego, Calif).

Histology

For histologic analysis, jejunal tissues collected on day 57 were fixed in 20% formalin neutral buffer solution. Mast cells in jejunal sections were detected by chloroacetate esterase staining by using a Naphthol AS-D Chloroacetate (Specific Esterase) Kit (Merck). The stained tissues were then visualized with an Axio Lab.A1 microscope (Zeiss, Jena, Germany). Mast cells were counted by using AxioVision software (Zeiss) and are expressed in cells/mm².

Preparation of single-cell suspensions from mesenteric lymph nodes, spleens, and colons

Mesenteric lymph nodes (MLNs) and spleens were collected 24 hours after the first or second OFC. Thereafter, they were prepared in suspensions by filtering through 70- μ m cell strainers. Single-cell suspensions of spleens were hemolyzed with ammonium-chloride-potassium buffer for 1 minute. LP and intraepithelial cells of colons were isolated as described previously.^{E2} Briefly, tissues collected on days 43 or 57 were cleared of Peyer patches, cut longitudinally, minced on ice, and then incubated in 1 $\times$ HBSS containing 5 mM EDTA, 5% FCS, and 1 mM dithiothreitol for 20 minutes at 37°C under continuous rotation to isolate epithelial cells. The remaining LP tissue pieces were digested by using an LP dissociation kit and a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. LP and epithelial cells were resuspended with 40% Percoll (GE Healthcare, Buckinghamshire, England), after which 75% Percoll was added to the bottom of the tube to form 75% (lower) and 40% (upper) Percoll layers. After Percoll density gradient centrifugation, LP mononuclear cells and intraepithelial cells were collected at the Percoll gradient interface.

In vitro antigen restimulation

Splenocytes or colonic LP mononuclear cells were seeded in 48-well plates at 4×10^6 cells/well or in 96-well plates at 4×10^5 cells/well and cultured for 6 days in culture medium (RPMI 1640 medium [Sigma-Aldrich, St Louis, Mo] supplemented with 10% heat-inactivated FCS [Thermo Fisher Scientific, Waltham, Mass]), 100 μ g/mL of streptomycin, 100 U/mL of penicillin, 10 mM sodium pyruvate, 100 μ M 2-mercaptoethanol, and 10 μ M minimum essential medium [MEM] nonessential amino acid solution [Thermo Fisher Scientific]) in the presence of 50 μ g/mL of ovalbumin at 37°C in a humidified atmosphere in the presence of 5% CO₂.

Flow cytometric analysis

EDTA-treated whole blood cells were hemolyzed with an ammonium-chloride-potassium buffer to obtain white blood cells. After the Fc receptors were blocked with anti-CD16/CD32 (clone 2.4G2) (BD Biosciences, Franklin Lakes, NJ), white blood cells, MLN cells, hemolyzed spleen cells, and

intestinal LP and intraepithelial cells were stained with the following antibodies and reagents: fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (clone M1/70) (Tonbo Biosciences, San Diego, Calif), IFN- γ (XMG1.2) (BD Biosciences, San Jose, Calif), Alexa Fluor 488-anti-IL-4 (11B11) (BioLegend), allophycocyanin (APC)-anti-Gr-1 (RB6-8C5), CD25 (PC61.5) (Tonbo Biosciences), phycoerythrin (PE)-anti-Ly-6G (1A8) (Tonbo Biosciences), IL-4 (11B11) (eBioscience, Waltham, Mass), IL-10 (JES5-16E3) (BD Biosciences), peridinin chlorophyll protein complex (PerCP)/Cy5.5-anti-Ly-6C (HK1.4) (BioLegend), CD4 (RM4-5) (Tonbo Biosciences), APC/Cy7-anti-CD45 (30-F11) (Tonbo Biosciences), Ghost Dye Violet 450, and cell viability reagents (Tonbo Biosciences). Splenic or intestinal Treg cells were detected by using a Treg cell detection kit (mouse) (Miltenyi Biotec). Intracellular cytokine staining of antigen-restimulated cells was performed following a 4-hour stimulation in culture medium containing Cell Stimulation Cocktail (Thermo Fisher Scientific) and Brefeldin A solution (BioLegend) according to their manufacturer's protocols. Cells were fixed and permeabilized with a Cyto-Fast Fix/Perm Buffer Set (BioLegend). Flow cytometry was performed on a FACSVerse (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences).

