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Notch Signaling Enhances FceRI-Mediated Cytokine Production by Mast Cells through Direct and Indirect Mechanisms

Nobuhiro Nakano,* Chiharu Nishiyama,**† Hideo Yagita,‡ Mutsuko Hara,* Yasutaka Motomura,§,¶ Masato Kubo,§,¶ Ko Okumura,*,** and Hideoki Ogawa*

Th2-type cytokines and TNF-α secreted by activated mast cells upon cross-linking of FceRI contribute to the development and maintenance of Th2 immunity to parasites and allergens. We have previously shown that cytokine secretion by mouse mast cells is enhanced by signaling through Notch receptors. In this study, we investigated the molecular mechanisms by which Notch signaling enhances mast cell cytokine production induced by FceRI cross-linking. FceRI-mediated production of cytokines, particularly IL-4, was significantly enhanced in mouse bone marrow–derived mast cells by priming with Notch ligands. Western blot analysis showed that Notch signaling augmented and prolonged FceRI-mediated phosphorylation of MAPKs, mainly JNK and p38 MAPK, through suppression of the expression of SHIP-1, a master negative regulator of FceRI signaling, resulting in the enhanced production of multiple cytokines. The enhancing effect of Notch ligand priming on multiple cytokine production was abolished by knockdown of Notch2, but not Notch1, and FceRI-mediated production of multiple cytokines was enhanced by retroviral transduction with the intracellular domain of Notch2. However, only IL-4 production was enhanced by both Notch1 and Notch2. The enhancing effect of Notch signaling on IL-4 production was lost in bone marrow–derived mast cells from mice lacking conserved noncoding sequence 2, which is located at the distal 3′ element of the Il4 gene locus and contains Notch effector RBP-J binding sites. These results indicate that Notch2 signaling indirectly enhances the FceRI-mediated production of multiple cytokines, and both Notch1 and Notch2 signaling directly enhances IL-4 production through the noncoding sequence 2 enhancer of the Il4 gene. The Journal of Immunology, 2015, 194: 000–000.

Mast cells are key effector cells in IgE-mediated immune responses, including protection against parasites (1) and allergic diseases (2). Mast cell activation induced by Ag- and IgE-dependent cross-linking of FceRI leads to the secretion of inflammatory cytokines and lipid mediators. In mucosal tissues, IL-4, IL-6, IL-13, and TNF-α from activated mast cells contribute to the development and maintenance of Th2 immunity to helminths and allergens (1–6). Mast cell cytokine production is controlled by the activation of intracellular signaling pathways and the chromatin-based transcriptional regulation of cytokine genes. In FceRI-triggered cytokine production, the MAPK family members JNK, p38, and ERK are essential signaling molecules that are activated by phosphorylation and mediate the activation of transcription factors (7, 8). The transcription factors activated by FceRI signaling bind to cis-regulatory elements at cytokine gene loci, leading to the transcription of cytokine genes. Mast cells produce Th2-type cytokines, such as IL-4 and IL-13, in response to FceRI-mediated stimuli, because chromatin structure and histone modification patterns in the Il4/Ill13 locus of mast cells are similar to those of IL-4–producing Th2 cells (9, 10). Additionally, previous studies have shown that conserved noncoding sequence (CNS)2 located at the distal 3′ element of the Il4 gene is an important enhancer for Il4 gene transcription in mast cells (10).

Mast cells are distributed throughout most tissues, especially in barrier tissues such as skin and mucosa. The pattern and amount of cytokines produced by mast cells are variable among tissue-resident mast cell populations. The ability of mast cells to produce cytokines in response to stimuli is highly influenced by microenvironmental factors such as local cytokines and cell surface molecules (11–14). However, the molecular mechanism by which the ability of mast cells to produce cytokines modulated by microenvironmental factors is largely unknown. Previously, we reported that FceRI-mediated cytokine production by mouse mast cells is enhanced by signaling through the transmembrane receptor Notch (15). Mammals have four different Notch family members, Notch 1, 2, 3, and 4. Mouse mast cells constitutively express Notch1 and Notch2 (15). Notch signaling is initiated by interaction of the extracellular domain with its ligands Jagged (Jag1), Jag2, Delta-like (Dll1), and Dll4. Sequential cleavage by proteases releases the Notch intracellular domain (NICD) from the mem-
brane, allowing it to translocate into the nucleus. The N<sup>ICD</sup> forms a complex with the DNA-binding protein, RBP-J, leading to the transactivation of target genes. Previous studies have demonstrated that Notch2-mediated signaling is an important mediator of mast cell development from myeloid progenitors and is required for intraepithelial localization of intestinal mast cells and anti-parasite immunity (16, 17). Because the Notch ligands are expressed on various tissue cells, including intestinal epithelial cells, epidermis keratinocytes, and vascular endothelial cells (17–20), they are inferred to act as a microenvironmental factor for tissue-resident mast cells. Therefore, we investigated the mechanism by which Notch signaling enhances FcerI-mediated cytokine production by mast cells.

