

Pharmacologic inhibition of Notch signaling suppresses food antigen–induced mucosal mast cell hyperplasia

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Background: Mucosal mast cells (MMC) play a central role in the development of symptoms associated with IgE-mediated food allergy. Recently, Notch2-mediated signaling was shown to be involved in proper MMC distribution in the intestinal tract. **Objective:** This study aimed to clarify the mechanism by which Notch signaling regulates MMC distribution in the intestinal mucosa. Furthermore, pharmacologic inhibition of Notch signaling was evaluated as a treatment for symptoms associated with experimental food allergy.

Methods: Bone marrow–derived mast cells generated from mice were cultured with Notch ligands, and then expression of genes associated with MMCs was measured in the cells. In addition, the effect of an inhibitor of Notch signaling on food antigen–induced allergic reactions was examined in a mouse model of food allergy.

Results: Notch signaling induced MMC differentiation through upregulation of expression of genes characteristic of MMCs in the presence of IL-3. Some lamina propria cells isolated from the mouse small intestine expressed Notch ligands and were able to upregulate MMC markers in bone marrow–derived mast cells through Notch signaling. In a mouse model of food allergy, administration of a Notch signaling inhibitor led to suppression of food antigen–induced hyperplasia of intestinal MMCs, resulting in alleviation of allergic diarrhea and systemic anaphylaxis.

Conclusion: Notch signaling contributes to differentiation and accumulation of MMCs in the intestinal mucosa. Thus inhibition of Notch signaling alleviates symptoms associated with experimental food allergy. These results raise the

possibility that Notch signaling in mast cells is a novel target for therapy in patients with food allergy. (*J Allergy Clin Immunol* 2016;■■■:■■■-■■■.)

Key words: Notch signaling, mucosal mast cells, food allergy, mast cell hyperplasia

The prevalence of food allergy has increased over the past few decades in developed countries. Recent epidemiologic studies estimate food allergy rates approaching 5% in adults and 8% in children.¹ Therefore it is extremely important to develop prophylactic and therapeutic agents for food allergies.

Immune-mediated adverse food reactions, including food allergies, are categorized into 4 groups: IgE-mediated, non-IgE-mediated, mixed, and cell-mediated reactions. Patients with IgE-mediated food allergy can develop any of the following disorders within minutes to a few hours after ingestion of a specific food: urticaria, oral allergy syndrome, rhinitis, asthma, gastrointestinal hypersensitivity, or anaphylaxis.¹ These disorders are caused by activation of mast cells, which are the major effector cells that mediate hypersensitivity.

Mast cells are classified into 2 subtypes in rodents: mucosal mast cells (MMC) located in mucosal tissues and connective tissue–type mast cells (CTMCs) located in skin and blood vessels. IgE-mediated food allergy is frequently accompanied by mast cell hyperplasia in the intestinal mucosa. In murine models of food allergy, the occurrence of allergic diarrhea and severity of systemic anaphylaxis are correlated with increased numbers of intestinal MMCs.^{2–5} Therefore intestinal MMCs are critically involved in the development of food-induced allergic disorders. MMCs are characterized by intracellular expression of mouse mast cell protease (mMCP) 1 and mMCP-2 and cell-surface expression of the high-affinity IgE receptor FcεRI, c-Kit, and αE-integrin/CD103.⁶ During inflammation induced by food allergy and parasite infection, mast cell progenitors are mobilized from the bone marrow to the intestinal mucosa, or CTMCs move from the submucosa to the mucosa.^{7,8} After migration, these cells are converted into mature MMCs in the intestinal mucosa, suggesting that the intestinal mucosa can induce MMC maturation. TGF-β has been reported previously as an inducer of MMC maturation.^{6,9} Recently, Sakata-Yanagimoto et al¹⁰ have shown that Notch2-null mast cells are rarely detected in the epithelial layer in the small intestinal mucosa, which is in contrast to wild-type mast cells, accompanied by impaired eradication of infectious parasites. This report indicates that Notch2-mediated signaling plays an important role in MMC distribution and mucosal immunity in the intestine.

Notch family receptors, which mediate communication between adjacent cells, play an important role in various cell fate

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Abbreviations used

BMMC:	Bone marrow–derived mast cell
CHO:	Chinese hamster ovary
CTMC:	Connective tissue–type mast cell
DAPT:	N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester
Dll1:	Delta-like 1
Dll4:	Delta-like 4
IQDMA:	N1-(11H-indolo[3,2-c]quinolin-6-yl)-N2,N2-dimethylethane-1,2-diamine
Jag1:	Jagged 1
Jag2:	Jagged 2
LP:	Lamina propria
MLN:	Mesenteric lymph node
MMC:	Mucosal mast cell
mMCP:	Mouse mast cell protease
OVA:	Ovalbumin
PE:	Phycoerythrin
SCF:	Stem cell factor
STAT5:	Signal transducer and activator of transcription 5
TLR4:	Toll-like receptor 4

determinations.¹¹ Notch receptor–mediated signaling is initiated by interactions between Notch receptors on a receiving cell and Notch ligands on a contacting cell, 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.¹² Previous reports have shown that Notch1 and Notch2 are constitutively expressed on mouse mast cells, and Notch signaling induces MHC class II expression on mast cell surfaces and enhances Fc ϵ RI-mediated cytokine production by mast cells.^{13–15} However, the mechanism by which Notch signaling regulates MMC distribution in the intestine remains unclear.

In the present study we show that Notch signaling promotes differentiation of MMCs through induction of MMC-specific gene expression in the presence of IL-3. Mature intestinal MMCs expressed some adhesion molecules to stay in the intestinal mucosa. Thus inhibition of MMC maturation is anticipated to prevent MMC accumulation in mucosal tissues. In a murine model of food allergy, pharmacologic inhibition of Notch signaling was demonstrated to suppress food antigen–induced mast cell hyperplasia in the intestinal tract, resulting in alleviation of symptoms associated with food allergy.

METHODS

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

Reagents

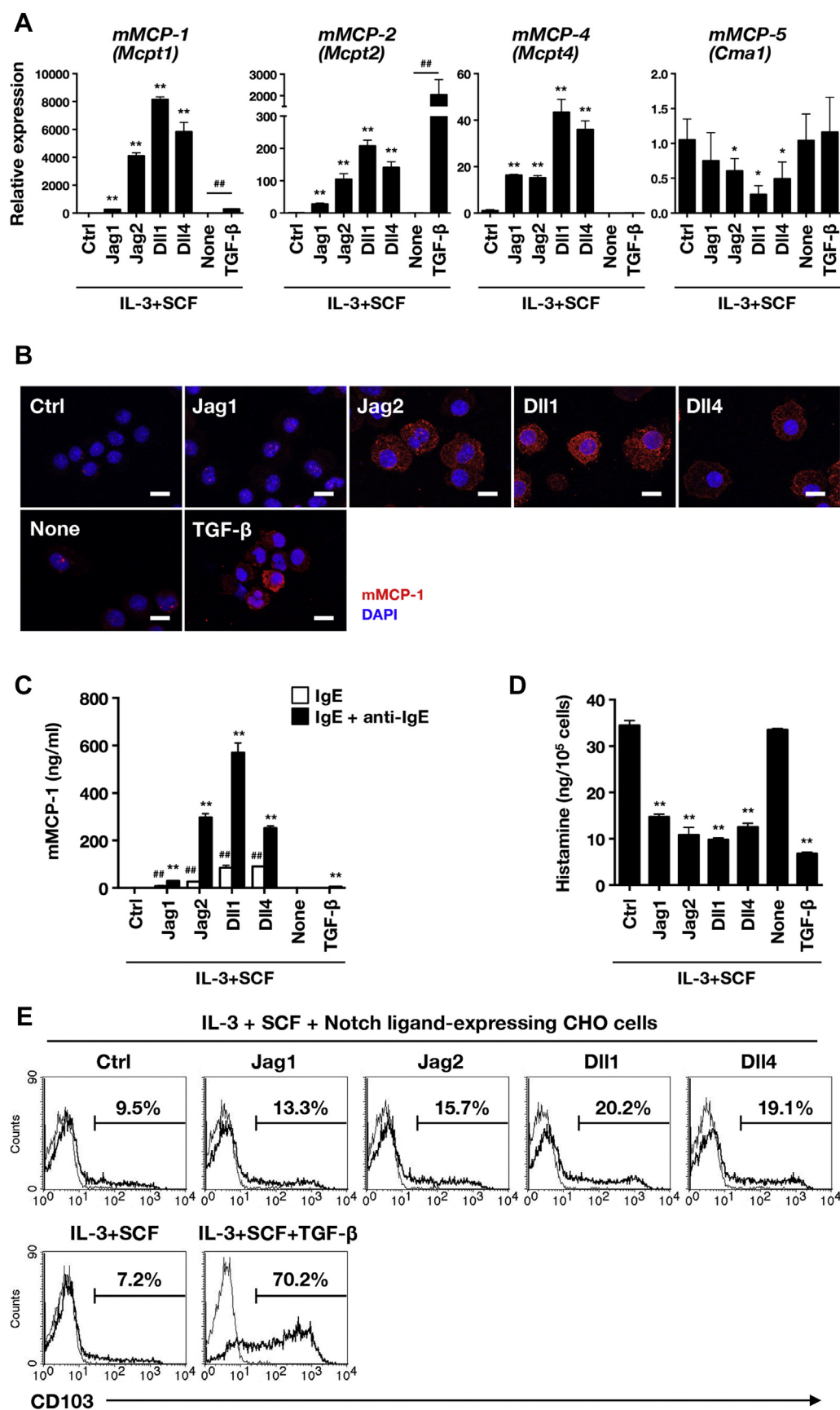
N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT), a γ -secretase inhibitor, and N1-(11H-indolo[3,2-c]quinolin-6-yl)-N2,N2-dimethylethane-1,2-diamine (IQDMA), a signal transducer and activator of transcription 5 (STAT5) inhibitor, were purchased from Chemscene (Monmouth Junction, NJ) and Merck Millipore (Billerica, Mass), respectively.