Analysis of in vitro CD4⁺ T-cell response to antigens

CD4⁺ T cells were isolated from the splenocytes of each group of mice on day 57 by magnetic activated cell sorting (MACS) (Miltenyi Biotec) by using a CD4⁺ T-cell isolation kit (mouse) (Miltenyi Biotec) and then stained with CytoTell UltraGreen (1:3000 dilution; AAT Bioquest, Sunnyvale, Calif) for 20 minutes at 37°C, according to the manufacturer's instructions. CD11c⁺ dendritic cells were isolated from naive BALB/c mouse splenocytes by MACS using CD11c MicroBeads (mouse) (Miltenyi Biotec). CytoTell UltraGreen-labeled purified CD4⁺ T cells were seeded in 96-well plates at 1×10^6 cells/well and cocultured in the culture medium with 1×10^5 cells/well purified CD11c⁺ dendritic cells in the presence of 20 μ g/mL of ovalbumin for 5 days. After 5 days of culture, cells were stained with PerCP-Cy5.5-anti-CD4 (RM4-5) and analyzed by using a FACSVerse for CytoTell dilution of CD4⁺ T cells, after which supernatants were collected.

Analysis of in vitro immunosuppressive function of CD11b⁺ cells

Splenocytes from each group of mice on day 44 were stained with FITC-anti-CD11b (clone M1/70) (Tonbo Biosciences), and CD11b⁺ cells were isolated from the splenocytes by MACS (Miltenyi Biotec) by using anti-FITC MicroBeads (Miltenyi Biotec). CD4⁺ T cells were isolated from naive BALB/c mouse splenocytes by MACS by using a CD4⁺ T-cell isolation kit (mouse) (Miltenyi Biotec) and then stained with CytoTell UltraGreen (1:3000 dilution; AAT Bioquest) for 20 minutes at 37°C, according to the manufacturer's instructions. CytoTell UltraGreen-labeled purified CD4⁺ T cells were seeded in 96-well plates at 4×10^5 cells/well and cocultured in the culture medium with or without 4×10^5 cells/well of purified CD11b⁺ cells in the presence or absence of 1 μ g/mL each of plate-bound anti-CD3e (clone 145-2C11) (Thermo Fisher Scientific) and 2 μ g/mL soluble anti-CD28 (clone 37.51) (Thermo Fisher Scientific) for 72 hours. After 72 hours of culturing, cells were stained with PerCP-Cy5.5-anti-CD4 (clone RM4-5) (BD Biosciences) and analyzed by using a FACSVerse for CytoTell dilution of CD4⁺ T cells.

Quantitative real-time PCR

Total RNA from splenocytes, MLN cells, and splenic CD11b⁺ cells were extracted by using an RNeasy Plus Mini (Qiagen, Valencia, Calif). Peyer patches were removed from the jejunums, after which the pieces of jejunum were stored in RNeasy RNA stabilization reagent (Qiagen). Fixed tissues were disrupted and homogenized by using a TissueLyser (Qiagen) for 3 minutes at 28 Hz, after which total RNA was extracted by using an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed by using a ReverTra Ace qPCR RT kit

(Toyobo, Osaka, Japan). Real-time PCR was performed with the Step One Plus Real-Time PCR System (Thermo Fisher Scientific) using TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression products for *Il4* (Mm00445259_m1), *Il10* (Mm01288386_m1), *Il17a* (Mm00439618_m1), *Ifng* (Mm01168134_m1), *Tgfb1* (Mm01178820_m1), and *Nos2* (Mm00440502_m1), all of which were purchased from Thermo Fisher Scientific. The mRNA expression levels were quantified by the comparative cycle threshold method using StepOne Software and then normalized against the housekeeping gene *Gapdh*.

Coculture of bone marrow–derived MDSCs with Notch ligand–expressing cells

Bone marrow cells were isolated from the femora of BALB/c mice. The mouse Notch ligand Delta-like 1 (DLL1)-expressing Chinese hamster ovary (CHO) cell line (DLL1-CHO) was donated by Shigeru Chiba (University of Tsukuba, Tsukuba, Japan).^{E3} DLL1-CHO cells or CHO cells were seeded at a density of 2.2×10^4 cells/cm² in plates and treated with 3 μ g/mL of mitomycin C (Merck) for 3 hours. Bone marrow cells were plated at a density of 1.2×10^5 cells/cm² and cocultured with DLL1-CHO cells or CHO cells for 4 days in MEM α medium (FUJIFILM Wako Pure Chemical Corp, Osaka, Japan) supplemented with 10% heat-inactivated FCS (Biosera, Nuaille, France), 100 U/mL of penicillin (Meiji Seika Pharma, Tokyo, Japan), 100 μ g/mL of streptomycin (Meiji Seika Pharma), 100 μ M 2-mercaptoethanol (FUJIFILM Wako Pure Chemical Corp), 10 mM sodium pyruvate (Merck), 10 μ M MEM nonessential amino acid solution (Thermo Fisher Scientific), 20 ng/mL of recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ), and 20 ng/mL of recombinant murine IL-6 (Tonbo Biosciences) in the presence or absence of 10 μ M DAPT (ChemScene).

Repeated oral challenges following OIT

OVA-sensitized mice were challenged intragastrically with 20 mg of unheated ovalbumin 6 times every other day from day 40 to day 50 following

OIT treatment. On days 40, 50, and 64, OVA-induced allergic diarrhea and hypothermia were evaluated in the mice.