In the present study, we show that Notch signaling augments and sustains FcerI-mediated phosphorylation of MAPKs through the suppression of SHIP-1, which is a negative regulator of FcerI signaling, resulting in enhanced production of IL-4, IL-6, IL-13, and TNF-α. Furthermore, our data indicate that IL-4 production is directly upregulated by binding of the N<sup>ICD</sup>RBP-J complex to the CNS2 region of the Il4 gene locus.

Materials and Methods

**Mice**

DNase I-hypersensitive site (HS)-2 or CNS2-deleted mice were described in our previous report (21). Wild-type C57BL/6 and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). All 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 and the guidelines of the RIKEN Yokohama Institute or Tokyo University of Science.

**Generation of bone marrow–derived mast cells**

Bone marrow–derived mast cells (BMMCs) were generated from the femoral bone marrow cells of mice as described previously (22). Cells were incubated for 3–4 wk in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Life Technologies, Carlsbad, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM 2-ME, 10 mM sodium pyruvate, 10 μM MEM nonessential amino acid solution (Life Technologies), 10 ng/ml recombinant murine IL-3 (Wako Pure Chemical Industries, Osaka, Japan), and 10 ng/ml recombinant murine stem cell factor (SCF) (Wako Pure Chemical Industries) at 37°C in a humidified atmosphere in the presence of 5% CO<sub>2</sub>. Mast cells were identified by flow cytometric analysis of the cell surface expression of c-Kit and the FcerI α-chain.

**Coculture of BMMCs with Chinese hamster ovary cell lines expressing Notch ligands**

Mouse Notch ligand–expressing Chinese hamster ovary cell lines (CHO-Jag1, Jag2, -DII1, and -DII4) were a gift from Dr. S. Chiba (University of Tsukuba, Tsukuba, Japan) (23, 24). BMMCs were cocultured with a Notch ligand–expressing CHO cell line or control CHO cells as described previously (15). In brief, the CHO cells were seeded at a density of 2.2 × 10<sup>5</sup> cells/cm<sup>2</sup> in plates and treated with 3 μg/ml mitomycin C (Sigma-Aldrich) for 3 h. BMMCs were plated at a density of 1.2 × 10<sup>5</sup> cells/cm<sup>2</sup> into the plates and cocultured with the CHO cells for 6 d in coculture medium (MEM nonessential amino acid solution, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM 2-ME, 10 mM sodium pyruvate, and 10 μM MEM nonessential amino acid solution) containing 10 ng/ml IL-3 and 10 ng/ml SCF in the presence or absence of a γ-secretase inhibitor, N-[3,5-difluorophenyl]acetyl]-l-alanyl-2-phenylglycine-1,1-dimethylallyl ester (DAPT) (Wako Pure Chemical Industries). After coculture with the CHO cells, c-Kit BMMCs (purity > 95%) were purified by magnetic cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) using a magnetic microbead–conjugated anti-mouse CD117/c-Kit mAb (Miltenyi Biotec) according to the manufacturer’s instructions.

**RNA interference**

Transient silencing of the Ship1 gene was achieved using small interfering RNAs (siRNA) targeted against Ship1 (no. 1, Stealth RNA interference siRNA MSS326924; no. 2, MSS326925), which were purchased from Invitrogen. A nontargeting siRNA (Invitrogen, no. 12935–300 or 4390843) was used as a negative control. BMMCs (2 × 10<sup>5</sup>) were transfected with 500 nM siRNA using a mouse macrophage Nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer’s instructions with a Nucleofector II device (Lonza).