RESULTS**Notch signaling induces MMC marker expression**

We reported previously that Notch1 and Notch2 are highly expressed during the development of bone marrow–derived mast cells (BMMCs).¹³ Sakata-Yanagimoto et al¹⁶ have shown that Notch2 signaling promotes cell fate determination of myeloid progenitors toward mast cell progenitors. However, little is known about the role of Notch signaling in mast cell maturation. In this study we first investigated whether mast cell maturation is influenced by Notch signaling *in vitro* using BMMCs cultured in the presence of IL-3 and stem cell factor (SCF), which are more than 95% pure populations of immature mast cells. BMMCs contain a high level of mMCP-5 but undetectable levels of mMCP-1, mMCP-2, and mMCP-4, serine proteases that are expressed in mature mast cells. In particular, mMCP-1 and mMCP-2 are only detected in mature MMCs.¹⁷ We cocultured BMMCs with Notch ligand (Jagged 1 [Jag1], Jagged 2 [Jag2], Delta-like 1 [Dll1], or Delta-like 4 [Dll4])–expressing Chinese hamster ovary (CHO) cells or control CHO cells for 6 days. The presence of Notch ligands markedly upregulated the transcription of *Mcpt1* (encoding mMCP-1), *Mcpt2* (encoding mMCP-2), and *Mcpt4* (encoding mMCP-4) genes in BMMCs but downregulated *Cma1* (encoding mMCP-5) gene transcription (Fig 1, A). mRNA levels of *Mcpt1*, *Mcpt2*, and *Mcpt4* were significantly increased by coculture of BMMCs with Dll1 for 3 hours (see Fig E1 in this article's Online Repository at www.jacionline.org), indicating that the transcriptions of these 3 protease genes were upregulated in BMMCs immediately after culturing with Notch ligands.

TGF- β 1 is known to induce expression of some MMC-specific genes in BMMCs.^{6,9} As shown in Fig 1, A, TGF- β 1 upregulated the transcription of *Mcpt1* and especially *Mcpt2* in BMMCs, although it did not affect the transcription of *Mcpt4* and *Cma1*. The results suggest that the mechanism of action of Notch ligands on MMC-specific gene expression is different from that of TGF- β 1. mMCP-1 (Fig 1, B, and see Fig E2, A, in this article's Online Repository at www.jacionline.org) and mMCP-4 (see Fig E2, A) proteins were clearly detected by means of immunocytochemistry, Western blotting, or both in BMMCs cultured with Jag2, Dll1, Dll4, and TGF- β 1. mMCP-1 protein was released from these BMMCs during degranulation induced by Fc ϵ RI cross-linking (Fig 1, C).

In addition to accumulation of these proteases in the granule, MMCs are characterized by a low content of histamine in comparison with CTMCs^{18,19} and by cell-surface expression of α E-integrin/CD103.⁶ The presence of Notch ligands significantly decreased the cellular content of histamine, as well as TGF- β 1 (Fig 1, D), and increased the rate of CD103-expressing cells, although with lower efficiency than TGF- β 1 (Fig 1, E). Although it has been reported that MMCs have little heparin in the granules^{18,20} and modestly lower levels of cell-surface expression of Toll-like receptor 4 (TLR4),²¹ the presence of Notch ligands slightly increased the cellular level of heparin (see Fig E2, B) and had little effect on the expression level of TLR4 (see Fig E2, C). In addition, expression of the IL-33 receptor ST2, which is suppressed in mast cells by TGF- β 1,²² was not affected by either Notch ligands or TGF- β 1 (see Fig E2, D). These results indicate that Notch signaling promotes a phenotypic change in BMMCs toward MMC-like cells. Under our experimental conditions, the ability of



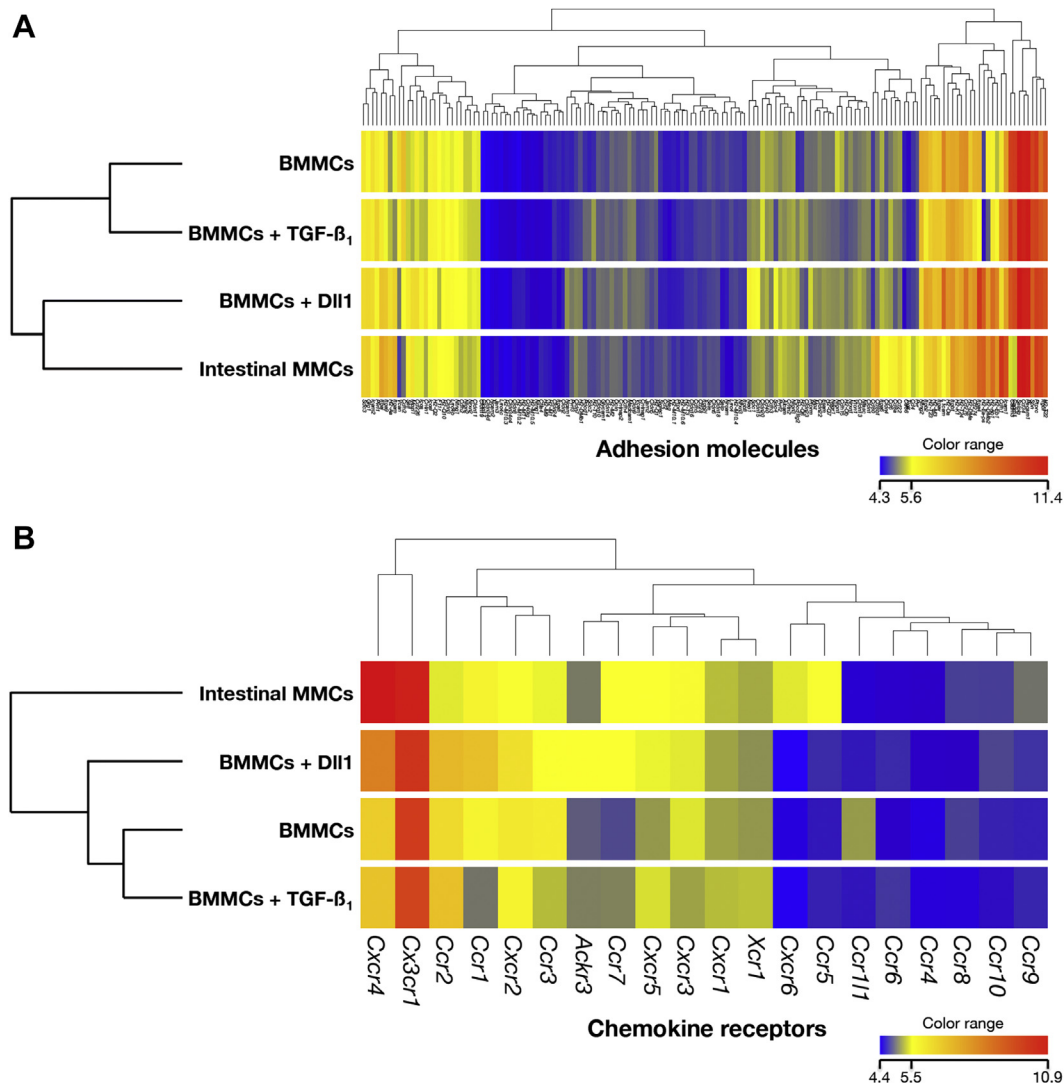


FIG 2. Comparison of gene expression profiles by using microarray analysis. Hierarchical clustering and heat map of normalized intensity values for genes encoding adhesion molecules (**A**) and chemokine receptors (**B**) in naive BMMCs, BMMCs cultured with TGF- β_1 , BMMCs cocultured with Dll1-expressing CHO cells, and intestinal MMCs isolated from the small intestines of naive mice.

Notch ligand to promote maturation of MMC-like cells was in the following order: Dll1 > Jag2 = Dll4 > Jag1.

BMMCs cultured with a Notch ligand display a gene expression pattern similar to that of intestinal MMCs

Although MMC marker expression was induced in BMMCs by both Notch ligands and TGF- β_1 , the expression pattern of MMC markers was different between Notch ligand-treated cells and

TGF- β_1 -treated cells (Fig 1 and see Fig E2). Therefore to determine the similarity among naive BMMCs, TGF- β_1 -treated BMMCs, Notch ligand Dll1-treated BMMCs, and intestinal MMCs, we analyzed gene expression patterns of these cells using microarray analysis. Gene expression data were analyzed by means of unsupervised hierarchical clustering with a Euclidean distance metric. The global gene expression pattern of intestinal MMCs was more similar to that of Dll1-treated BMMCs than that of TGF- β_1 -treated BMMCs (see Fig E3 in this article's Online Repository at www.jacionline.org). In unsupervised

FIG 1. Notch signaling upregulates MMC marker expression in BMMCs. BMMCs were cultured for 6 days in the presence of IL-3 and SCF with the indicated Notch ligand-expressing CHO cells, control CHO cells, or TGF- β_1 . **A**, Expression of mRNA for the indicated proteases in BMMCs. **B**, Immunofluorescence staining for mMCP-1 in BMMCs. Red, mMCP-1; blue, 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bars = 10 μ m. **C**, IgE-mediated release of mMCP-1 by BMMCs. **D**, Cellular histamine contents. **E**, Flow cytometric analysis of CD103 expression (bold line) on BMMCs (gated on Fc ϵ RI⁺c-Kit⁺). Negative control cells are plotted in a thin line. Values represent means $\pm$ SDs (n = 3-4). * P < .05 and ** P < .005 between control mice and others. ## P < .005 between indicated treatments (Fig 1, A and D). ## P < .005 compared with IgE-treated control mice and ** P < .005 compared with IgE + anti-IgE-treated control mice (Fig 1, C).

