Notch2 signaling is required for proper mast cell distribution and mucosal immunity in the intestine

Running title: Notch2 plays a role in mucosal immunity

by

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Footnote

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Abstracts

Notch receptor-mediated signaling is involved in developmental process and functional modulation of lymphocytes, as well as mast cell differentiation. Here we investigated whether Notch signaling is required for anti-pathogen host defense regulated by mast cells. Mast cells were rarely found in the small intestine of wild-type C57BL/6 mice, while abnormally accumulated in the lamina propria of the small intestinal mucosa of the *Notch2* conditional knockout mice in naïve status. When transplanted into mast cell-deficient W^{sh}/W^{sh} mice, *Notch2*-null bone marrow-derived mast cells (BMMCs) were rarely found within the epithelial layer but abnormally localized to the lamina propria, whereas control BMMCs were mainly found within the epithelial layer. After the infection with L3 lavae of *Strongyloides venezuelensis* (*SV*), the abundant number of mast cells was rapidly mobilized to epithelial layer in the control mice. In contrast, mast cells were massively accumulated in the lamina propria of the small intestinal mucosa in *Notch2* conditional knockout mice, accompanied by impaired eradication of *SV*. These findings indicate that cell-autonomous Notch2 signaling in mast cells is required for proper localization of intestinal mast cells, and further imply a critical role of Notch signaling in host-pathogen interface in the small intestine.

Introduction

Mast cells are important in a wide variety of physiologic and pathologic processes including protective immune responses to parasites and allergic disorders [1,2]. In intestinal parasite infection, mast cells play a central role in the immune response [3]. During the induction phase of parasite-induced inflammation, mast cells move from the submucosa to the tip of the villi, accompanying the serial changes in the protease expression pattern. Initially, they are positive for mouse mast cell protease-5 (mMCP-5) but negative for mMCP-1 and mMCP-2; eventually, they become positive for mMCP-1 and mMCP-2 but negative for mMCP-5, demonstrating convergence from connective tissue-type mast cells (CTMCs) to mature mucosal-type mast cells (MTMCs) [4]. The parasite-infected mice consequently experience jejunal mast cell hyperplasia[5], and the serum concentration of mMCP-1, an activation marker of small intestinal mast cells, is increased by >1000 fold compared to that in the naïve status [5].

In the mammalian immune system, we and other groups have demonstrated that Notch signaling is involved in the commitment and differentiation of T cells, the development of splenic marginal zone B cells, and the differentiation and functional modulation of mature T cells, including T-helper type I (Th1)/Th2 polarization [6,7] and differentiation of CD8-positive cytotoxic T cells [8]. Regarding the Notch signaling in mast cells, bone marrow-derived mast cells (BMMCs) highly express Jagged1 [9] and Notch2 [10] among the Notch ligands and the receptors, respectively. We have

previously shown that signaling through the Notch2 receptor induces mast cell development from myeloid progenitors by means of transcriptional upregulation of Hes-1 and GATA3 [11]. Induction of antigen-presenting potential of mast cells by Notch signaling is also demonstrated [12]. A question yet to be solved is how Notch signaling affects mast cell properties *in vivo*.

In this report, we examined the effect of Notch2 signaling in *in vivo* mast cells using *Notch2* conditional knockout mice[13]. We show that Notch2 signaling is specifically required for intra-epithelial localization of intestinal mast cells and anti-parasite immunity. In contrast, Notch2 is dispensable for either distribution or development of CTMCs.

Materials and Methods

Mice

The generation of *Notch2*$^{flox/flox}$ mice was described previously[13]. Mx-*Cre* transgenic mice [14] were crossed with *Notch2*$^{flox/flox}$ mice and the progeny were injected with pIpC (Sigma-Aldrich) 7 times every other day from 3 d after birth (25 μg/g body weight) or 3 times between 4 and 6 wk of age (20 μg/g body weight). *N2*-MxcKO mice were further crossed with C57BL/6-Ly5.1 mice (a kind gift from Dr. H. Nakauchi) to generate Ly5.1-*N2*-MxcKO mice. *Notch2* deletion in bone marrow was examined by polymerase chain reaction and 3% agarose gel electrophoresis ([13] and data not shown). W^{sh}/W^{sh} mice were purchased from Jackson Laboratory. All experiments were done in accordance

with our institutional guidelines.

Staining

Sections, fixed with Carnoid's fluid, were stained with 0.5% toluidine blue (Sigma-Aldrich), pH 0.3, followed by eosin. Small intestine was embedded in OCT compound and cut with cryostat (LEICA, CM1850). The section was fixed with 4% paraformaldehyde, washed with phosphate buffered saline (PBS), blocked in 10% horse serum, and 0.1 % Triton-PBS, and then stained with 1:100 goat anti-Jagged1 antibody (C-20, Santa Cruz), or goat anti-Delta1 antibody (GT Minneapolis), or control goat IgG (Santa Cruz) overnight at 4 degree. The sections were washed with PBS, stained with anti-goat Alexa 594 (Invitrogen). Sections were analyzed by fluorescence microscope (Zeiss, Axioplan2), x200.

BMMCs

Bone marrow cells from each mouse strain were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 50 ng/ml SCF (PeproTech) and 10 ng/ml IL-3 (PeproTech) for 4 wks. Generation of BMMCs was confirmed by staining with lineage markers, c-Kit and IgE, as previously described [11]. Briefly, the cells were incubated with purified IgE (BD Biosciences) after blocking the Fcγ receptors with purified anti-CD16/32 antibody

(BD Biosciences), stained with anti-IgE-fluorescein isothiocyanate (FITC, BD Biosciences), anti-Gr-1-phycoerythrin (PE), anti-Mac1-PE (eBioscience), and anti-c-Kit-allophycocyanin (APC, eBioscience), and then analyzed by FACSCalibur (BD Biosciences).

Peritoneal mast cells

Five mL ice-cold PBS was injected into peritoneal cavity and then three mL PBS was recovered. c-Kit and IgE receptor (FcεRI) expression was used to define the cells as peritoneal mast cells. Ly5.1 and Notch2 was stained with anti-Ly5.1-PE (BD Biosciences) and biotinylated anti-Notch2 antibody (clone HMN2-35)[8] followed by streptavidin PE (eBioscience), respectively.

Bone marrow transplantation

C57BL/6 mice and W^{sh}/W^{sh} mice were lethally irradiated with a total dose of 9.5 Gy and then transplanted with $1x10^7$ whole bone marrow cells from either *N2*-MxcKO-Ly5.1 mice or $Notch2^{flox/flox}$-Ly5.1 mice from tail vein. Tissues of transplanted mice were assessed after 3 to 4 months after transplantation. Donor cell engraftment was assessed by FACS analysis of peripheral blood, stained by anti-Ly5.2-FITC (BD Biosciences) and anti-Ly5.1-PE.