**Stable lentiviral transduction of BMMCs with short hairpin RNAs**

Stable knockdown of Notch1 or Notch2 expression was achieved by transduction with the lentiviral vector plKO.1-puro expressing a short hairpin RNA (shRNA) targeting mouse Notch1 (no. 1, TRCN0000025935; no. 2, TRCN0000362592) or Notch2 (no. 1, TRCN0000340451; no. 2, TRCN0000340513), which were purchased from Sigma-Aldrich. A nontargeting shRNA (SHC002V) was used as a negative control. The lentiviral transduction particles (1.5 × 10<sup>6</sup> transduction units) were incubated for 5 h at 37°C in RetroNectin (Takara Bio, Shiga, Japan)-coated plates to attach the virus particles onto the RetroNectin. After removing the supernatants, BMMCs (3 × 10<sup>5</sup> cells) were plated onto the virus-attached plates. The transduced cells were selected by additional culture in the presence of 1.6 μg/ml puromycin for 14 d.

**Stable retroviral transduction of BMMCs with Notch ICD**

Murine Notch1 ICD (N<sup>ICD</sup>) cDNA (25) or Notch2 ICD (N<sup>ICD</sup>) cDNA (26), a gift from Dr. S. Chiba (University of Tsukuba), was subcloned into the retroviral vector pMXs-puro (27), a gift from Dr. T. Kitamura (University of Tokyo, Tokyo, Japan). A retrovirus packaging cell line, PLAT-E, was transfected with each retroviral vector by using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) to generate recombinant retroviruses. Forty-eight hours after transfection, the virus-containing medium was collected and centrifuged. The supernatants were incubated for 6 h at 37°C in RetroNectin (Takara Bio)-coated plates to attach the virus particles onto the RetroNectin. After removing the supernatants, BMMCs were plated onto the virus-attached plates. The transduced cells were selected by additional culture in the presence of 1.6 μg/ml puromycin for 14 d.

**Analyses of cytokine production**

BMMCs were sensitized with mouse IgE as described previously (15). To assess cytokine production, IgE–sensitized cells were incubated at 1 × 10<sup>6</sup> cells/ml in the presence or absence of 1 μg/ml anti-mouse IgE mAb (R35-72; BD Biosciences, San Jose, CA) or 10 ng/ml PMA plus 100 ng/ml ionomycin (Sigma-Aldrich) for 6 h. Cytokine concentrations in culture supernatants were quantified using a corresponding ELISA kit (R&D Systems, Minneapolis, MN).

**Western blot analysis**

BMMCs were collected at the indicated time points and lysed by direct addition of sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glyceral, 2% SDS, 0.1 mg/ml bromophenol blue dye, and 10% 2-ME). The cell lysates were electrophoretically resolved in a 7.5% or 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA). Abs against phosphorylated-p44/p42 MAPK (Thr<sup>183</sup>/Tyr<sup>185</sup>), p38 MAPK, SAPK/JNK, SHIP-1 (Cell Signaling Technology, Danvers, MA), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary Abs. Alexa Fluor 680– or IRDye 800–conjugated anti-mouse or rabbit IgG Abs (Life Technologies) were used as secondary Abs. Infrared fluorescence on the membrane was detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). The immunoreactive bands were analyzed by densitometric scanning by using an Odyssey application software (LI-COR Biosciences).

**Real-time quantitative PCR**

Total cellular RNA was purified from BMMCs using an RNasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 100 ng total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time quantitative PCR (real-time PCR) was performed with the Eco real-time PCR system (Illumina, San Diego, CA) using TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression products for Il4 (product no. Mm00445259_m1), Tnf (Mm00446190_m1), Il17 (Mm00434204_m1), Tgif (Mm00447559_m1), Notch1 (Mm00435249_m1), Notch2 (Mm00803077_m1), Ship1/hopp5d (Mm00494987_m1), or Hes1 (Mm01342805_m1), which were purchased from Life Technologies. The mRNA expression levels were quantified with
Statistical analyses

The unpaired Student t test was used as appropriate for parametric differences. One-way ANOVA with Tukey’s method or two-way ANOVA with Dunnett’s or Sidak’s method was used for multiple testing of data. A p value <0.05 was considered significant.