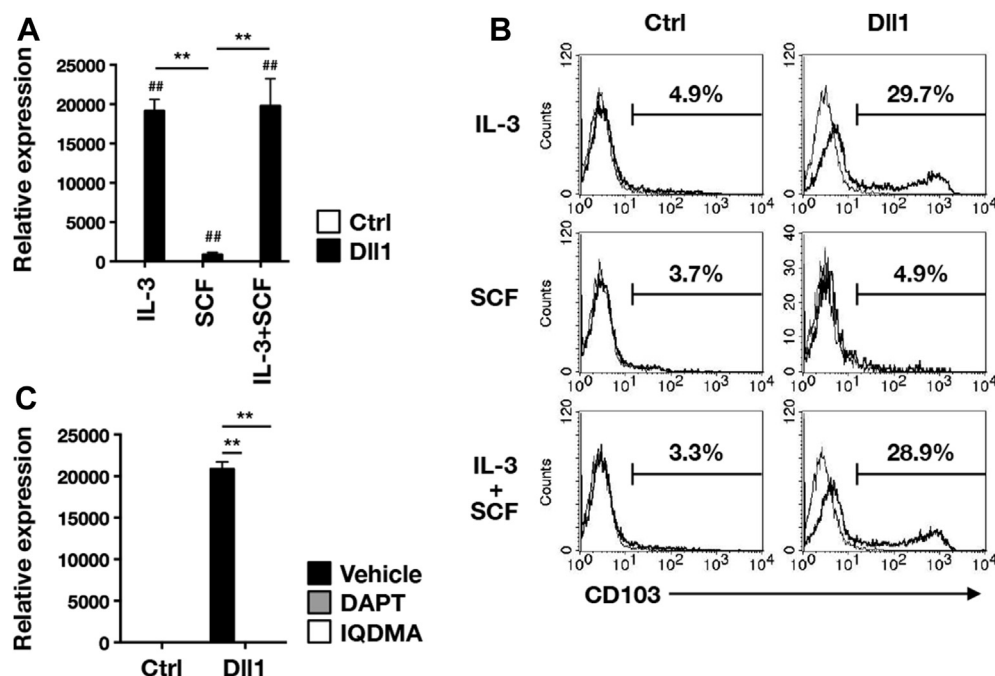


FIG 3. Notch and IL-3 signaling act coordinately to upregulate MMC marker expression. BMMCs were cocultured with Dll1-expressing CHO cells or control CHO cells in the presence of IL-3, SCF, or both for 6 days. **A**, Expression of *Mcpt1* mRNA. **B**, Flow cytometric analysis of CD103 expression (bold line) on BMMCs (gated on FcεRI⁺c-Kit⁺). Negative control cells are plotted in a thin line. **C**, Expression of *Mcpt1* mRNA in BMMCs cocultured with Dll1-expressing CHO cells or control CHO cells in the presence or absence of either 10 μmol/L DAPT or IQDMA for 3 hours. Values represent means ± SDs (n = 3-4). ***P* < .005 between indicated treatments (Fig 3, A and C). ##*P* < .005 between each control and others (Fig 3, A).

hierarchical clustering analysis focused on gene expression of adhesion molecules, Dll1-treated BMMCs clustered closely with intestinal MMCs (Fig 2, A). In particular, gene expression levels of integrin αL, integrin α9, integrin αM, CD113, claudin 7, and MHC class II-related molecules were higher in intestinal MMCs and Dll1-treated BMMCs than in TGF-β1-treated BMMCs and control BMMCs, and expression levels of versican and cadherin 2 were lower in intestinal MMCs and Dll1-treated BMMCs than in the others (Fig 2, A). Furthermore, intestinal MMCs and Dll1-treated BMMCs expressed high levels of the chemokine receptors CXCR4 and CX3CR1 (Fig 2, B). These data indicate that Notch signaling upregulates the expression of some gene characteristic of MMCs, including adhesion molecules and chemokine receptors.

Notch and IL-3 signaling act coordinately to upregulate MMC marker expression

To determine whether Notch signaling is sufficient by itself to induce a phenotypic change toward MMCs, we next examined the involvement of cytokines in Notch-induced expression of MMC markers. A remarkable upregulation of *Mcpt1* transcription and CD103 expression induced by Notch priming was observed only in BMMCs cultured in medium containing IL-3. In contrast, the presence of SCF had no influence on upregulation of these markers (Fig 3, A and B). IL-3 receptor-mediated signaling is transduced through activated Janus kinase 2 and STAT5.²³ Upregulation of *Mcpt1* transcription in BMMCs cultured for 3 hours in the presence of Dll1 was completely abolished by treatment with either the γ-secretase inhibitor DAPT, as an inhibitor of Notch signaling, or

the STAT5 inhibitor IQDMA (Fig 3, C). Although STAT5 is an essential molecule for mast cell survival and proliferation, cell viability was not affected by treatment of BMMCs with either DAPT or IQDMA for 3 hours (see Fig E4 in this article's Online Repository at www.jacionline.org). These results indicate that both Notch signaling and IL-3 receptor-mediated STAT5 activation are necessary for MMC marker expression in BMMCs.

Small intestinal lamina propria cells induce MMC marker expression in BMMCs through Notch signaling

The expression of multiple MMC markers was induced in BMMCs by coculturing with Notch ligand-expressing CHO cells (Figs 1 and 2). We next investigated whether the cells that constitute the intestinal mucosa have the ability to induce MMC marker expression on mast cells. Cell-surface expression of Notch ligands, such as Jag1, Jag2, Dll1, and Dll4, was detected in part of the lamina propria (LP) cells isolated from the small intestines of naive mice (Fig 4, A). Transcription of *Mcpt1*, *Mcpt2*, and *Mcpt4*, but not *Cma1*, was significantly upregulated in BMMCs by means of coculturing with LP cells for 18 hours (Fig 4, B). Upregulation of expression of these genes was suppressed by treatment with DAPT (Fig 4, B), suggesting that Notch signaling contributes to differentiation of MMCs in the intestinal mucosa.

Inhibition of Notch signaling alleviates symptoms associated with experimental food allergy

We showed *in vitro* that Notch signaling induces MMC marker expression and decreases intracellular histamine content in

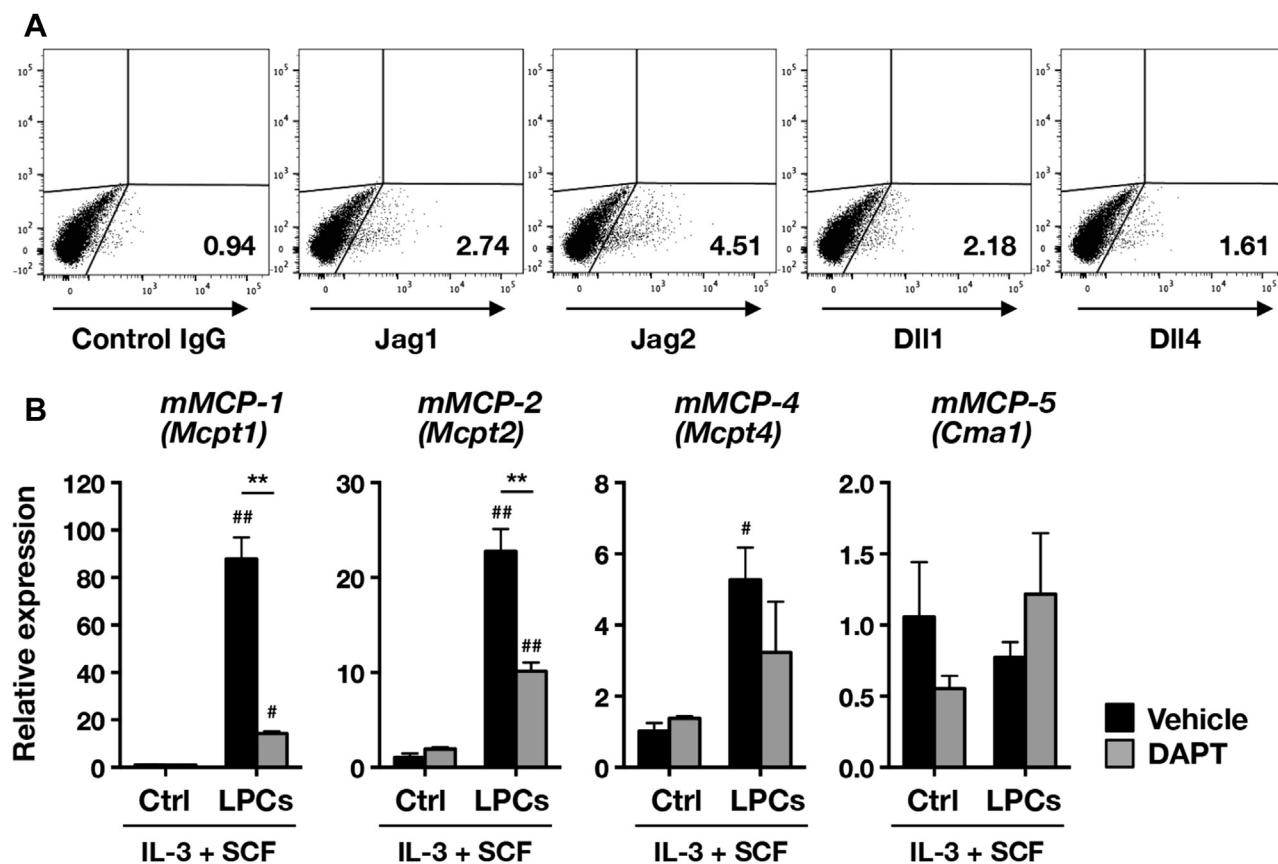


FIG 4. Expression of MMC markers is induced in BMMCs cocultured with mouse small intestinal LP cells through Notch signaling. **A**, Expression of the indicated Notch ligands on LP cells isolated from the mouse small intestine. **B**, BMMCs were cocultured with or without LP cells for 18 hours in the presence or absence of 10 μ mol/L DAPT. Values represent means $\pm$ SDs ($n = 3$). ** $P < .005$, # $P < .05$, and ## $P < .005$ between each control and others.

BMMCs. In addition, we previously reported that Notch signaling enhances Fc ϵ RI-mediated T_H2 cytokine production.¹⁵ These data suggest that immature mast cells undergo a phenotypic change toward mature MMCs by means of Notch signaling. Thus inhibition of Notch signaling is anticipated to alleviate some symptoms associated with food allergy that are caused by activated MMCs. To test this hypothesis, we examined the effect of treatment with the γ -secretase inhibitor DAPT as an inhibitor of Notch signaling during the effector phase of experimental food allergy on food antigen-induced hypersensitivity reactions (Fig 5, A). We observed no difference in body weight during the experimental period (see Fig E5, A, in this article's Online Repository at www.jacionline.org) or in levels of serum total IgE and ovalbumin (OVA)-specific IgE before OVA challenge (see Fig E5, B) between the vehicle-treated and DAPT-treated groups.