***S. venezuelensis* (*SV*) infection**

Mice were infected by subcutaneous injection of third-stage infective larvae of *SV*. The degree of infection was monitored by counting the number of eggs per gram of feces. Mast cells were counted and presented as the number per 10 villus crypt units. BMMCs were washed with PBS twice and then cultured with 10 ng/mL interleukin (IL) -4 and 10 ng/mL IL-10 for 3 days. These Th2-conditioned BMMCs were injected at day 3 and day 6 of experiments [15].

Statistical analysis

The data of the number of mast cells and the *SV* infection data were analyzed by the t-test. *P* values <0.05 were considered significant.

Results

Notch signaling affects the number and localization of mast cells in the small intestine

We have previously reported that Notch2 regulates mast cell differentiation *in vitro* [11]. In order to examine whether Notch2 controls the differentiation or development of MTMCs *in vivo*, we examined intestinal mast cells by toluidine blue staining in C57BL/6 mice carrying *Notch2*$^{flox/flox}$ allele with or without Mx1-*Cre* transgene (*N2*-MxcKO mice or *Notch2*$^{flox/flox}$ mice, respectively) after pIpC treatment [13]. Mast cells were only sparsely detected in the small intestine of *Notch2*$^{flox/flox}$

mice, mainly within the epithelium. However, the total number of mast cells in the small intestine of *N2*-MxcKO mice was unanticipatedly greater than that of *Notch2*$^{flox/flox}$ mice. Furthermore, those mast cells were mainly localized to the lamina propria, and very few mast cells were found within the epithelium (Fig. 1A, B).

Localization of MTMCs is abnormal in wild-type mice transplanted with *N2*-MxcKO bone marrow cells, reminiscent of that in *N2*-MxcKO mice.

Because the Mx-*Cre*-based conditional knockout system deletes target genes not only in the bone marrow cells but also, albeit partially, those in the intestinal cells [14], there was a possibility that *Notch2* deletion in the intestinal cells was responsible for the distinct distribution pattern or increased number of mast cells in *N2*-MxcKO mice compared with control mice. To exclude this possibility, we transplanted *Notch2*-null bone marrow cells carrying the Ly5.1 marker to irradiated wild-type C57BL/6-Ly5.2 mice. A chimerism of donor-derived Ly5.1 positive fraction was more than 70% in the peripheral blood (data not shown). The recipients of bone marrow cells from *Notch2*$^{flox/flox}$ mice showed that the intestinal mast cell distribution was virtually the same as that in wild-type mice, whereas the recipients of *Notch2*-null bone marrow cells showed increase in mast cells mainly in the lamina propria, in an indistinguishable manner from the *N2*-MxcKO mice (Fig. 2A, B). This result indicates that deletion of *Notch2* in bone marrow-derived cells alters the

distribution pattern and increases the number of mast cells in the small intestine.

Notch ligand expression in the small intestine.

Notch signaling is known to be activated through Notch ligand-receptor binding [16]. We examined the expression pattern of Notch ligands in the small intestine with antibodies against Notch ligands, Jagged1 and Delta1 and found that the epithelial layer was clearly stained with anti-Jagged1 but not anti-Delta1 antibody (Fig. 3). The staining with the anti-Jagged1 antibody was confined to the surface of epithelial cells, especially at their basal side rather than the apical side (Fig. 3). The Jagged1 expression pattern suggests a possibility that Jagged1-Notch2 interaction between the basal side of the epithelial cells and mast cells has an important role for mast cell migration from lamina propria across basement membrane towards the epithelium (Fig. 3). Furthermore, the ligand-receptor binding itself might contribute to mast cell-epithelial cell adhesion to some extent, based on our observation that *Notch2*-expressing BMMCs attached to the Jagged1-expressing CHO cells, while *Notch2*-null BMMCs did not (Supplementary Fig. 1).

***Notch2* is dispensable for the CTMC development and distribution.**

We next investigated the roles of Notch2 in the development of CTMCs. The localization and the number of CTMCs in the skin and peritoneal cavity were not significantly different between

N2-MxcKO and littermate *Notch2*$^{flox/flox}$ mice more than four weeks after the treatment with pIpC (Fig. 4A and data not shown). This observation might simply indicate that the Mx-*Cre* system was inefficient in the tissue-resident mast cells, as a great majority of peritoneal mast cells of pIpC-treated *N2*-MxcKO mice still expressed Notch2 (Fig. 4B, lower panel). Therefore, to clarify the requirement of *Notch2* in the CTMC development, we examined peritoneal mast cells of W^{sh}/W^{sh} mice that lack mast cells, after transplantion of *Notch2*-null bone marrow cells carrying the Ly5.1 marker. In this system, mast cells exclusively develop from transplanted bone marrow progenitors, in which the *Cre* recombinase under the Mx-promoter is quite effective ([14] and data not shown). In this experiment, we found that the proportion and absolute number of peritoneal mast cells was not significantly different between those developed from the *N2*-MxcKO-Ly5.1 bone marrow cells and those developed from littermate *Notch2*$^{flox/flox}$-Ly5.1 bone marrow cells (Fig. 4C, D). Notch2 was not expressed in the peritoneal mast cells derived from *N2*-MxcKO-Ly5.1 bone marrow cells while expressed in those derived from littermate *Notch2*$^{flox/flox}$-Ly5.1 bone marrow cells (Fig. 4C, Middle), indicating that *Notch2* was deleted efficiently. These results suggest that Notch2 is dispensable for the development and distribution of CTMCs.

Cell-autonomous Notch2 signaling in mast cells is important for mast cell migration across the basement membrane in the small intestine

We then asked a question whether aberrant mast cell migration in the small intestine in *N2*-MxcKO mice is dependent on Notch2 signaling in mast cells per se. We intravenously infused *Notch2*-null or control BMMCs into W^{sh}/W^{sh} mice after *SV* infection, because it is reported that BMMCs could only transiently reconstitute intestinal mast cells in mast-cell deficient mice if these recipient mice are in naïve status [17]. In tissue sections, we found that the distribution of mast cells in the small intestine was different between control BMMCs-reconstituted mice and *Notch2*-null BMMCs-reconstituted mice; control BMMCs were mainly migrated into the epithelial layer, while a majority of *Notch2*-null BMMCs remained in the lamina propria. This observation indicates that mast cell-autonomous Notch2 expression contributes to mast cell migration across the basement membrane from lamina propria into the epithelial layer (Fig. 5A, B). Even in the control BMMC-infused mice, however, substantial proportion of mast cells still remained in the lamina propria, submucosa, and smooth muscle layers, and the distribution of mast cells within the epithelium was confined to the basement membrane side of the epithelial layer (Fig. 5B, C). This mast cell localization pattern was different from that in the $Notch2^{flox/flox}$ mice with *SV* infection, in which mast cells were present mainly at the mid to apical side of the epithelial layer (Fig. 5C). The numbers of *SV* eggs in the stool were virtually the same in the *SV*-infected W^{sh}/W^{sh} mice infused with *Notch2*-null and control BMMCs, and in the *SV*-infected W^{sh}/W^{sh} mice without any BMMC infusion throughout the period after infection (Fig. 5D).