Anti-Gr1 depletion

Depletion of Gr1⁺ cells was conducted as previously described.^{E4} A total of 200 μ g of anti-Gr1 (RB6-8C5) per mouse or isotype control rat IgG2b κ (RTK4530) (BioLegend) was injected into the mice intraperitoneally on days 29, 32, 35, 38, and 41 of the protocol as shown in Fig 7, A or Fig E5.

Statistical analyses

Data are presented as means plus or minus SEMs in all figures in which error bars are shown. The unpaired Student *t* test was used as appropriate for parametric differences. One-way ANOVA or 2-way ANOVA with the Dunnett method was used for multiple testing of data. *P* values less than .05 were considered significant.

REFERENCES

- E1. Ochi H, Takai T, Shimura S, Maruyama N, Nishioka I, Kamijo S, et al. Skin treatment with detergent promotes protease allergen-dependent epicutaneous sensitization in a manner different from tape stripping in mice. *J Invest Dermatol* 2017; 137:1578-82.
- E2. Honjo A, Nakano N, Yamazaki S, Hara M, Uchida K, Kitaura J, et al. Pharmacologic inhibition of Notch signaling suppresses food antigen-induced mucosal mast cell hyperplasia. *J Allergy Clin Immunol* 2017;139:987-96.e10.
- E3. Shimizu K, Chiba S, Hosoya N, Kumano K, Saito T, Kurokawa M, et al. Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. *Mol Cell Biol* 2000;20: 6913-22.
- E4. Ma C, Kapanadze T, Gamrekashvili J, Manns MP, Korangy F, Greten TF. Anti-Gr-1 antibody depletion fails to eliminate hepatic myeloid-derived suppressor cells in tumor-bearing mice. *J Leukoc Biol* 2012;92:1199-206.