Because diarrhea is a representative symptom in this mouse model of food allergy,^{2,24} we first assessed stool consistency after intragastric OVA challenge. After the fifth OVA challenge, allergic diarrhea was observed in 30% of DAPT-treated mice compared with 89% of vehicle-treated mice (Fig 5, B). Moreover, hypothermia after the fourth OVA challenge was significantly alleviated in DAPT-treated mice compared with vehicle-treated mice (Fig 5, C).

Notch signaling is well known to regulate thymic T-cell lineage commitment and maturation, marginal zone B-cell development,

and cell fate decisions in myeloid lineages.^{12,25,26} Because administration of a γ -secretase inhibitor to mice causing the development of thymus atrophy has been reported,²⁷ we examined the effect of DAPT treatment on lymphocyte differentiation and function. DAPT administration every other day for 14 days to mice during the effector phase seemingly did not affect the development of lymphoid and other hematopoietic lineages in the thymus (see Fig E6, A, in this article's Online Repository at www.jacionline.org) and spleen (see Fig E6, B). Secretion of IFN- γ , IL-3, IL-4, and IL-6 after *in vitro* antigen restimulation of mesenteric lymph node (MLN) cells (see Fig E6, C) and splenocytes (see Fig E6, D) from mice receiving their fourth challenge with OVA showed no remarkable differences between vehicle- and DAPT-treated mice. Furthermore, the increase in total IgE and OVA-specific IgE levels in serum induced by repeated OVA challenge also showed no significant difference between vehicle- and DAPT-treated mice (Fig 5, D). Therefore DAPT is considered to act on effector cells in this mouse model.

Inhibition of Notch signaling suppresses food antigen-induced mast cell hyperplasia in the intestinal tract

To confirm the influence of Notch signaling inhibition on intestinal MMCs in this mouse model, we quantified mast cells

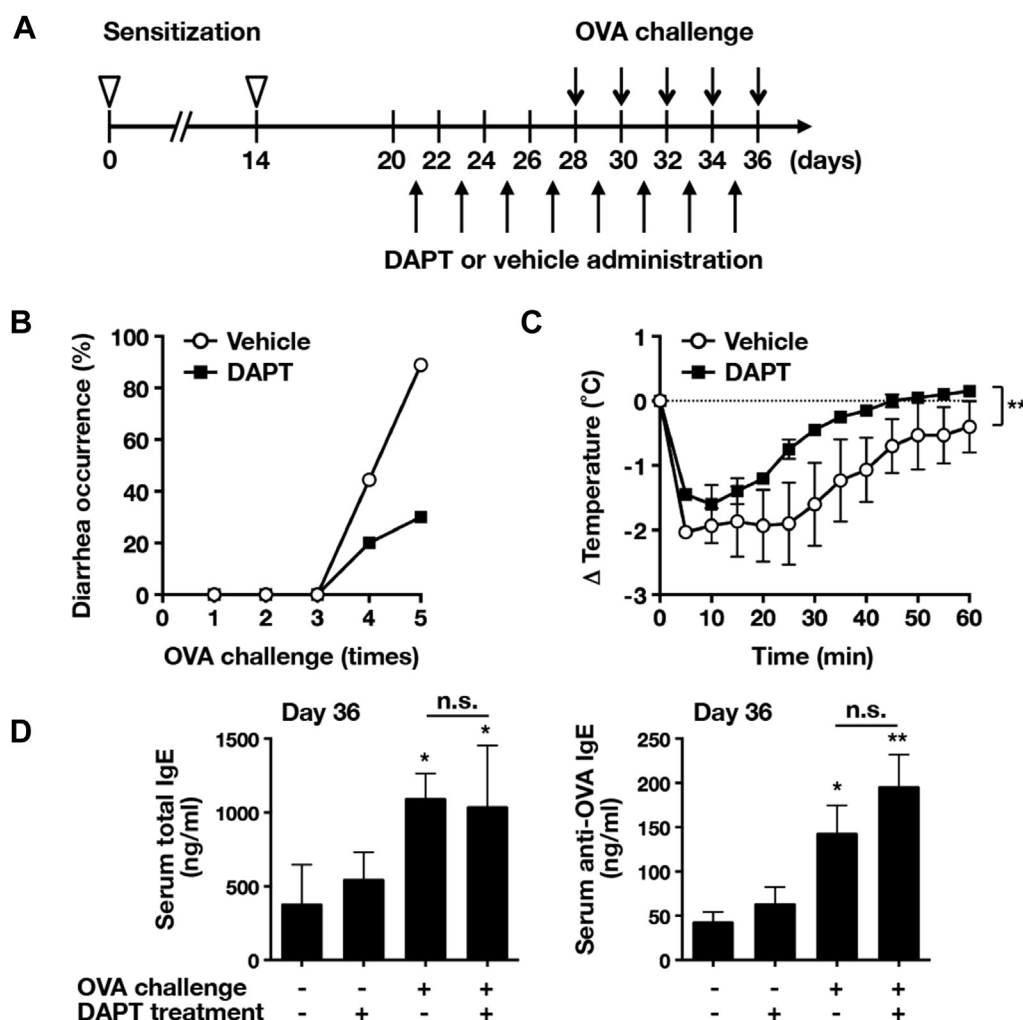


FIG 5. Pharmacologic inhibition of Notch signaling attenuates food allergy. **A**, Experimental outline. **B**, Diarrhea occurrence in vehicle- or DAPT-treated mice after each intragastric OVA challenge ($n = 9-10$). **C**, Rectal temperature in vehicle- or DAPT-treated mice after the fourth intragastric OVA challenge ($n = 3-4$). **D**, Serum total IgE (left panel) and OVA-specific IgE (right panel) levels in mice after the fifth intragastric OVA challenge ($n = 5$). Values represent means $\pm$ SEMs. * $P < .05$ and ** $P < .005$ between indicated treatments (Fig 5, C) or between OVA(-)/DAPT (-) and other treatments (Fig 5, D). *n.s.*, Not significant.

staining positive for toluidine blue in the small intestine and ascending colon. After the fifth intragastric OVA challenge, the number of mast cells was markedly increased in these tissues of vehicle-treated mice. The accumulation of mast cells in both tissues was significantly suppressed by treatment of mice with DAPT (Fig 6, A and B). mRNA expression of the MMC marker *Mcpt1* was increased remarkably in both tissues in vehicle-treated mice by using repeated OVA challenge. In agreement with the results of mast cell measurement shown in Fig 6, B, treatment with DAPT significantly attenuated the increase in *Mcpt1* mRNA expression (Fig 6, C). Serum mMCP-1 levels after the fifth OVA challenge were also significantly lower in DAPT-treated than in vehicle-treated mice (Fig 6, D). In contrast, there were no differences in the number of CTMCs in the ear auricle between vehicle- and DAPT-treated mice (see Fig E7 in this article's Online Repository at www.jacionline.org). Therefore these data show that administration of DAPT to mice suppresses food antigen-induced hyperplasia of MMCs in the intestinal tract.

Upregulated expression of the degranulation marker CD63 on the intestinal mast cells of mice after intragastric OVA challenge was little affected by administration of DAPT (see Fig E8 in this article's Online Repository at www.jacionline.org). This result indicates that alleviation of food antigen-induced allergic diarrhea and hypothermia in DAPT-treated mice was due to suppression of MMC hyperplasia but not mast cell activation.

DISCUSSION

Repeated challenge with food antigens leads to a dramatic increase in MMC numbers in the intestinal tract. Increased numbers of MMCs in inflamed mucosa are supplied by infiltration of mast cell progenitors, CTMCs, or both. Infiltrated immature cells, CTMCs, or both convert to mature MMCs in the inflamed mucosa, suggesting that mucosal tissues can promote the phenotypic change of mast cells. TGF- β , which is abundant in the intestinal tract, is a potent inducer of MMCs.^{6,9} In addition, Chen et al²⁸ have recently reported that T_H2 cells and IL-4 are