Taken together, the BMMC-W^{sh}/W^{sh} transplantation model demonstrated that Notch2 in the mast cells indeed determines their intra-epithelial migration from lamina propria; nevertheless, this model was not adequate to examine the physiologic mast-cell distribution pattern and subsequent parasite expulsion that depends on mast cells.

Notch2 signaling regulates anti-parasite immunity of mast cells in the intestine

The BMMC-W^{sh}/W^{sh} reconstitution model could not completely reflect physiologic mast cell distribution pattern in the small intestine. Therefore, in order to further assess the effect of Notch2 signaling on mucosal immune response of intestinal mast cells under a pathologic condition, *N2*-MxcKO or control *Notch2*$^{flox/flox}$ mice were infected with *SV*. Total mast cell number was increased in *Notch2*$^{flox/flox}$ mice much more than *N2*-MxcKO mice, especially in the epithelium in both cypts and villi 8 days after infection (Fig. 6A, B). Thirteen days after infection, mast cells in the epithelium in *Notch2*$^{flox/flox}$ mice were still more abundant than those in *N2*-MxcKO mice (Fig. 6C and D), while mast cell accumulation in the lamina propria in *N2*-MxcKO mice was more prominent in both villi and crypt than earlier stage of infection (Fig. 6A and C). In particular, dense aggregation of mast cells was prominent in the lamina propria of *N2*-MxcKO mice, at the tip of the villi (Fig. 6D). As a consequence, the total number of mast cells in the intestine of *N2*-MxcKO mice became

equivalent to those of *Notch2*$^{flox/flox}$ mice 13 days after infection (Fig. 6C and E). The number of *SV* eggs in the stool was gradually decreased during day8-10 in control *Notch2*$^{flox/flox}$ mice but not in N2-MxcKO mice (Fig. 6F). Furthermore, the worms were still observed in *N2*-MxcKO mice but not in *Notch2*$^{flox/flox}$ mice 12 days after infection (Fig. 6G). These data suggest that Notch2 deficiency alters distinct distribution pattern of mast cells in the small intestine, which is responsible for the defective eradication of *SV*.

Discussion

There is a growing body of evidence that Notch signaling modulates cellular migration and adhesion in endothelial, neural, and lymphoid lineage cells, as well as cancer cells [18]. Our group has shown that Notch2 signaling induces the development of mast cells [11]. However, it has remained unclear whether Notch2 signaling is involved in the distribution of mast cells in the intestinal mucosa or connective tissues, or in controlling the functions of mast cells against microorganisms. We here investigated the role of Notch2 signaling in mast cells in terms of their distribution and functions by using cell-specific *Notch2*-deficient mice. We found that in *N2*-MxcKO mice, mast cells were abnormally accumulated in the lamina propria of the small intestine, suggesting that *Notch2*-null mast cells have some defect in the migration towards the epithelium. Furthermore,

N2-MxcKO mice failed to eradicate *SV* and exhibited a distinct mast cell migration pattern in the intestine as compared with control mice, suggesting that mast cells regulate the host-microbial interface in the intestine through Notch2 signaling.

Mast cell number was rather increased in the intestinal mucosa of *N2*-MxcKO mice compared with control mice in naïve status. Mast cell progenitors were supposed to reside in the submucosa and gradually move towards the villi, accompanied by their differentiation into mature mast cells. Based on our observation in an *SV* infection model, mast cells increase in number in the epithelium in control *Notch2*$^{flox/flox}$ mice, while they abnormally aggregate in lamina propria in *N2*-MxcKO mice, especially in the later stage of infection. This suggests that mast cell migration from lamina propria towards the epithelium across the basement membrane is impaired in *N2*-MxcKO mice. Consequently, mast cell turnover might be prolonged in *N2*-MxcKO mice. Given that the mechanism of mast cell migration from lamina propria towards the epithelium is common in naïve status and infection status, such migration defect may also explain the mast cell increase in *N2*-MxcKO mice in naïve status that we observed.

The defect of mast cell migration towards intra-epithelium of the small intestine in *N2*-MxcKO mice is very similar to that in integrin β6-deficient mice [19], in which activation of transforming growth factor (TGF)-β signaling is impaired [20]. A crosstalk between Notch signaling and TGF-β signaling might occur in intestinal mast cells as well as the cases of other cell types [21].

Alternatively, Notch signaling might directly regulate a downstream target of TGF-β1 in intestinal mast cell migration; for example, the induction of integrin αE expression [19,22]. Integrin αE, forming an integrin αEβ7 complex on mast cells, binds to E-cadherin on epithelial cells and is involved in mast cell localization in the epithelium [22]. The expression level of integrin αEβ7, measured by flow cytometric analysis, however, was not affected by Notch-ligand stimulation in BMMCs (unpublished data).

In the previous paper we showed that Notch signaling facilitates mast cell lineage development at the expense of granulocyte/macrophage development from both common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) *in vitro*[11]. Mast cells, however, were not depleted in *N2*-MxcKO mice in naïve status *in vivo*, but rather slightly increased in the small intestine of *N2*-MxcKO mice. This clearly indicates that Notch2 signaling is dispensable for steady-state mast cell generation *in vivo*. However, the dynamic increase of mast cells during the early phase of intestinal parasite infection was markedly impaired in *N2*-MxcKO mice. The mechanisms underlying the Notch2 signaling requirement only in parasite-infected mice remain to be clarified. Nevertheless, rapidly increasing intestinal mast cells have to be supplied by mast cell progenitors. The pathways and mechanisms responsible for mast cell progenitor recruitment and trafficking are likely to be dynamic and susceptible to modification during inflammation [1]. Such a modulation of the mast cell generation pathway during intestinal infection might underlie the

requirement of *Notch2* only during parasite infection. It is really similar to IL-3-deficient mice. IL-3 is essential for mast cell differentiation *in vitro*; however, IL-3-deficient mice have the normal number of mast cells at the steady state, whereas mast cell hyperplasia is impaired upon intestinal parasite infection [23].

Our data showed that parasite expulsion was impaired in *N2*-MxcKO mice. We could not exclude the possibility that the *Notch2* deletion in immune cells other than mast cells modulate the response against the nematode infection. If we could show that Th2-conditioned wild-type BMMCs successfully eradicate *SV* in W^{sh}/W^{sh} mice and that *Notch2*-null BMMCs do not, it would be more clear that Notch2 signaling in mast cells per se but not that in other immune cells should be critically important for defense against *SV* infection. The failure of rescue experiments may be caused by abnormal mast cell distribution pattern of wild-type BMMCs in W^{sh}/W^{sh} mice. Nevertheless, the result of this experiment supported the previous finding that the proper epithelial migration of mast cells is required for efficient expulsion of *SV* [24], and thus, provides an insight that the impaired *SV* expulsion in *N2*-MxcKO mice is attributed to the mast cell-autonomous deletion of *Notch2*.