Results

Priming with Notch ligands enhances cytokine production by mast cells

We have previously shown that FcεRI-mediated cytokine production is enhanced in Dll1-primed BMMCs (Dll1-BMMCs) compared with control cells (15). In this study, we first examined the effects of various Notch ligands on cytokine production by BMMCs. After coculture with various Notch ligand–expressing CHO cell lines for 6 d, BMMCs were activated by FcεRI cross-linking or PMA plus ionomycin. Flow cytometric analysis confirmed that all CHO transfectants expressed similar levels of the Notch ligand (data not shown). As shown in Fig. 1, the production of IL-4, IL-6, IL-13, and TNF-α by BMMCs upon FcεRI cross-linking or stimulation with PMA plus ionomycin was markedly enhanced by priming with Jag2, Dil1, and Dil4. Alternatively, Jag1 only slightly enhanced the production of IL-4 and IL-13, but not IL-6 or TNF-α (Fig. 1). Although activated BMMCs produce little IL-4 under normal culture conditions, interestingly, the ability to produce IL-4 was markedly enhanced by priming with Notch ligands. Additionally, although BMMCs stimulated with PMA plus ionomycin generally produce higher levels of cytokines than did BMMCs upon FcεRI cross-linking, the level of IL-4 produced by FcεRI cross-linking was higher than that produced by stimulation with PMA plus ionomycin in Notch ligand–primed BMMCs. Therefore, in this study, we focused on the enhancing effect of Notch signaling on FcεRI-mediated production of IL-4 and other cytokines by BMMCs.

Furthermore, FcεRI-mediated cytokine production by control BMMCs was significantly enhanced in the presence of IL-9 and TGF-β, and the production of IL-4, IL-6, and IL-13 by Dil1-BMMCs was markedly increased in the presence of IL-9 and TGF-β (Supplemental Fig. 1A). This result indicates that Notch and cytokines, such as IL-9 and TGF-β, act synergistically to enhance FcεRI-mediated production of IL-4, IL-6, and IL-13 by BMMCs. FcεRI cross-linking leads to not only cytokine production but also degranulation in mast cells. Histamine released from degranulating mast cells is an important mediator responsible for allergic symptoms. Therefore, we measured levels of FcεRI-mediated degranulation in BMMCs primed with Notch ligands by both β-hexosaminidase release assay and histamine release assay. The level of β-hexosaminidase release was measured as a marker of mast cell degranulation. Although there was no significant difference in the cellular content of β-hexosaminidase among all groups (Supplemental Fig. 2A), the rate of β-hexosaminidase released by BMMCs upon FcεRI cross-linking was significantly higher in Notch ligand–primed cells than in control cells (Supplemental Fig. 2B). Interestingly, the cellular content of histamine and the amount of histamine released into the medium from activated cells were significantly lower in Notch ligand–primed cells than in control cells (Supplemental Fig. 2C). However, the rate of histamine released by BMMCs upon FcεRI cross-linking was significantly increased by Notch priming (Supplemental Fig. 2D), consistent with the result of the release rate of β-hexosaminidase. These results indicate that Notch priming enhances not only FcεRI-mediated cytokine production but also FcεRI-mediated degranulation of BMMCs.

Notch priming enhances FcεRI-mediated phosphorylation of MAPKs

In FcεRI-mediated signaling events, MAPK activation plays a critical role in cytokine production (7, 8). To elucidate the mechanism by which Notch priming enhances FcεRI-mediated cytokine production, we examined the phosphorylation state of MAPKs induced by FcεRI cross-linking in BMMCs. JNKs, p38 MAPK, and ERK1/2 were transiently phosphorylated following FcεRI cross-linking (Fig. 2A). The levels of their transient phosphorylation were significantly augmented in Dil1-BMMCs at all time points compared with control BMMCs (Fig. 2B). It is notable that the phosphorylation states of JNKs and p38 MAPK in Dil1-BMMCs were maintained at 60 min after the FcεRI cross-linking, whereas those in control BMMCs nearly disappeared at 60 min (Fig. 2). FcεRI-mediated IL-4, IL-6, IL-13, and TNF-α production by Dil1-BMMCs was significantly inhibited by treatment with the JNK inhibitor SP600125, and FcεRI-mediated IL-6 production by Dil1-BMMCs was also significantly inhibited by treatment with the p38 inhibitor SB203580, but not by treatment with the ERK inhibitor U0126 (Supplemental Fig. 3C). Thus, augmented and sustained phosphorylation of JNK and p38 MAPK may contribute to the enhancement of FcεRI-mediated cytokine production in Dil1-BMMCs. The augmentation of FcεRI-mediated phosphorylation of MAPKs was also observed in BMMCs primed with other Notch ligands, including Jag1, Jag2, or Dil4 (Supplemental Fig. 3A, 3B). Despite priming with Jag1, Jag2 and Dil4 appeared to have similar effects on the phosphorylation of JNKs, p38, and ERK1/2 (Supplemental Fig. 3A, 3B), and they had different effects on the enhancement of cytokine production by BMMCs (Fig. 1). The cause for this discrepancy cannot be explained only by these data.
Downregulation of SHIP-1 expression contributes to the enhancement of FcεRI-mediated cytokine production by Notch signaling