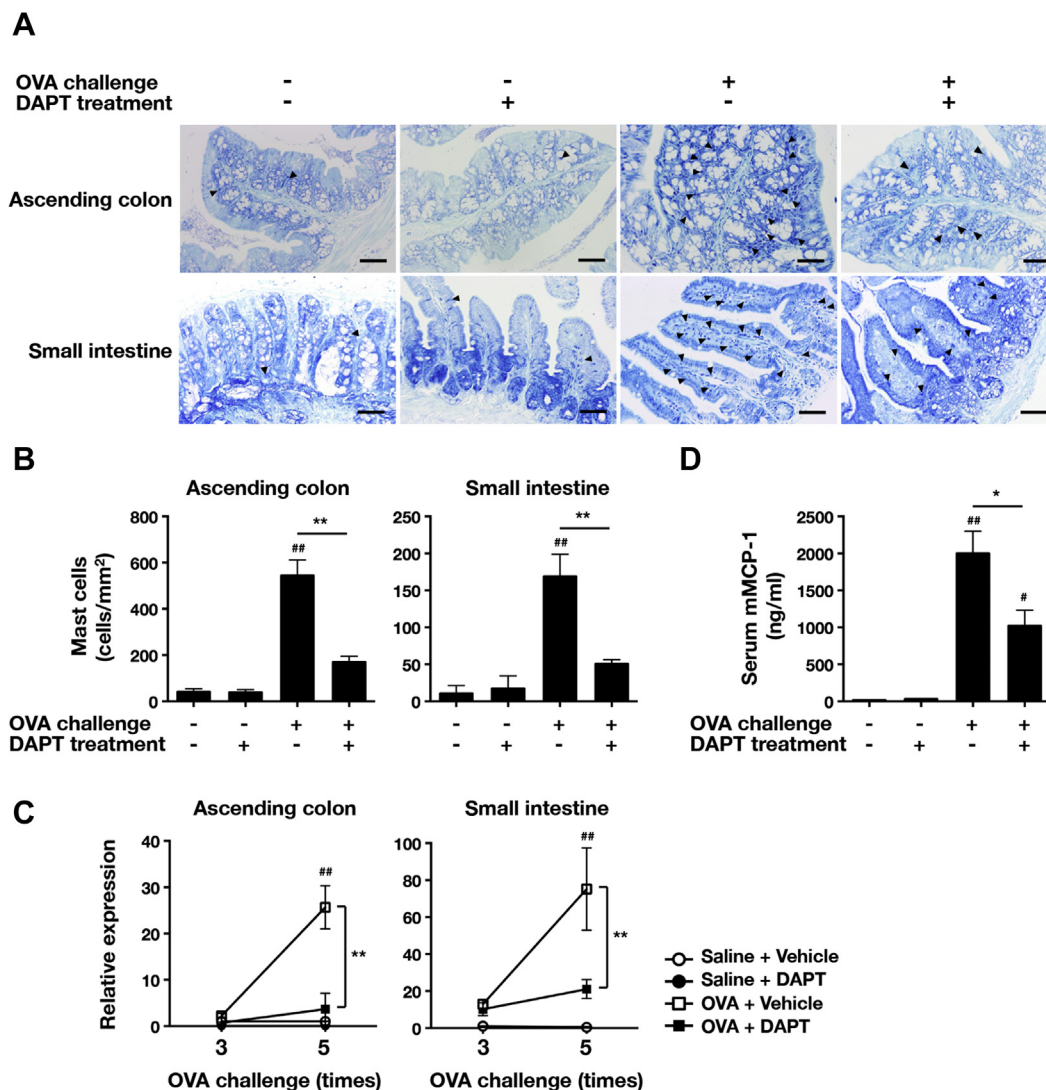


FIG 6. Pharmacologic inhibition of Notch signaling suppresses food antigen-induced mast cell hyperplasia. **A**, Toluidine blue staining of ascending colon (*upper panels*) and small intestine (*lower panels*) of mice after the fourth intragastric saline or OVA challenge. Scale bars = 50 μ m. **B**, Mean number of mast cells of Fig 6, **A**, are expressed in numbers per square millimeter ($n = 3-4$). **C**, Expression of *Mcpt1* mRNA in the ascending colon (*left panel*) and small intestine (*right panel*) of mice after the third and fifth intragastric saline or OVA challenge ($n = 5$). **D**, Serum mMCP-1 concentration of mice 5 hours after the fifth intragastric saline or OVA challenge ($n = 5$). Values represent means $\pm$ SEMs. * $P < .05$ and ** $P < .005$ between indicated treatments (Fig 6, **B** and **D**). # $P < .05$ and ## $P < .005$ between OVA(-)/DAPT(-) and other treatments (Fig 6, **B**, **C**, and **D**).

required for induction of IL-9-producing MMCs. In this study we showed that Notch signaling is also a powerful inducer of MMCs in the intestinal mucosa. The expression of MMC markers was remarkably upregulated in BMMCs by priming with the Notch ligands Jag2, Dll1, and Dll4 but modestly upregulated by priming with Jag1 (Fig 1, A-C). This difference might reflect the differential affinity of Notch receptors for ligands caused by glycosylation of the Notch extracellular domain.²⁹ Notch ligands were less effective than TGF- β 1 in induction of CD103-expressing BMMCs. This might indicate a difference in the mechanism of action between Notch and TGF- β signaling, or the signal might not be transmitted in all cells because of nonuniform cell-cell contact in this coculture system. In either case microarray analysis shows that the expression of many genes characteristic of MMCs was

induced in BMMCs by Notch signaling (Fig 2 and see Fig E3). In particular, BMMCs cultured with Dll1, but not TGF- β 1, expressed MHC class II molecules, as well as intestinal MMCs.

We have previously shown that Notch1 signaling induces cell-surface expression of MHC class II molecules on BMMCs.^{13,14} Interestingly, both intestinal MMCs and BMMCs cultured with Dll1 expressed unique adhesion molecules, including CD103 and CX3CR1 (Fig 2). CD103, which is expressed as a heterodimer by associating with β 7-integrin, is a receptor for E-cadherin. CX3CR1 is a receptor for CX3CL1/fractalkine. Both ligands are expressed on intestinal epithelial cells, and thus these receptors play an important role in the anchoring of cells to the epithelial layer of intestinal mucosa. It is known that CD103⁺ dendritic cells and CX3CR1⁺ macrophages are retained in the

epithelial layer of the intestinal tract through interactions between these receptors and their ligands.³⁰⁻³³ Therefore these receptors are probably important for the anchoring of MMCs to the intestinal mucosa. On the other hand, some molecules, such as CD80, CD86, CXCR6, and CCR5, which were expressed in intestinal MMCs, were not detected in BMMCs cultured with Dll1 or TGF- β 1 and control BMMCs (Fig 2). This result indicates that some factors other than Notch ligands or TGF- β are necessary to generate the mature MMC phenotype. In addition, it has been shown that upregulation of MMC markers requires the presence of IL-3 and activated STAT5, a downstream molecule of IL-3 receptor (Fig 3). Because a major source of IL-3 *in vivo* appears to be activated T cells, the present results agree with those of previous reports showing that the development of MMCs depends on T cells.^{34,35}

Food allergy is frequently accompanied by MMC hyperplasia in the intestinal mucosa. These MMCs are probably supplied by infiltration of mast cell progenitors and CTMCs. Our data show that small intestinal LP cells express Notch ligands and have the ability to induce expression of MMC markers in BMMCs through Notch signaling (Fig 4), suggesting that Notch signaling contributes to the differentiation of MMCs in the intestinal mucosa. Therefore blockade of Notch signaling can lead to inhibition of the phenotypic change to MMCs, resulting in a deficiency of adhesion molecules to anchor the cells in the mucosa, such as CD103 and CX3CR1. Studies using a mouse model of food allergy showed that the inhibition of Notch signaling suppressed accumulation of mast cells in the small intestine induced by food antigen administration (Fig 6).

This observation agrees with a previous report by Sakata-Yanagimoto et al¹⁰ showing that Notch2-null mast cells did not accumulate in the epithelial layer of the small intestine. Because intestinal mast cells play a critical role in the development of some symptoms associated with experimental food allergy, such as allergic diarrhea and systemic anaphylaxis,^{2,24,36} suppression of MMC hyperplasia is effective to alleviate these symptoms (Fig 5). Notch signaling is known to control cell fate decisions and function in lymphocytes. In this study short-term treatment of mice with DAPT did not apparently affect the development of lymphoid lineage (see Fig E6, A and B); antigen-specific production of cytokines, including IL-3, by MLN cells and splenocytes (see Fig E6, C and D); or IgE production (Fig 5, D). Thus it is unlikely that MMC differentiation was inhibited by insufficiency of IL-3. Recent reports have demonstrated that macrophages, basophils, and/or neutrophils are also involved in food antigen-induced anaphylaxis.³⁶ Administration of DAPT to mice might also affect the differentiation or function of those cells in addition to mast cells. Even so, in this study the inhibition of Notch signaling in DAPT-treated mice suppressed the effector phase of food antigen-induced allergic response.

In summary, we showed *in vitro* that the differentiation of MMCs is promoted by Notch signaling through the induction of expression of MMC-specific proteases and adhesion molecules. Some small intestinal LP cells express Notch ligands and are able to induce MMC marker expression on mast cells through Notch signaling. Therefore pharmacologic inhibition of Notch signaling in mice suppresses food antigen-induced MMC hyperplasia in the intestinal tract, resulting in alleviation of allergic diarrhea and systemic anaphylaxis in a model of food allergy. These results raise the possibility that Notch signaling in mast cells is a novel target for therapy of food allergies.

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Clinical implications: Notch signaling in MMCs might become a target for therapy of IgE-mediated food allergies.

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METHODS

Mice

Female BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). 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.

Generation of BMMCs

BMMCs were generated from femoral bone marrow cells of BALB/c mice, as described previously.^{E1} Cells were incubated for 3 to 4 weeks in RPMI 1640 medium (Sigma-Aldrich, St Louis, Mo) supplemented with 10% heat-inactivated FCS (Thermo Fisher Scientific, Waltham, Mass), 100 μ g/mL streptomycin, 100 U/mL penicillin, 10 mmol/L sodium pyruvate, 100 μ mol/L 2-mercaptoethanol, 10 μ mol/L MEM nonessential amino acid solution (Thermo Fisher Scientific), 10 ng/mL recombinant murine IL-3 (Wako Pure Chemical Industries, Osaka, Japan), and 10 ng/mL recombinant murine SCF (Wako Pure Chemical Industries) at 37°C in a humidified atmosphere in the presence of 5% CO₂. Mast cells were identified by means of flow cytometric analysis of the cell-surface expression of c-Kit and the Fc ϵ RI α -chain.

Culture of BMMCs with TGF- β 1

Three-week-cultured BMMCs were incubated for 6 days in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 μ g/mL streptomycin, 100 U/mL penicillin, 10 mmol/L sodium pyruvate, 100 μ mol/L 2-mercaptoethanol, 10 μ mol/L MEM nonessential amino acid solution, 10 ng/mL IL-3, 10 ng/mL SCF, and 5 ng/mL recombinant human TGF- β 1 (Wako Pure Chemical Industries) at 37°C in a humidified atmosphere in the presence of 5% CO₂.

Isolation of LP cells and LP mononuclear cells

LP cells of mouse small intestine were isolated, as described previously.^{E2} Briefly, after removal of Peyer patches, small intestines were cut longitudinally and incubated in 1 $\times$ HBSS containing 5 mmol/L EDTA, 5% FCS, and 1 mmol/L dithiothreitol for 20 minutes at 37°C under continuous rotation to isolate epithelial cells. The remaining LP tissue pieces were digested with an LP dissociation kit and a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. LP cells were resuspended with 40% Percoll (GE Healthcare, Buckinghamshire, United Kingdom), and then 75% Percoll was added to the bottom of the tube to form 75% (lower)/40% (upper) Percoll layers. After Percoll density gradient centrifugation, LP mononuclear cells were collected at the Percoll gradient interface.