In conclusion, our data clearly indicate that Notch2 receptor signaling is specifically required for proper intestinal mast cell distribution, and further imply a critical role of Notch signaling for mucosal immunity, particularly for eradication of infected nematodes at least partly dependent on mast cells.

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M.S.-Y. designed the research, performed the research, analyzed data, and wrote the paper. T.Sakai, Y.Miyake, and Y. Morishita performed research. T.Saito, H.M. and H.Y. contributed new reagents. E.N.-Y., K.K., M.F., S.O. and M.K. provided vital discussion. K.Y. designed the research. S.C designed the research, analyzed data and wrote the paper.

Reference

1. Gurish MF, Boyce JA. Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. J Allergy Clin Immunol. 2006;117:1285-1291.
2. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol. 2005;6:135-142.
3. Maruyama H, Yabu Y, Yoshida A, Nawa Y, Ohta N. A role of mast cell glycosaminoglycans for the immunological expulsion of intestinal nematode, Strongyloides venezuelensis. J Immunol. 2000;164:3749-3754.
4. Friend DS, Ghildyal N, Gurish MF, et al. Reversible expression of tryptases and chymases in the jejunal mast cells of mice infected with Trichinella spiralis. J Immunol. 1998;160:5537-5545.
5. Miller HR, Pemberton AD. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. Immunology. 2002;105:375-390.
6. Tsukumo S, Yasutomo K. Notch governing mature T cell differentiation. J Immunol. 2004;173:7109-7113.
7. Radtke F, Wilson A, Mancini SJ, MacDonald HR. Notch regulation of lymphocyte development and function. Nat Immunol. 2004;5:247-253.
8. Maekawa Y, Minato Y, Ishifune C, et al. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. Nat Immunol. 2008;9:1140-1147.
9. Singh N, Phillips RA, Iscove NN, Egan SE. Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. Exp Hematol. 2000;28:527-534.
10. Jonsson JI, Xiang Z, Pettersson M, Lardelli M, Nilsson G. Distinct and regulated expression of Notch receptors in hematopoietic lineages and during myeloid differentiation. Eur J Immunol. 2001;31:3240-3247.
11. Sakata-Yanagimoto M, Nakagami-Yamaguchi E, Saito T, et al. Coordinated regulation of transcription factors through Notch2 is an important mediator of mast cell fate. Proc Natl Acad Sci U S A. 2008;105:7839-7844.
12. Nakano N, Nishiyama C, Yagita H, et al. Notch signaling confers antigen-presenting cell functions on mast cells. J Allergy Clin Immunol. 2009;123:74-81 e71.
13. Saito T, Chiba S, Ichikawa M, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. Immunity. 2003;18:675-685.
14. Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice.

Science. 1995;269:1427-1429.
15. Fukao T, Yamada T, Tanabe M, et al. Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. Nat Immunol. 2002;3:295-304.
16. Kopan R, Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell. 2009;137:216-233.
17. Tanzola MB, Robbie-Ryan M, Gutekunst CA, Brown MA. Mast cells exert effects outside the central nervous system to influence experimental allergic encephalomyelitis disease course. J Immunol. 2003;171:4385-4391.
18. Nam DH, Jeon HM, Kim S, et al. Activation of notch signaling in a xenograft model of brain metastasis. Clin Cancer Res. 2008;14:4059-4066.
19. Brown JK, Knight PA, Pemberton AD, et al. Expression of integrin-alphaE by mucosal mast cells in the intestinal epithelium and its absence in nematode-infected mice lacking the transforming growth factor-beta1-activating integrin alphavbeta6. Am J Pathol. 2004;165:95-106.
20. Munger JS, Huang X, Kawakatsu H, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell. 1999;96:319-328.
21. Kluppel M, Wrana JL. Turning it up a Notch: cross-talk between TGF beta and Notch signaling. Bioessays. 2005;27:115-118.
22. Wright SH, Brown J, Knight PA, Thornton EM, Kilshaw PJ, Miller HR. Transforming growth factor-beta1 mediates coexpression of the integrin subunit alphaE and the chymase mouse mast cell protease-1 during the early differentiation of bone marrow-derived mucosal mast cell homologues. Clin Exp Allergy. 2002;32:315-324.
23. Lantz CS, Boesiger J, Song CH, et al. Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. Nature. 1998;392:90-93.
24. Abe T, Nawa Y. Localization of mucosal mast cells in W/Wv mice after reconstitution with bone marrow cells or cultured mast cells, and its relation to the protective capacity to Strongyloides ratti infection. Parasite Immunol. 1987;9:477-485.

Figure legend

Fig. 1 Mature mast cells were abnormally accumulated in the lamina propria of the small intestine of *Notch2*-deficient mice.

A, Sections of the small intestine of *N2*-MxcKO or littermate control *Notch2*$^{flox/flox}$ mice. Toluidine blue staining, followed by eosin. Original magnification, x 200.

B, The numbers of mast cells per 10 villus crypt unit (vcu) distributing to various layers of the small intestine. Data are presented as means +/- SEM; *Notch2*$^{flox/flox}$ (n=10) vs *N2*-MxcKO (n=8), p=0.000461 [total], p=0.000261 [villus, lamina propria], p=0.001918 [crypt, lamina propria], p=0.046874 [submucosa]

Fig.2 Localization of intestinal mast cells is abnormal in wild-type mice transplanted with *N2*-MxcKO-Ly5.1 bone marrow cells, reminiscent of that in *N2*-MxcKO mice.

A, Bone marrow cells either from *N2*-MxcKO- Ly5.1 mice or littermate *Notch2*$^{flox/flox}$- Ly5.1 mice were transplanted into lethally irradiated (9.5 Gy) C57BL/6-Ly5.2 mice. Toluidine blue staining, followed by eosin. Original magnification, x 200.

B, The numbers of mast cells per 10 villus crypt unit (vcu) distributing to various layers of the small intestine. Data are presented as means +/- SEM; Mast cells in C57BL/6-Ly5.2 mice transplanted with *Notch2*$^{flox/flox}$-Ly5.1 (n=3) vs *N2*-MxcKO-Ly5.1 (n=3). p=0.020594 [total] and 0.030123 [villus,

lamina propria].

Fig. 3 Jagged1 is strongly expressed on the surface of the epithelial cells, especially at their basal side.

A section of small intestine prepared using cryostat was stained with goat anti-Jagged1 and goat anti-Delta1 antibodies followed by anti-goat Alexa594. Original magnification x200.

Fig. 4 Notch2 is not required for peritoneal mast cell development.

A, The proportion (Left) and the absolute number (Right) of peritoneal mast cells were comparable between *N2*-MxcKO mice and control *Notch2*$^{flox/flox}$ mice.