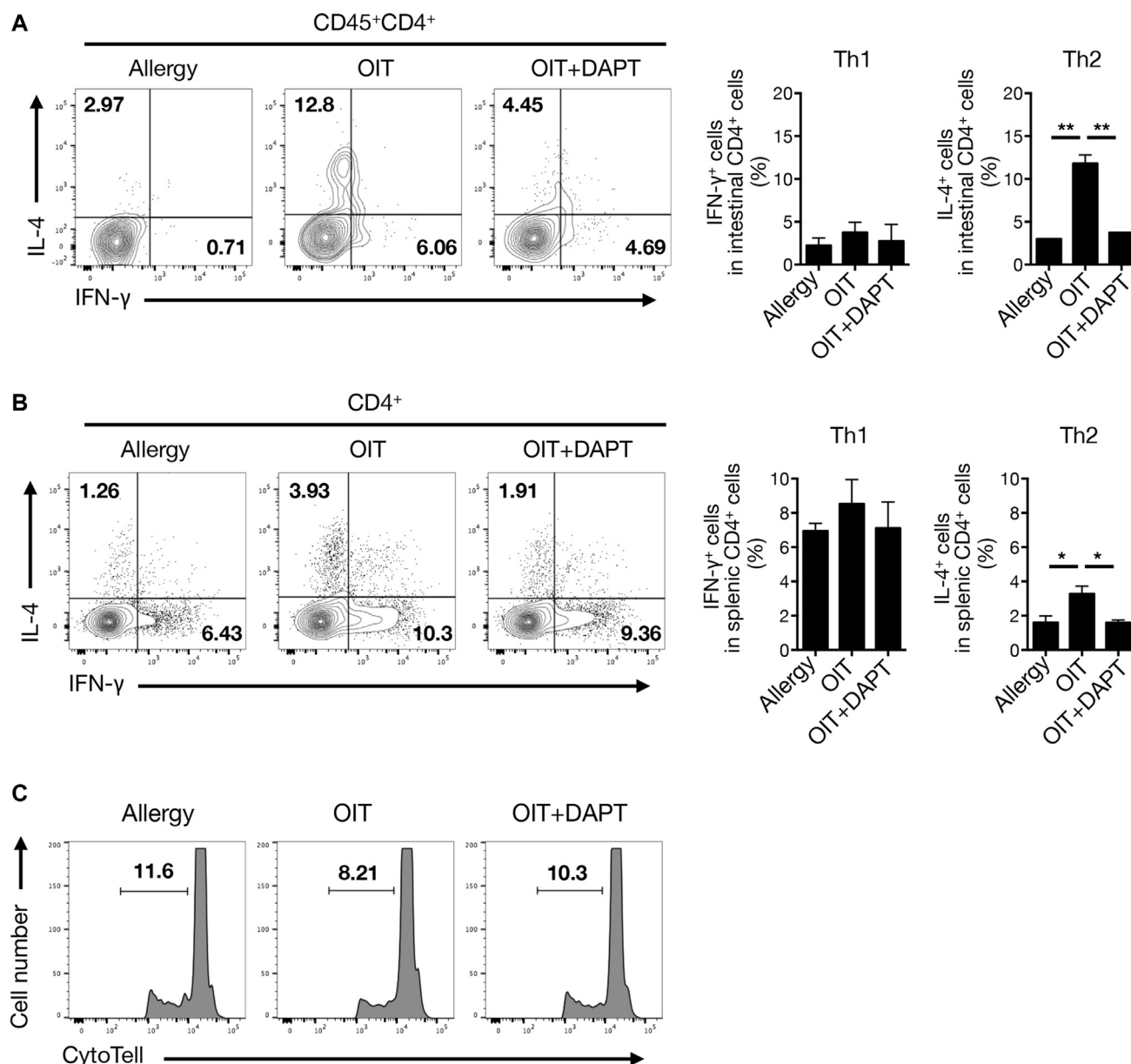


FIG E1. Inhibition of Notch signaling prevents OIT-induced Th₂ cell expansion and suppression of T-cell activation. Flow cytometric analysis (*left*) and frequencies (*right*) of intracellular staining of colonic (**A**) or splenic (**B**) $CD4^+$ T cells for IFN- γ and IL-4. Cells were isolated from the mice on day 57 and were cultured with ovalbumin for 6 days ($n = 3$). * $P < .05$; ** $P < .005$. **C**, Flow cytometric analysis