Coculture of BMMCs with CHO cell lines expressing Notch ligands or LP cells

Mouse Notch ligand-expressing CHO cell lines (CHO-Jag1, CHO-Jag2, CHO-Dll1, and CHO-Dll4) were donated by Professor Shigeru Chiba (University of Tsukuba, Tsukuba, Japan).^{E3,E4} BMMCs were cocultured with a Notch ligand-expressing CHO cell line or control CHO cells, as described previously.^{E5} In brief, the CHO cells were seeded at a density of 2.2×10^4 cells/cm² in plates and treated with 3 μ g/mL mitomycin C (Sigma-Aldrich) for 3 hours. Three-week-cultured BMMCs were plated at a density of 1.2×10^5 cells/cm² onto plates and cocultured with CHO cells for 6 days in coculture medium (MEM Alpha [Thermo Fisher Scientific] supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 100 μ mol/L 2-mercaptoethanol, 10 mmol/L sodium pyruvate, and 10 μ mol/L MEM nonessential amino acid solution) with 10 ng/mL IL-3, 10 ng/mL SCF, or both in the presence or absence of 10 μ mol/L DAPT or IQDMA. LP cells isolated from the small intestines of naive BALB/c mice were seeded at a density of 3.3×10^4 cells/cm² on plates. After incubation for 1.5 hours, nonadherent cells were removed. Three-week-cultured BMMCs were placed at a density of 1.2×10^5 cells/cm² onto plates and cocultured with adherent LP cells for 18 hours in coculture medium with 10 ng/mL IL-3, SCF, or both in the presence or absence of 10 μ mol/L

DAPT. After coculture with CHO or LP cells, c-Kit⁺ BMMCs (purity >98%) were purified by means of magnetic cell sorting (Miltenyi Biotec) with a magnetic microbead-conjugated anti-mouse CD117/c-Kit mAb (Miltenyi Biotec), according to the manufacturer's instructions. Coculture was performed in MEM Alpha-based medium, whereas BMMCs were generated in RPMI 1640 media. We confirmed that the difference in media did not affect cell proliferation and survival, cell-surface expression of Fc ϵ RI and c-Kit, or mast cell degranulation (data not shown).

Quantitative real-time PCR

Total RNA was purified from BMMCs by using an RNeasy kit (Qiagen, Valencia, Calif) and purified from the tissues of mice 5 hours after intragastric OVA challenge with an RNeasy Lipid Tissue Kit (Qiagen) and an RNeasy kit, according to the manufacturer's instructions. First-strand cDNA was synthesized from 500 ng of total RNA by using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Real-time PCR was performed with the Eco Real-Time PCR System (Illumina, San Diego, Calif) by using TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression products for mouse mMCP-1 (*Mcpt1*; product no. Mm00656886_g1), mMCP-2 (*Mcpt2*; Mm00484932_m1), mMCP-4 (*Mcpt4*; Mm00487636_g1), and mMCP-5 (*Cma1*; Mm00487638_m1), which were purchased from Applied Biosystems (Waltham, Mass). mRNA expression levels were quantified by using the comparative method with Eco Software and normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Immunofluorescence cell staining

BMMCs were cytocentrifuged onto silane-coated glass slides (Muto Pure Chemicals, Tokyo, Japan) and air-dried. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.1% Triton X-100 for 3 minutes. Nonspecific binding in slides was blocked by means of incubation with the blocking solution Protein Block (Dako, Glostrup, Denmark) for 10 minutes at room temperature. Cells were then incubated with 5 μ g/mL anti-mMCP-1 antibody (Moredun Scientific, Penicuik, Scotland) overnight at 4°C and then incubated with 2 μ g/mL Alexa Fluor 594-conjugated anti-sheep antibody for 1 hour at room temperature. Nuclei were counterstained blue with 4'-6-diamidino-2-phenylindole dihydrochloride (Thermo Fisher Scientific). Stained cells were then visualized with a laser scanning microscope (LSM700; Zeiss, Jena, Germany).

Western blot analysis

BMMCs were collected and lysed through direct addition of sample buffer (62.5 mmol/L Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.1 mg/mL bromophenol blue dye, and 10% 2-mercaptoethanol). The cell lysates were electrophoretically resolved on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, Calif). Antibodies against mMCP-1 (285008; R&D Systems, Minneapolis, Minn), mMCP-4 (LifeSpan BioSciences, Seattle Wash), mMCP-5 (Proteintech, Rosemont, Ill), and β -actin (C4; Santa Cruz Biotechnology, Dallas, Tex) were used as primary antibodies. Alexa Fluor 680- or IRDye 800-conjugated anti-mouse or anti-rabbit IgG antibodies (Life Technologies) were used as secondary antibodies. Infrared fluorescence on the membrane was detected by using the infrared imaging system Odyssey (LI-COR Biosciences, Lincoln, Neb).

Staining and quantification of mast cells

Mouse tissue samples of the ascending colon, small intestine, and ear auricle were fixed and embedded in paraffin, and 4- μ m sections were cut. Sections were deparaffinized and then stained with 0.05% acidic toluidine blue, (pH 0.5). Mast cells were counted with AxioVision software (Zeiss) and expressed as numbers per square millimeter.

Isolation of MLN and spleen cells

MLNs and spleens were collected from mice after the fifth challenge with either OVA or saline and mechanically disrupted. Cells were filtered through

70- μ m cell strainers. Erythrocytes in splenic cell suspensions were lysed with ACK buffer.

Measurement of cytokines and mediators

BMMCs cocultured with Notch ligand-expressing or control CHO cells and BMMCs cultured in the presence or absence of TGF- β 1 were sensitized with mouse IgE, as described previously.^{E5} IgE-sensitized cells were resuspended in $1 \times$ Tyrode buffer at 1×10^6 cells/mL and incubated in the presence or absence of 1 μ g/mL anti-mouse IgE mAb (R35-72; BD Biosciences, Franklin Lakes, NJ) for 60 minutes, and then culture supernatants were collected. Mouse sera were collected from mice 5 hours after the third or fifth challenge with either OVA or saline. Concentrations of mMCP-1 in culture supernatants and mouse sera were quantified with the mMCP-1 ELISA kit (eBioscience, San Diego, Calif). Serum total IgE and anti-OVA IgE levels were quantified by using a corresponding ELISA kit (Chondrex, Redmond, Wash). MLN and spleen (2×10^5 cells) cells were restimulated with 20 μ g/mL OVA for 48 hours, and then culture supernatants were collected. Secreted cytokines in supernatants were quantified with ELISA kits for IFN- γ , IL-3, IL-4, and IL-6 (R&D Systems). Histamine and heparin contents in BMMC lysates were measured with a histamine EIA kit (Oxford Biomedical Research, Rochester Hills, Mich) and an unfractionated heparin ELISA kit (Lifespan Technologies, Salt Lake City, Utah), respectively. All ELISA procedures were performed according to the manufacturer's instructions.

Flow cytometric analysis and cell sorting

BMMCs were stained with fluorescein isothiocyanate-conjugated anti-mouse Fc ϵ RI α (MAR-1; eBioscience), peridinin chlorophyll protein complex/Cy5.5-conjugated anti-mouse c-Kit (2B8; BioLegend, San Diego, Calif), phycoerythrin (PE)-conjugated anti-mouse CD103 (2E7; BioLegend), biotinylated anti-mouse IL-33 receptor α (ST2; DIH9, BioLegend), biotinylated anti-mouse TLR4/MD2 (MTS510, BioLegend), and PE-labeled streptavidin (BioLegend) after blocking Fc receptors with anti-mouse CD16/CD32 (2.4G2; BD Biosciences). LP cells from the small intestines of naive mice were stained with biotinylated mAbs against mouse Notch1 (HMN1-12), Notch2 (HMN2-35), Notch3 (HMN3-133), and Notch4 (HMN4-14), which were originally generated in our laboratory^{E4} and used as the first antibodies after blocking Fc receptors. PE-labeled streptavidin was used in the second step of staining. LP mononuclear cells from the small intestines of mice 30 minutes after the fifth challenge with either OVA or saline were stained with fluorescein isothiocyanate-anti-Fc ϵ RI α , peridinin chlorophyll protein complex/Cy5.5-anti-c-Kit, and PE-anti-mouse CD63 (NVG-2; BioLegend) after blocking Fc receptors. The expression of cell-surface markers was analyzed on a FACSCalibur (BD Biosciences). Intestinal MMCs were obtained by sorting Fc ϵ RI⁺c-Kit⁺ cells from the small intestinal LP cells of naive BALB/c mice by using a JSAN cell sorter (Bay Bioscience, Hyogo, Japan).

Microarrays and gene expression analysis

Total RNA was purified from BMMCs, BMMCs cultured with TGF- β 1, BMMCs cocultured with Dll1-expressing CHO cells, and intestinal MMCs by using an RNeasy kit. Samples were processed at GeneticLab (Hokkaido, Japan) and then hybridized on a GeneChip Mouse Gene 2.0 ST Array

(Affymetrix, Santa Clara, Calif). Data were analyzed at Tohoku Chemical (Aomori, Japan). Genes were clustered by using a Euclidean distance matrix. For heat map analysis, colors indicate the expression value relative to the mean expression value per gene in the data set. Red indicates a maximum value, blue indicates a minimum value, and yellow indicates a mean value of normalized intensity values. Thus red indicates upregulation and blue indicates down-regulation relative to mean value. The complete data set is available at the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE80139).

Cell viability and cytotoxicity assays

Cell viability after treatment with DAPT or IQDMA was determined by using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol.

Food allergy model

Five-week-old female BALB/c mice were sensitized twice within a 2-week interval by means of intraperitoneal injection with 50 μ g of egg white albumin (OVA; Sigma-Aldrich) and 2 mg of aluminum hydroxide and magnesium hydroxide (Alum; Thermo Fisher Scientific) in saline. After sensitization, mice were challenged intragastrically with 50 mg of OVA in saline or vehicle 5 times every other day from day 28. Mice were injected intraperitoneally with 4 mg of DAPT in dimethyl sulfoxide or vehicle every other day from day 21 to day 35 to investigate the therapeutic effect of inhibiting Notch signaling in this model. Oral antigen-induced diarrhea was assessed by visually monitoring mice for up to 30 minutes after intragastric OVA challenge. Mice demonstrating loose or liquid stools were recorded as diarrhea-positive animals. Rectal temperature was measured with a digital thermometer (A&D Company, Tokyo, Japan) every 5 minutes after intragastric OVA challenge.

Statistical analyses

The unpaired Student *t* test was used as appropriate for parametric differences. One-way or 2-way ANOVA with the Dunnett method was used for multiple testing of data. *P* values of less than .05 were considered significant.