B, Peritoneal mast cells of *N2*-MxcKO mice showed normal mast cell proportion as those in littermate control *Notch2*$^{flox/flox}$ mice. Peritoneal mast cells were stained with anti-c-Kit-APC, IgE, and biotinylated anti-Notch2 antibody (clone HMN2-35), followed by anti-IgE-FITC and streptavidin-PE and then analyzed by FACSCalibur (BD Biosciences). Note that the cKit+FceRI+ fraction in N2-MxcKO mice was still positive for Notch2.

C, Bone marrow cells from *N2*-MxcKO-Ly5.1 mice or control *Notch2*$^{flox/flox}$-Ly5.1 mice were transplanted into lethally irradiated W^{sh}/W^{sh} mice. Peritoneal mast cells were stained with anti-c-Kit-APC, IgE, and biotinylated anti-Notch2 antibody (HMN2-35), or Ly 5.1 antibody,

followed by anti-IgE-FITC and streptavidin-PE and then analyzed by FACSCalibur (BD Biosciences).

D, The proportion (Left) and the absolute number (Right) of peritoneal mast cells were not significantly different between W^{sh}/W^{sh} mice transplanted with *Notch2*-WT bone marrow cells and those transplanted with *Notch2*- null bone marrow cells.

Fig. 5 Mast cell-autonomous Notch2 expression is required for mast cell migration towards the epithelium.

W^{sh}/W^{sh} mice infected with *S. venezuelensis* were intravenously infused with Th2-conditioned *Notch2*-null or control BMMCs on days 3 and 6 of infection.

A, *Notch2*-null BMMCs poorly migrated towards the epithelium compared to control BMMCs. Toluidine blue staining followed by eosin staining. Original magnification, x 200. Upper, control BMMCs; Lower, *Notch2*-null BMMCs.

B, The number of mast cells per 10 villus crypt unit (vcu) in the small intestine on day 12 after *S. venezuelensis* infection in W^{sh}/W^{sh} mice, with or without BMMC infusion. Data are presentated as means +/- SEM. n=3; p=0.008592 [total], 0.005695 [villus, epithelium], 0.000715 [villus, lamina propria], 0.005245 [crypt, epithelium], and 0.045466 [crypt, lamina propria]. Note that mast cells in W^{sh}/W^{sh} mice infused with *Notch2*-null BMMCs were abnormally resided in the lamina propria,

whereas most of those in W^{sh}/W^{sh} mice infused with control BMMCs were intra-epithelially migrated.

C, Mast cell number in mid to apical side of the epithelial layer was divided with that in the basal side of the epithelial layer.

D, Time course of *SV* egg numbers in the stool. The number of excreted eggs was not significantly different between W^{sh}/W^{sh} mice infused with *Notch2*-null and control BMMCs. Data are presentated as means +/- SEM.

Fig. 6 *Notch2* is essential for anti-parasite immunity of mast cells in the intestine.

N2-MxcKO or control $Notch2^{flox/flox}$ mice were subcutaneously injected with third-stage infective larvae of *S. venezuelensis*

A, The number of mast cells per 10 villus crypt unit (vcu) in the small intestine on day 8 after *S. venezuelensis* infection. Data are presented as means +/- SEM. The number of mast cells was much less in *N2*-MxcKO mice. n=3, p=0.008592 [total], 0.005695 [villus, epithelium], 0.000715 [villus, lamina propria], 0.005245 [crypt, epithelium], and 0.045466 [crypt, lamina propria]. Note that mast cells in *N2*-MxcKO mice were abnormally clustered in the lamina propria, whereas most of those in the control $Notch2^{flox/flox}$ mice were intra-epithelially migrated.

B, Toluidine blue staining followed by eosin staining of the small intestine on day 8; original

magnification, x200.

C, The number of mast cells per 10 villus crypt unit (vcu) in the small intestine on day 13 after *S. venezuelensis* infection. Data are presented as means +/- SEM. n=3, p= 0.026076 [villus, epithelium], 0.00194 [villus, lamina propria], 0.021177 [crypt, epithelium], and 0.019324 [crypt, lamina propria], 0.047445 [submucosa].

D, Toluidine blue staining followed by eosin staining of the small intestine on day 13. Original magnification, x200.

E, The total number of mast cells per 10 villus crypt unit (vcu) on day 0, day 8, and day 13 of infection. The total number of mast cells was significantly lower in *N2*-MxcKO mice at the early phase (day 8) and almost equal at the later phase (day 13) to that of control mice. Data are presented as means +/- SEM: n=10 and 8 (day0, *Notch2*$^{flox/flox}$and *N2*-MxcKO); n=3 and 3 (day8, *Notch2*$^{flox/flox}$and *N2*-MxcKO); n=4 and 4 (day13, *Notch2*$^{flox/flox}$and *N2*-MxcKO).

F, Time course of egg number in the stool. The number of excreted eggs was significantly greater in *N2*-MxcKO mice compared to those in *Notch2*$^{flox/flox}$ mice. Data are represented as means +/- SEM; n=4; p=0.0291 [day 8] and 0.0219 [day 9].

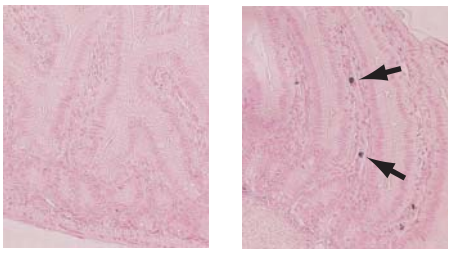
G, Hematoxylin-eosin staining of the small intestine on day 12. Original magnification, x200. Arrows indicate worms. Worms were still observed in the villi in the jejunum of *N2*-MxcKO, but not of *Notch2*$^{flox/flox}$ mice.

Supplementary Fig. 1 *Notch2*-expressing BMMCs adhered to Notch ligand-expressing CHO cells, while *Notch2*-null BMMCs did not.

BMMCs from *N2*-MxcKO mice and wild type mice were put on the CHO parent cells, Jagged-1- and Delta-1-expressing CHO cells (CHO p, CHO J1, CHO D1). After 2 hours, the supernatant was removed. The dish was observed by microscope. Origial Magnification, x 200.