of proliferation of splenic $CD4^+$ T cells that had been isolated from each group of mice, labeled with CytoTell UltraGreen, and cocultured with splenic $CD11c^+$ dendritic cells in the presence of ovalbumin for 5 days. Error bars indicate means $\pm$ SEMs. Data are from an individual experiment that represents 3 (**A** and **B**) or 2 (**C**) independent experiments with similar results.

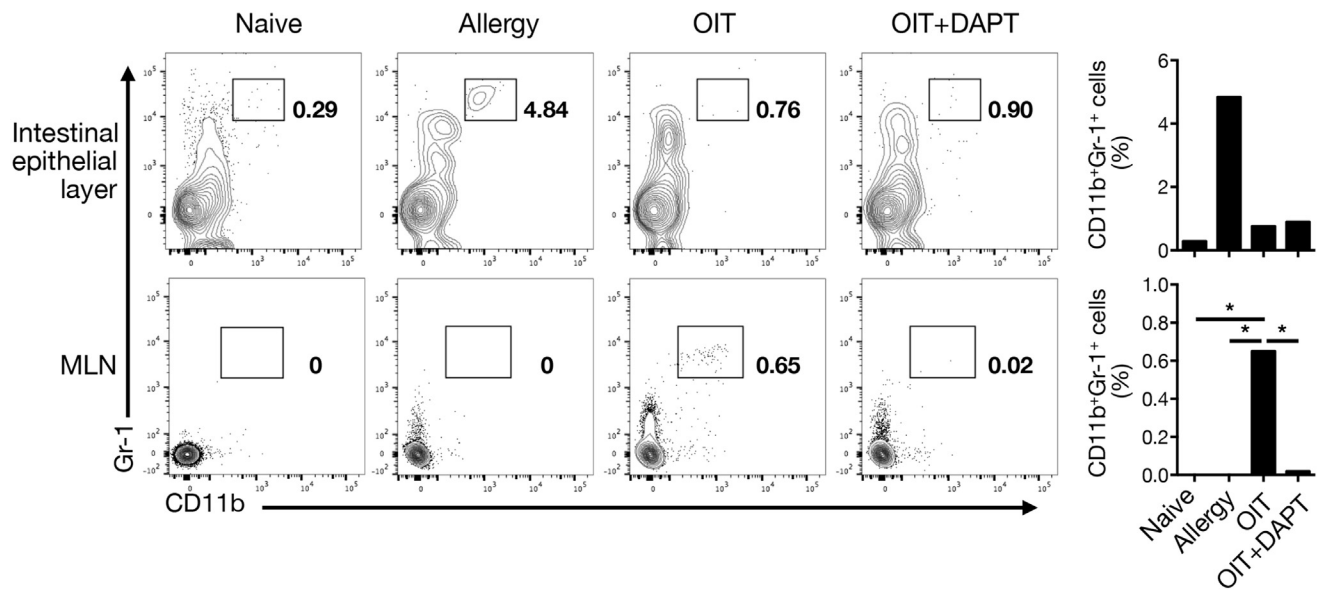
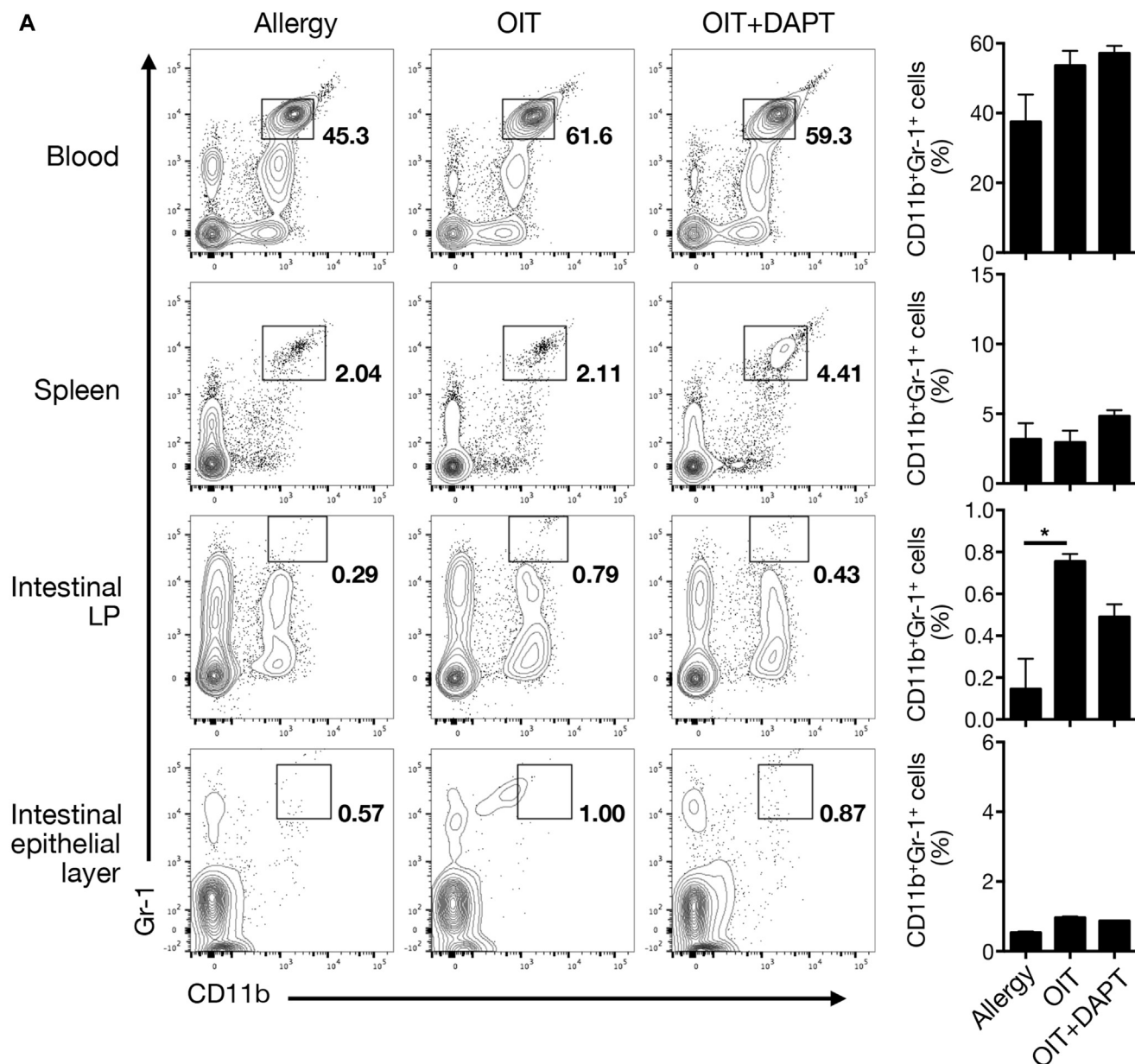