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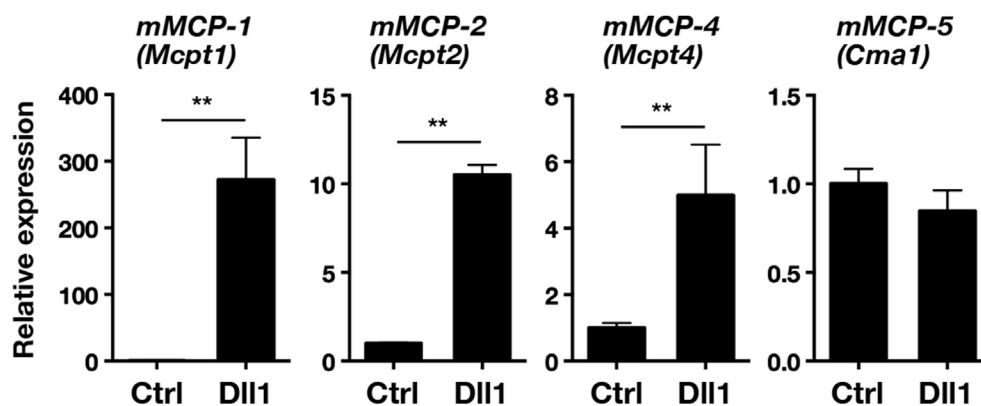


FIG E1. Expression of MMC-specific proteases is upregulated in BMMCs immediately after culturing in the presence of DII1. Expression of mRNA for the indicated proteases in BMMCs cultured for 3 hours in the presence or absence of DII1. Values represent means $\pm$ SDs ($n = 4$). ** $P < .005$ between indicated treatments.

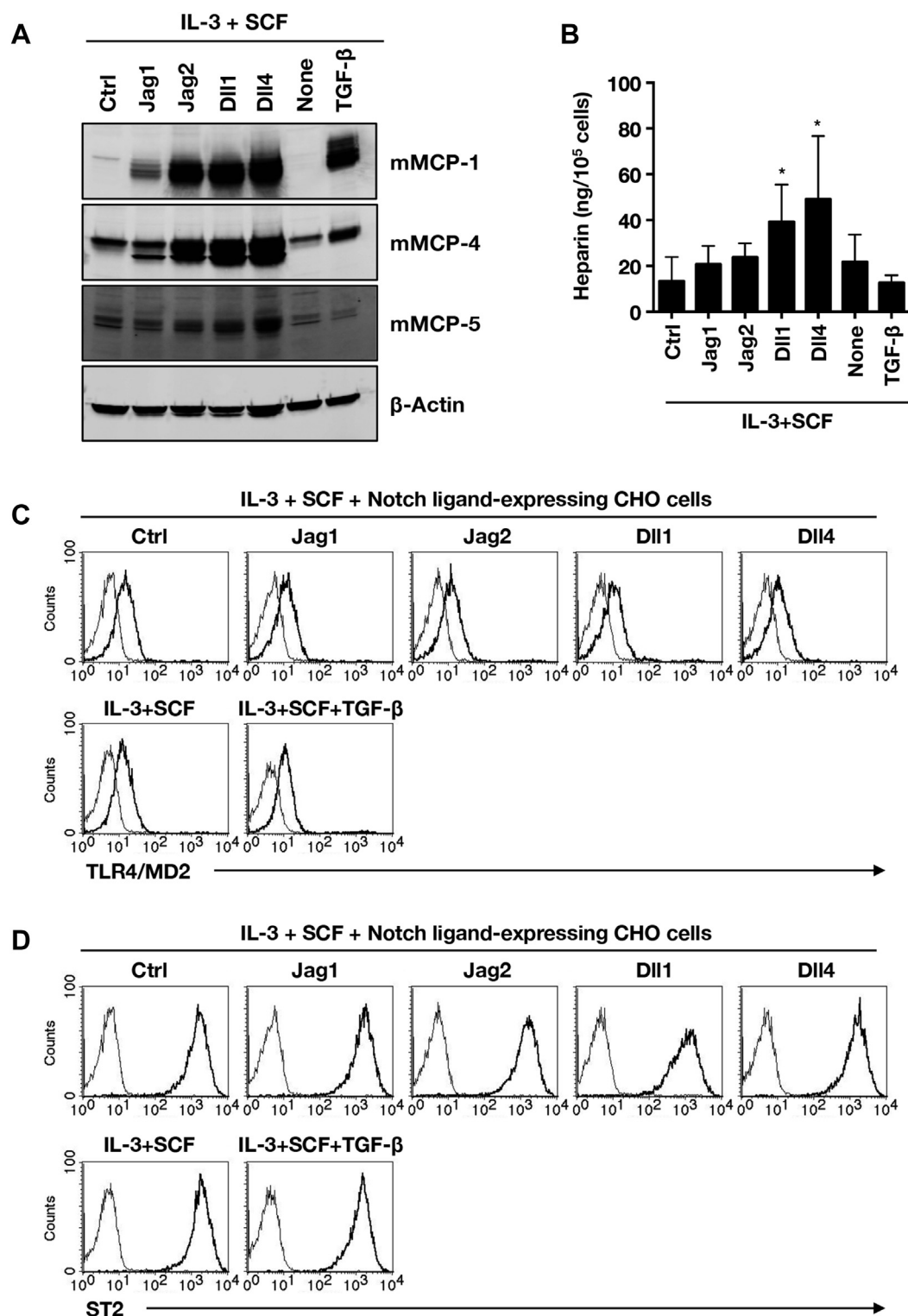


FIG E2. Expression of MMC-specific proteases and other markers in BMMCs. BMMCs were cultured for 6 days in the presence of IL-3 and SCF with the indicated Notch ligand-expressing CHO cells, control CHO cells, or TGF- β 1. **A**, Western blot analysis of BMMCs for the indicated protease. β -Actin was used as a loading control. **B**, Cellular heparin contents. * $P < .05$ between control and others. **C** and **D**, Flow cytometric analysis of TLR4/MD2 (Fig E2, **C**) and ST2 (Fig E2, **D**) expression (**bold line**) on BMMCs (gated on Fc ϵ R1⁺c-Kit⁺). Negative control cells are plotted in a **thin line**.

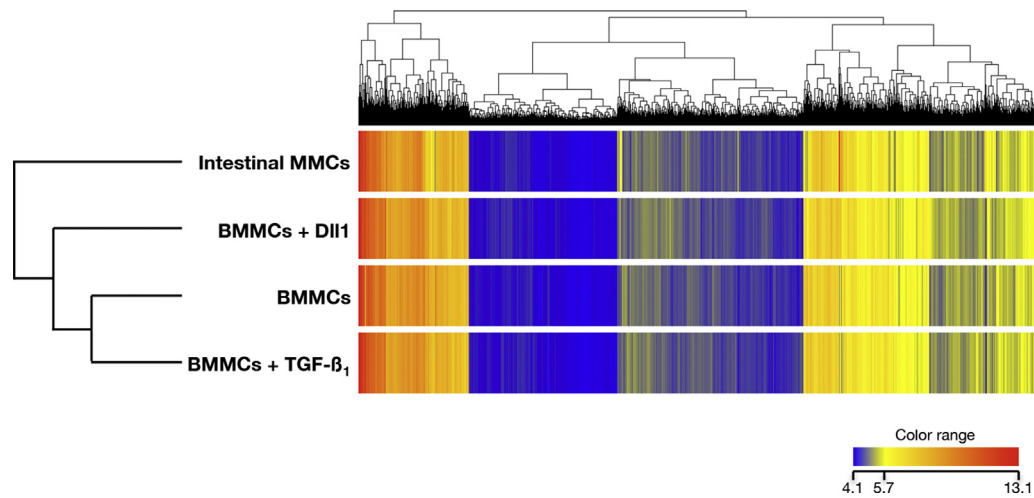


FIG E3. Comparison of gene expression profiles by using microarray analysis. Hierarchical clustering and heat map of normalized intensity values of 35,240 transcripts in naive BMMCs, BMMCs cultured with TGF- β_1 , BMMCs cultured with Notch ligand DII1, and intestinal MMCs isolated from the small intestines of naive mice.

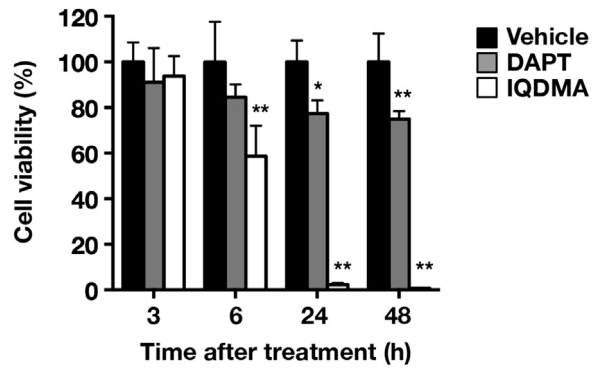


FIG E4. Effect of inhibitors on cell viability. BMMCs were cultured in the presence of 10 $\mu\text{mol/L}$ DAPT, 10 $\mu\text{mol/L}$ IQDMA, or vehicle (dimethyl sulfoxide) for the indicated time. Values represent means $\pm$ SDs ($n = 4$). * $P < .05$ and ** $P < .005$ compared with vehicle.