Fig.1

A



Notch2$^{flox/flox}$ *N2*-MxcKO

B

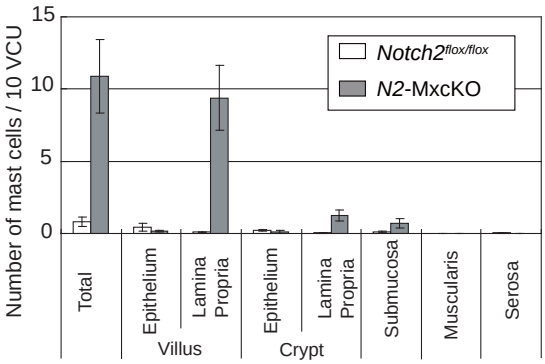
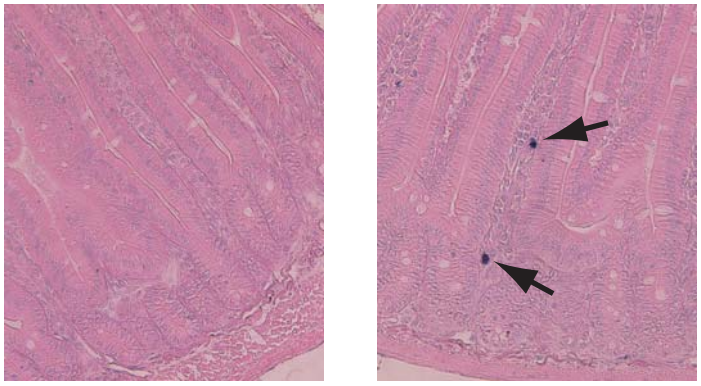


Fig. 2

A, Small Intestine



Notch2$^{flox/flox}$-Ly5.1 → C57BL/6-Ly5.2

N2-MxcKO-Ly5.1 → C57BL/6-Ly5.2

B

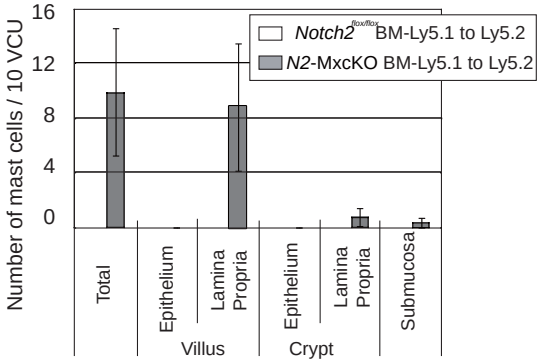
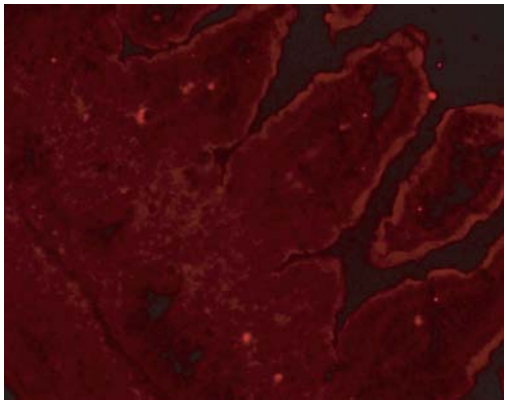
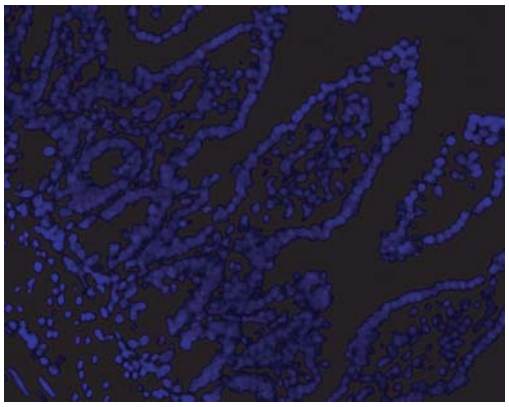


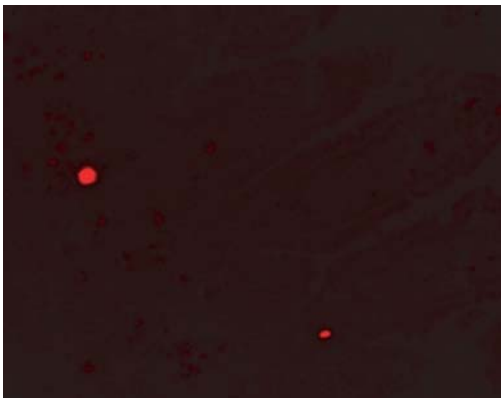
Fig. 3 Immunostaining of small intestine