FIG E2. Inhibition of Notch signaling suppresses OIT-induced MDSC expansion in MLNs. Flow cytometric analysis (*left*) and frequencies (*right*) of CD11b⁺Gr-1⁺ cells in intestinal epithelial layer and mesenteric lymph nodes (MLNs) of mice on day 57 (n = 2 or 3). **P* < .05. Error bars indicate means ± SEMs. Data are from an individual experiment that represents 3 independent experiments with similar results.



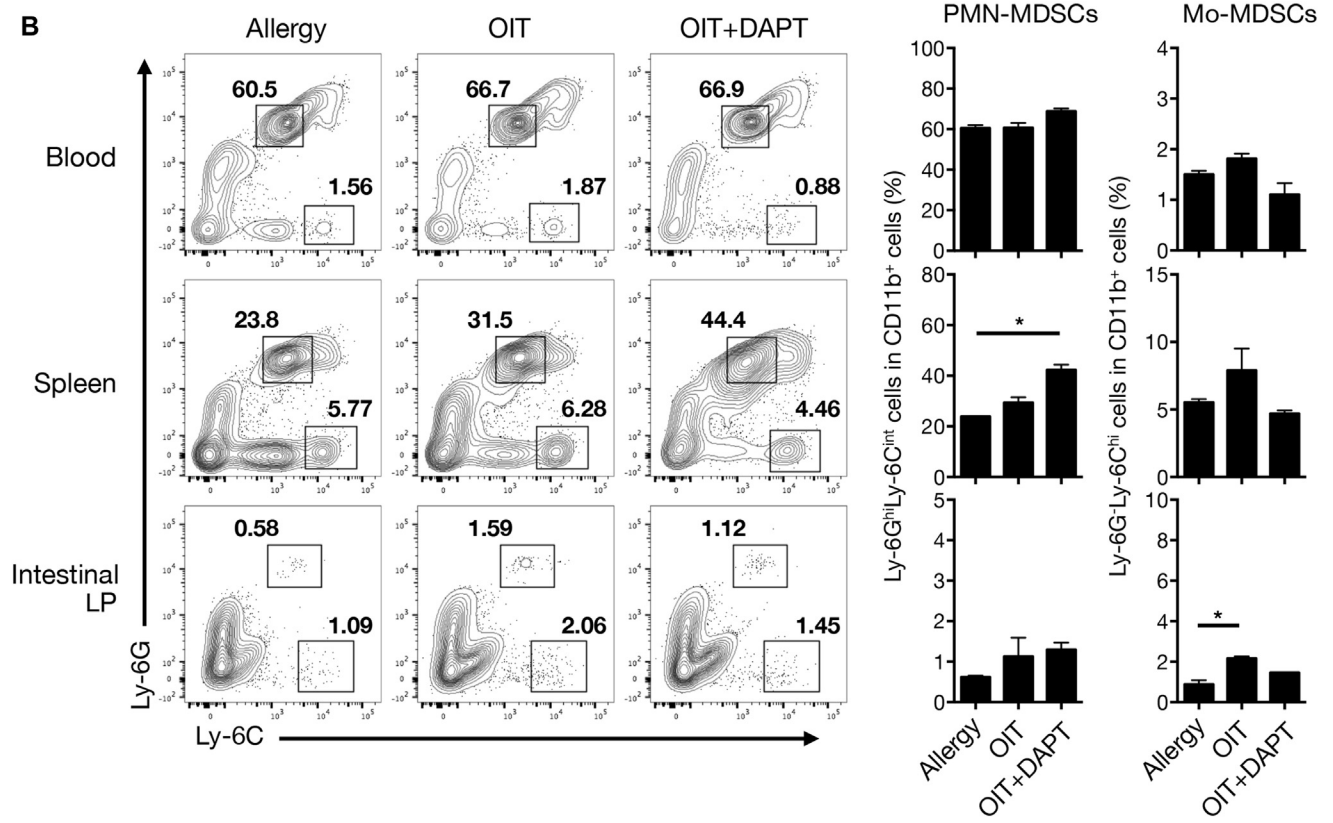


FIG E3. (Continued).

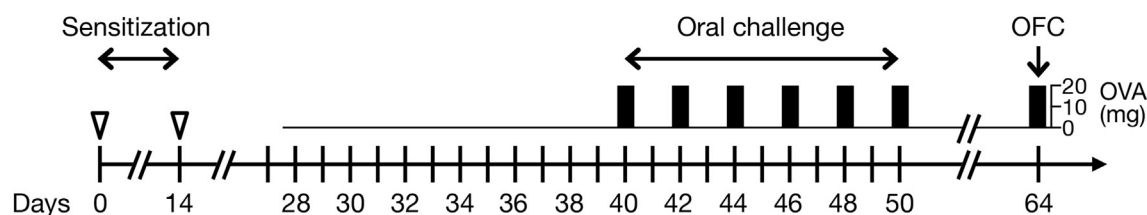
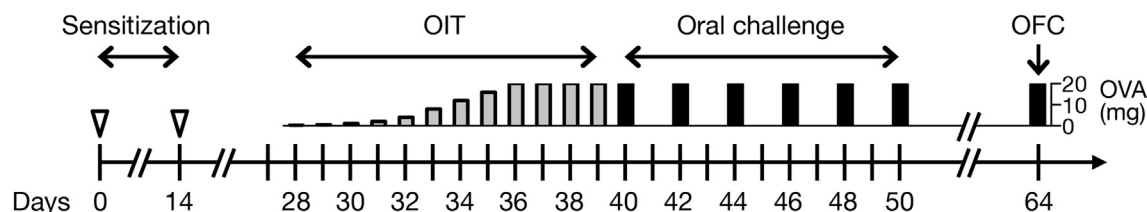
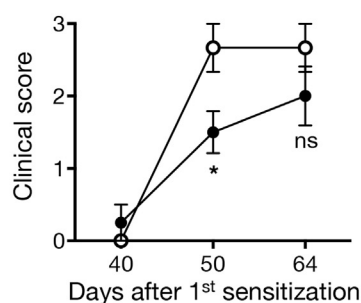
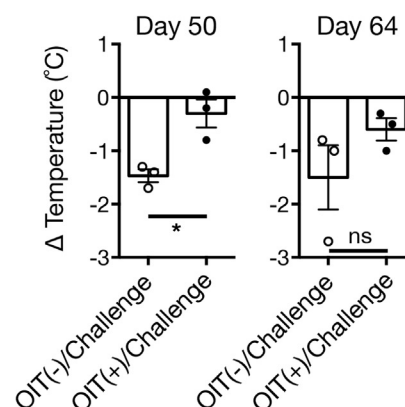
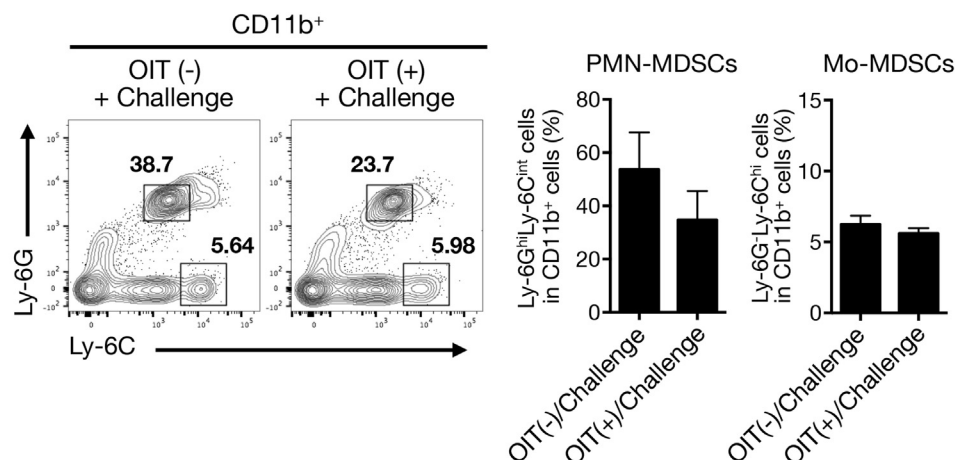
A OIT(-) / Oral challenge model**B** OIT(+) / Oral challenge model**C** ○ OIT(-)/Challenge
● OIT(+)/Challenge**D****E**