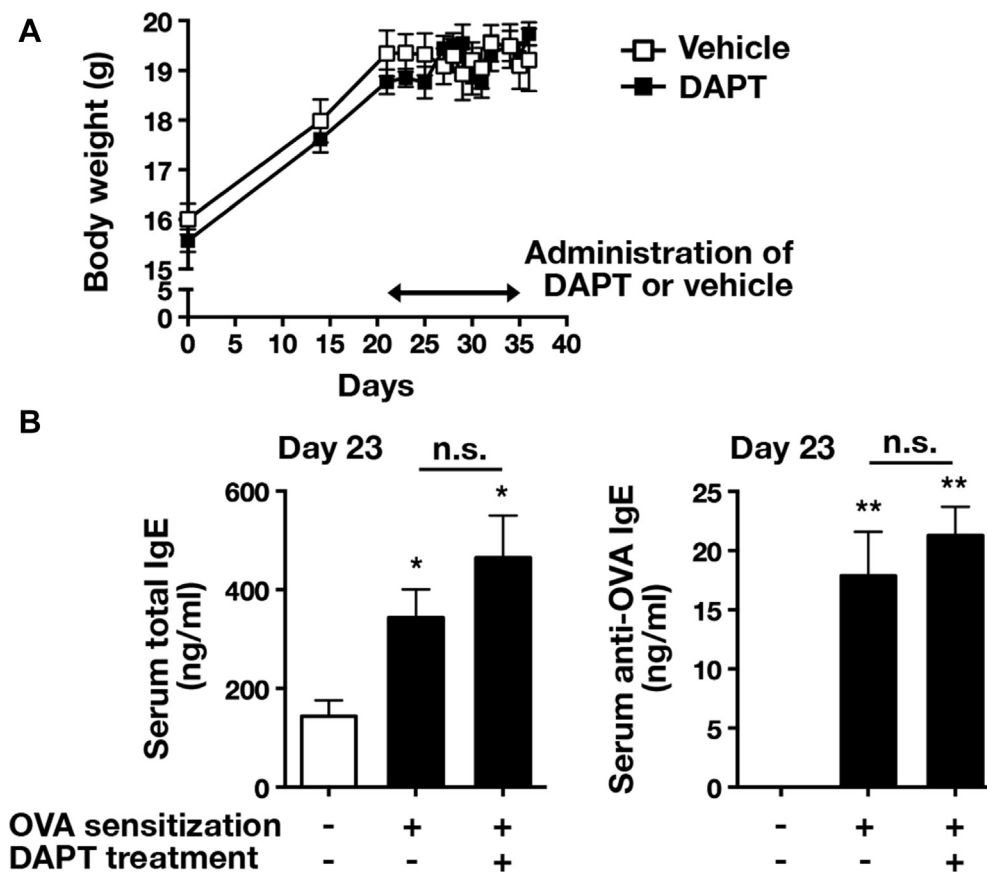


FIG E5. Administration of DAPT does not affect body weight of mice and serum IgE levels at day 23. **A**, Body weight change in the experimental period. DAPT or vehicle was administered intraperitoneally during the period indicated by the arrow. **B**, Serum total IgE (left panel) and OVA-specific IgE (right panel) levels in mice at day 23 ($n = 5$). Values represent means $\pm$ SEMs. * $P < .05$ and ** $P < .005$ compared with OVA-unsensitized and DAPT-untreated mice (Fig E5, B). n.s., Not significant.

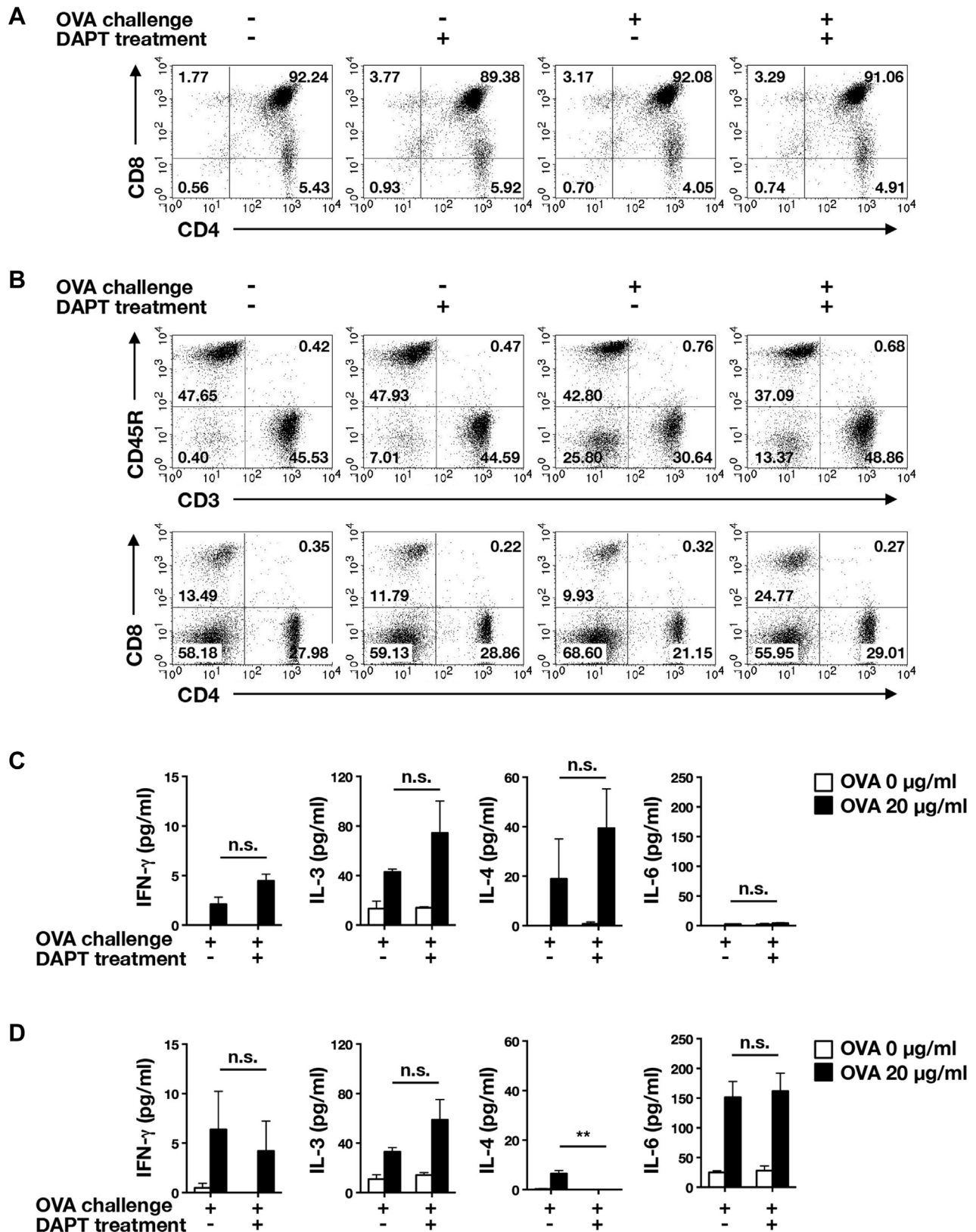


FIG E6. Administration of DAPT does not affect lymphocyte differentiation and functions. **A** and **B**, Flow cytometric analysis of CD4 and CD8 expression in thymocytes (Fig E6, **A**) and CD45R and CD3 expression (Fig E6, **B**, upper panels) and CD4 and CD8 expression (Fig E6, **B**, lower panels) in splenic cells of mice after the fifth intragastric saline or OVA challenge. **C** and **D**, Secretion of cytokines by MLN cells (Fig E6, **C**) and splenocytes (Fig E6, **D**), which were isolated from mice after the fifth intragastric saline or OVA challenge re-stimulated with 20 µg/mL OVA. Values represent means $\pm$ SEMs ($n = 3$). ** $P < .005$ between indicated treatments. *n.s.*, Not significant.

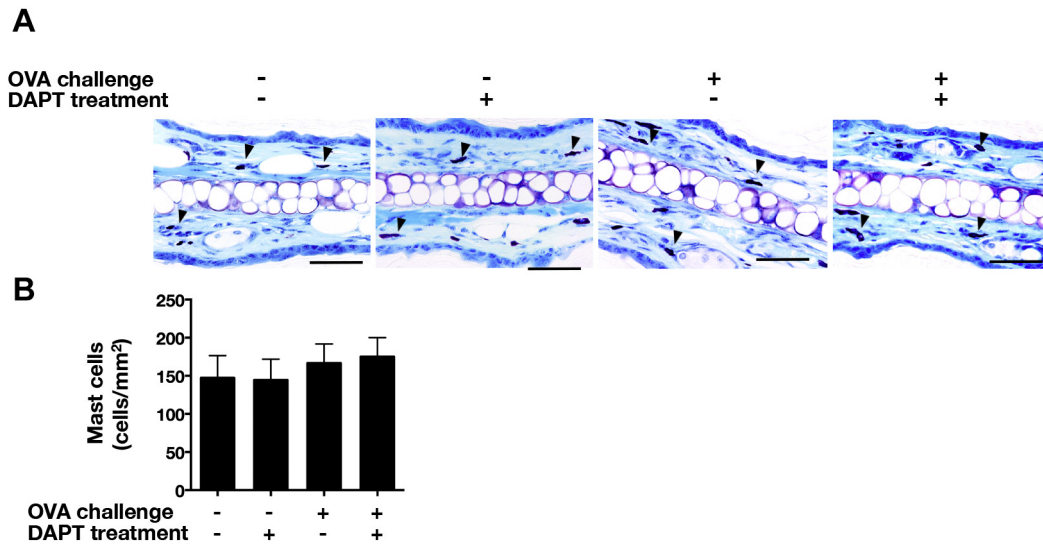


FIG E7. Administration of DAPT does not affect the number of CTMCs in the ear auricle. **A**, Toluidine blue staining of the ear auricle sections of mice after the fifth intragastric saline or OVA challenge. *Scale bars* = 50 μ m. **B**, Mean number of mast cells of Fig E7, **A**, expressed in numbers per square millimeter ($n = 3$).

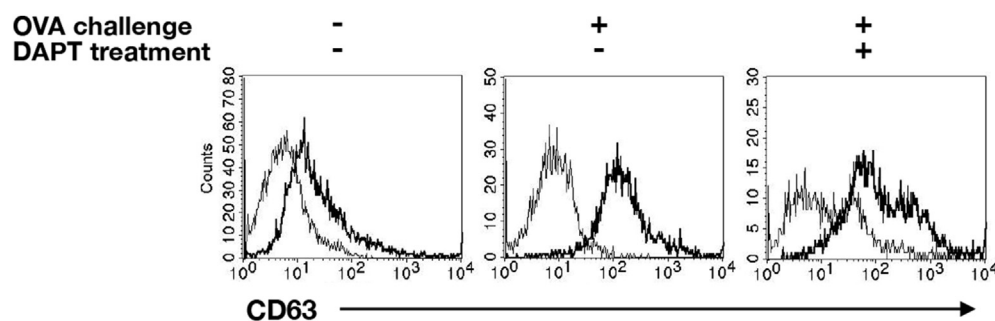


FIG E8. Degranulation of intestinal MMCs induced by intragastric OVA challenge is not affected by administration of DAPT. Flow cytometric analysis of the degranulation marker CD63 expression (*bold line*) on intestinal mast cells (gated on FcεRI⁺c-Kit⁺) in the small intestinal LP cells of mice 30 minutes after the fifth intragastric saline or OVA challenge. Negative control cells are plotted in a *thin line*.