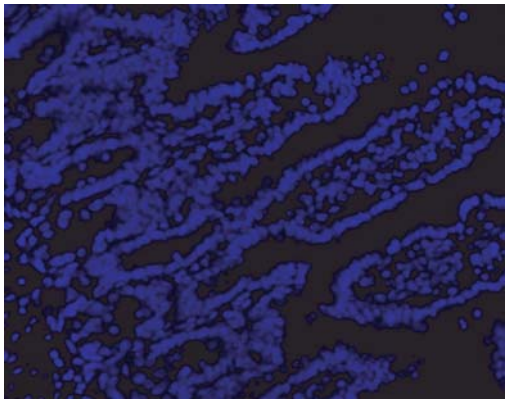
Control IgG



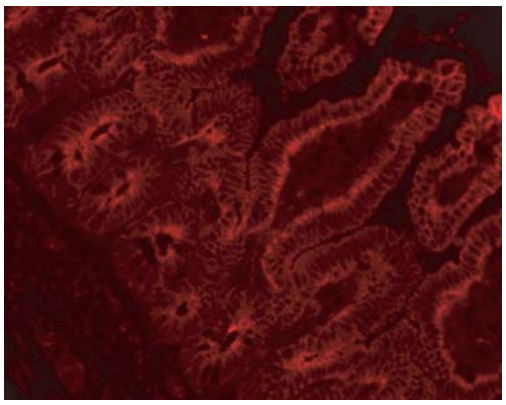


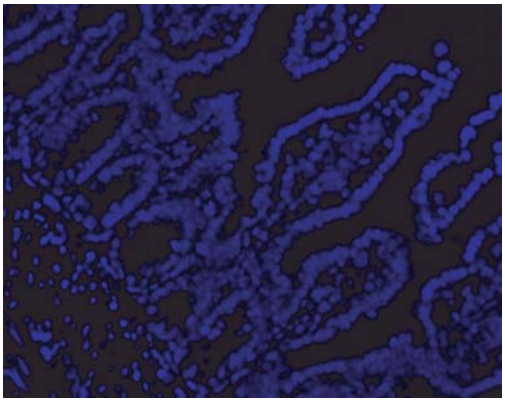
Delta1





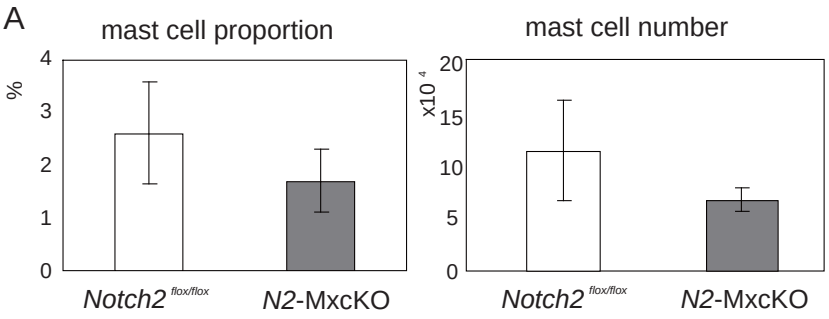
Jagged1

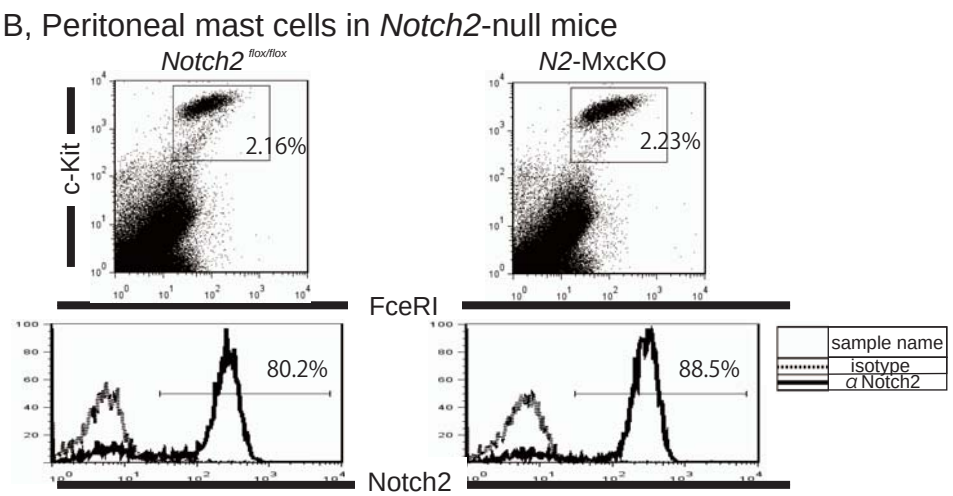


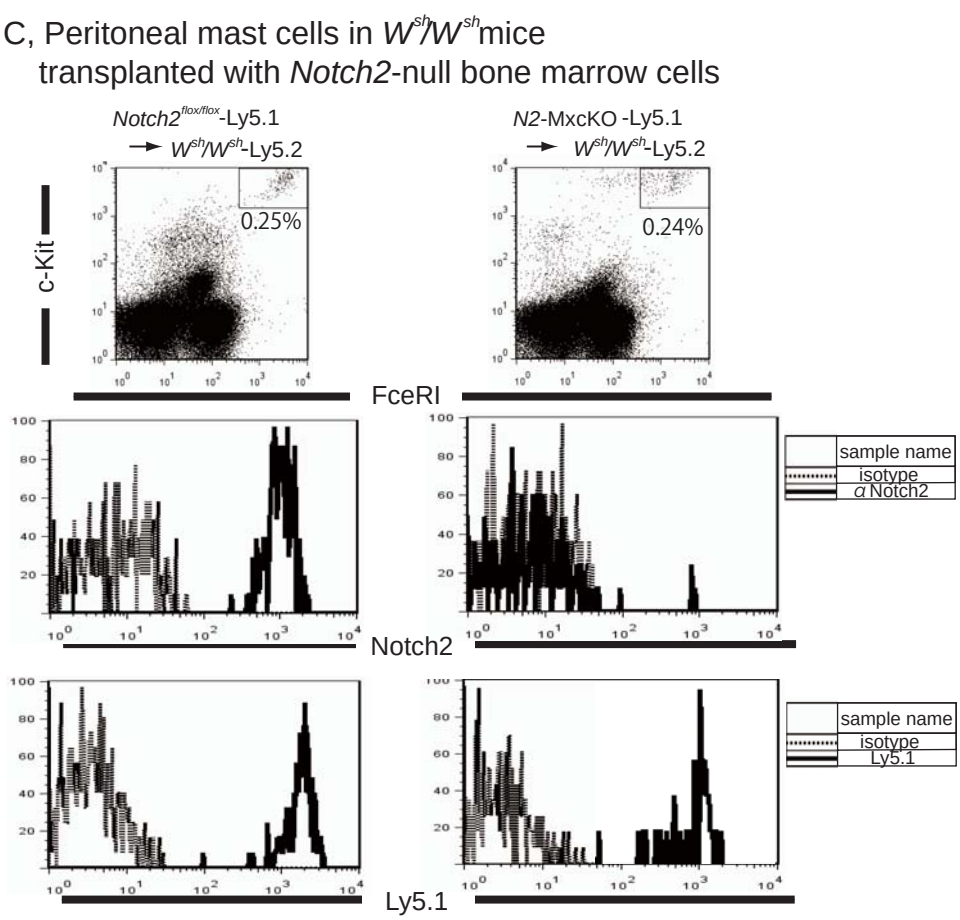


DAPI

Fig.4


A
mast cell proportion
%
4
3
2
1
0
Notch2 flox/flox
N2-MxcKO
mast cell number
x10 4
20
15
10
5
0
Notch2 flox/flox
N2-MxcKO


B, Peritoneal mast cells in Notch2-null mice
Notch2 flox/flox
N2-MxcKO
c-Kit
2.16%
2.23%
FceRI
80.2%
88.5%
Notch2
sample name
isotype
α Notch2


C, Peritoneal mast cells in W sh/W sh mice
transplanted with Notch2-null bone marrow cells
Notch2 flox/flox-Ly5.1
→ W sh/W sh-Ly5.2
N2-MxcKO -Ly5.1
→ W sh/W sh-Ly5.2
c-Kit
0.25%
0.24%
FceRI
Notch2
Ly5.1
sample name
isotype
α Notch2
Ly5.1

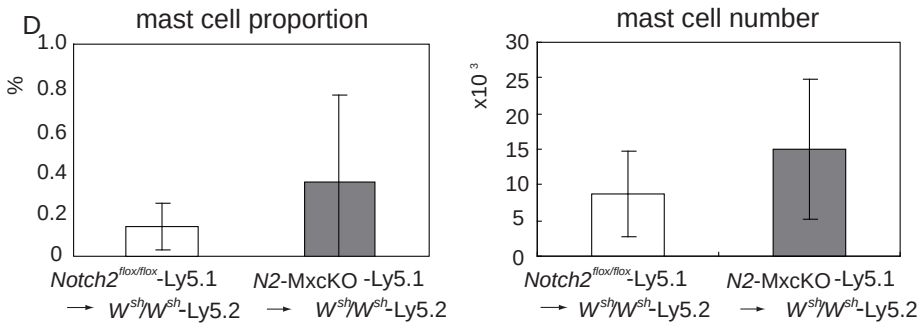
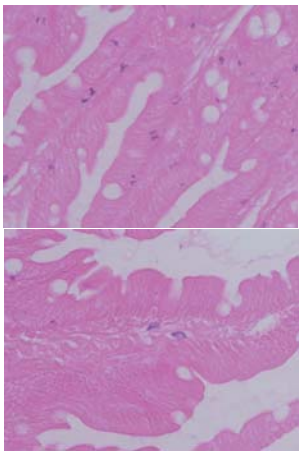