FIG E4. Induction of SU in ovalbumin (OVA)-desensitized mice is prevented by repeated oral challenges. Schematic procedures to establish the experimental food allergy model (**A**) and repeated oral challenges following the OIT model (**B**) are shown. Immediate hypersensitivity reactions to ovalbumin (OVA) were assessed by OFC on day 64. Black bar indicates unheated OVA; gray bar indicates heated OVA. **C**, The severity of allergic diarrhea induced by OFC ($n = 4$). * $P < .05$ compared with mice with food allergy. **D**, Changes in rectal temperature at 25 minutes after OFC. * $P < .05$. **E**, Flow cytometric analysis (left) and frequencies (right) of Ly6G⁺Ly6C^{int} cells and Ly6G⁺Ly6C^{hi} cells in spleen cells of mice on day 65 ($n = 3$). Error bars indicate means $\pm$ SEMs. Data are from an individual experiment that represents 2 independent experiments with similar results. *ns*, Not significant.

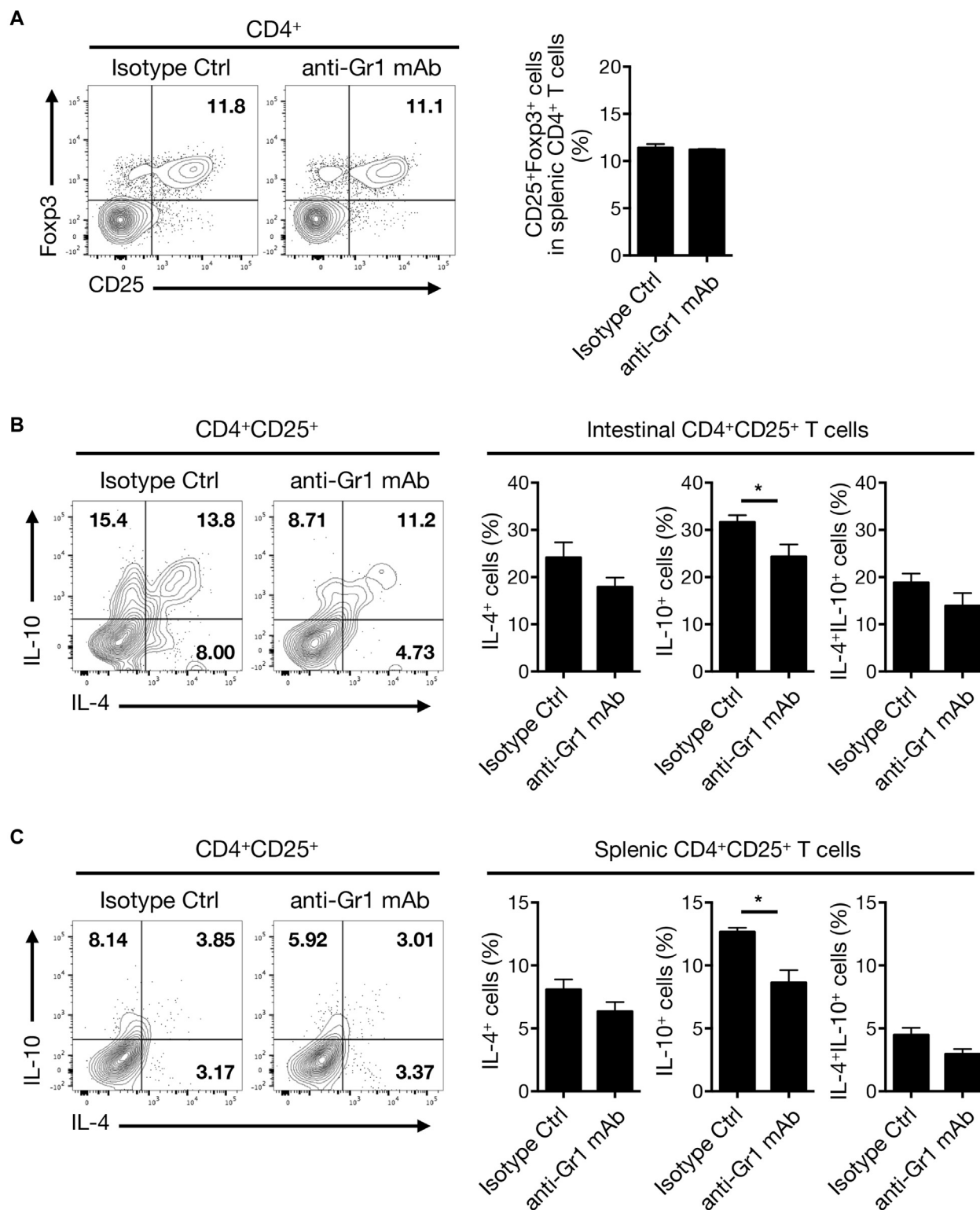


FIG E5. Depletion of Gr1⁺ cells during OIT treatment period does not affect splenic Treg cell frequencies, but it reduces IL-10-producing CD4⁺ T-cell frequencies locally and systemically. **A**, Flow cytometric analysis (left) and frequencies (right) of Treg cells (gated as CD4⁺CD25⁺Foxp3⁺) in the splenocytes of mice on day 57 (n = 3). Flow cytometric analysis (left) and frequencies (right) of intracellular staining in colonic (**B**) and splenic (**C**) activated CD4⁺ T cells (gated as CD4⁺CD25⁺) for IL-4 and IL-10. Cells were isolated from mice on day 57 and cultured with ovalbumin for 6 days for antigen restimulation (n = 3) (*P < .05). Error bars indicate means ± SEMs. Data are from an individual experiment that represents 2 independent experiments with similar results. Ctrl, Control.

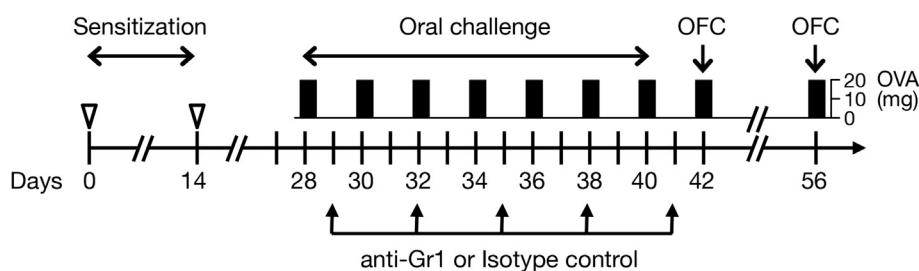
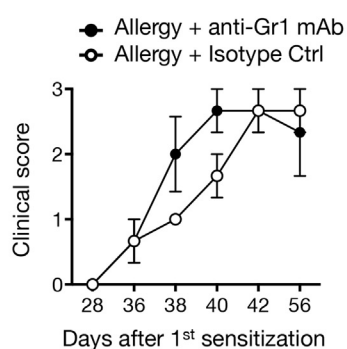
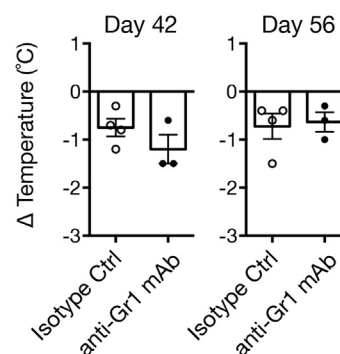
A Allergy + Gr1⁺ cell depletion**B****C**

FIG E6. Depletion of Gr1⁺ cells does not affect the development of allergic symptoms in mice with food allergy. **A**, Schematic procedures to establish the experimental food allergy model administered anti-Gr1-depleting antibody or isotype control (Ctrl). Immediate hypersensitivity reactions to ovalbumin (OVA) were assessed by OFC on days 42 and 56. Black bar indicates unheated OVA. **B**, The severity of allergic diarrhea induced by OFC ($n = 3$ or 4). **C**, Changes in rectal temperature at 25 minutes after OFC. Error bars indicate means $\pm$ SEMs. Data are from an individual experiment that represents 2 independent experiments with similar results.