D
mast cell proportion
%
1.0
0.8
0.6
0.4
0.2
0
Notch2 flox/flox-Ly5.1
→ W sh/W sh-Ly5.2
N2-MxcKO -Ly5.1
→ W sh/W sh-Ly5.2
mast cell number
x10 3
30
25
20
15
10
5
0
Notch2 flox/flox-Ly5.1
→ W sh/W sh-Ly5.2
N2-MxcKO -Ly5.1
→ W sh/W sh-Ly5.2

Fig.5

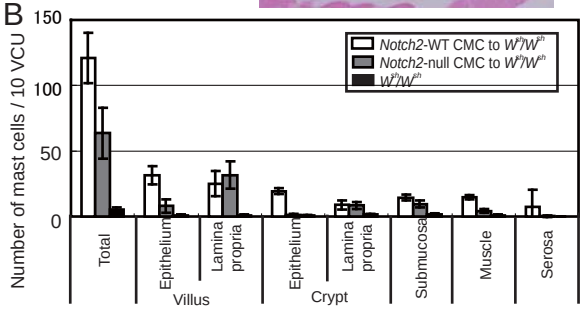
A

Notch2 -WT CMC to W^{sh}/W^{sh}

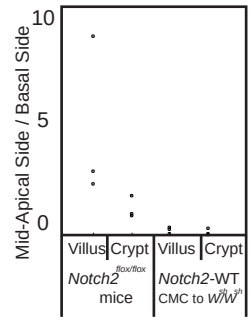
Notch2 -null CMC to W^{sh}/W^{sh}



B



C



D

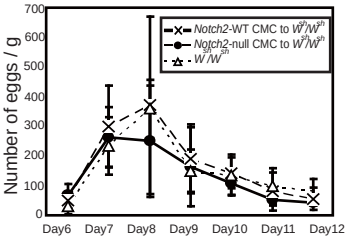
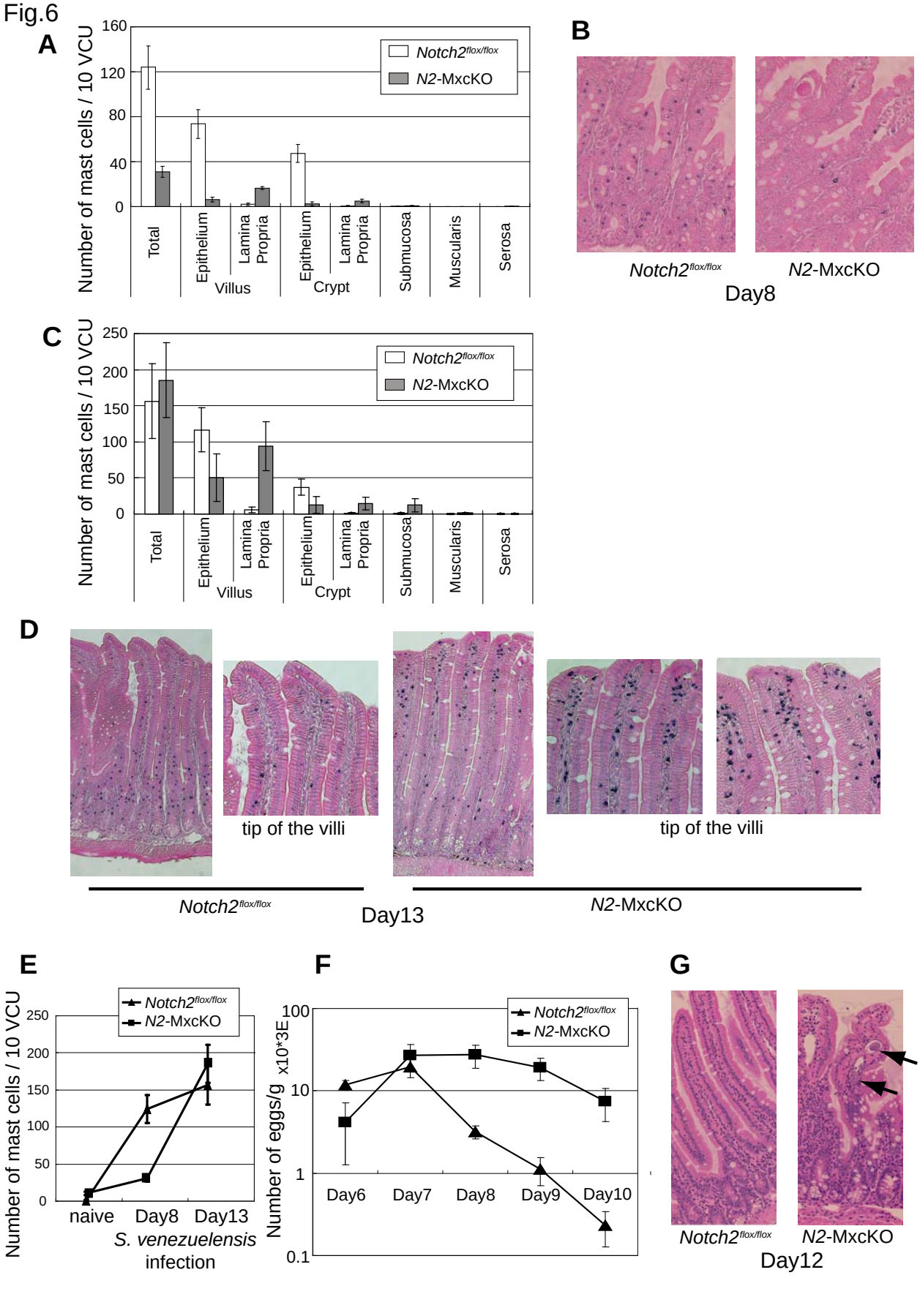


Fig.6


A
Number of mast cells / 10 VCU
160
120
80
40
0
Notch2flox/flox
N2-MxcKO
Total
Epithelium
Lamina Propria
Epithelium
Lamina Propria
Submucosa
Muscularis
Serosa
Villus
Crypt
B
Notch2flox/flox
N2-MxcKO
Day8
C
Number of mast cells / 10 VCU
250
200
150
100
50
0
Notch2flox/flox
N2-MxcKO
Total
Epithelium
Lamina Propria
Epithelium
Lamina Propria
Submucosa
Muscularis
Serosa
Villus
Crypt
D
tip of the villi
tip of the villi
Notch2flox/flox
Day13
N2-MxcKO
E
Number of mast cells / 10 VCU
250
200
150
100
50
0
Notch2flox/flox
N2-MxcKO
naive
Day8
Day13
S. venezuelensis infection
F
Number of eggs/g x10*3E
100
10
1
0.1
Notch2flox/flox
N2-MxcKO
Day6
Day7
Day8
Day9
Day10
G
Notch2flox/flox
N2-MxcKO
Day12