Because flow cytometric analysis indicated that the expression level of FcεRI on Dll1-BMMC surfaces were nearly equivalent to that on control BMMCs (15), the enhancement of MAPK phosphorylation appeared to be caused by an alteration in downstream signaling of FcεRI. Therefore, we focused on a negative regulator in FcεRI signaling. The inositol 5'-phosphatase SHIP-1 is well known as a master negative regulator of FcεRI-mediated mast cell activation. SHIP-1–deficient mast cells exhibit increased degranulation and cytokine production in response to FcεRI cross-linking compared with wild-type mast cells (28–30). Western blot analysis showed that the expression of SHIP-1 protein was clearly reduced in Jag2- and Dll1-BMMCs compared with control BMMCs (Fig. 3A). The expression level of SHIP-1 mRNA in Jag2-, Dll1-, and Dll4-BMMCs was significantly decreased by ∼46, 45, and 32%, respectively, compared with control BMMCs (Fig. 3B). The reduction of SHIP-1 was prevented by DAPT, a γ-secretase inhibitor that blocks activation of Notch receptors (Fig. 3C). These results indicate that Notch receptor–mediated signaling suppresses the transcription of the SHIP-1 gene in BMMCs. Transcription of the SHIP-1 gene was also significantly suppressed in the presence of IL-9 and TGF-β (Supplemental Fig. 1B), suggesting that the downregulation of SHIP-1 expression contributes to the enhancement of FcεRI-mediated cytokine production by BMMCs.

To ascertain whether the enhancement of FcεRI-mediated cytokine production in Dll1-BMMCs can be explained by the reduction in expression of SHIP-1, we next determined the cytokine production by SHIP-1 knockdown BMMCs. In this experiment, two siRNAs for SHIP-1 were used to exclude the possibility of any off-target effects of siRNAs. The expression of SHIP-1 mRNA was decreased by ∼70% in both Ship1-specific siRNA-transfected BMMCs (Fig. 4A). The production of IL-4, IL-6, IL-13, and TNF-α by BMMCs upon FcεRI cross-linking and stimulation with PMA plus ionomycin was significantly enhanced by knockdown of SHIP-1 (Fig. 4B, 4C). Additionally, knockdown of SHIP-1 resulted in marked augmentation of FcεRI-mediated phosphorylation of JNKs and p38 MAPK and slight but significant augmentation of ERK phosphorylation similarly to Notch priming (Fig. 4C, 4E). These results indicate that the reduced expression of SHIP-1 contributes to the enhancement of FcεRI-mediated cytokine production in Dll1-BMMCs through the augmentation and prolongation of phosphorylation of MAPKs.
FIGURE 4. Downregulation of SHIP-1 contributes to the enhancement of FcεRI-mediated cytokine production and MAPK phosphorylation. SHIP-1 gene was knocked down with Ship1-specific siRNA. In this experiment, two siRNAs of different sequences (nos. 1 and 2) were used to avoid off-target effects. A nontargeting siRNA was taken as negative control. (A) After 48 h of transfection with siRNA, total RNA was isolated from BMMCs. Real-time PCR analysis was performed for SHIP-1 mRNA. Data were normalized to the expression of GAPDH mRNA and are shown as means ± SD. **p < 0.005 compared with the result for negative control (Neg. Ctrl.), one-way ANOVA. (B and C) After 48 h of transfection with siRNA, BMMCs were sensitized with IgE, and then cells were stimulated with 1 μg/ml anti-IgE mAb (B) or PMA plus ionomycin (C) for 6 h. Cytokine concentrations in culture supernatants were measured by ELISA. Data are shown as means ± SD. *p < 0.05, **p < 0.005 compared with the result for the corresponding negative control (Neg. Ctrl.), one-way ANOVA. Similar results were obtained in three independent experiments. (D) After 48 h of transfection with siRNA, BMMCs were sensitized with IgE and stimulated with 1 μg/ml anti-IgE mAb for the indicated periods. SDS-lysed total cell lysates were subjected to Western blot analysis as indicated. (E) Densitometric analysis was performed on total and phosphorylated MAPKs and is represented as the ratio of (Figure legend continues)
Notch2 is critical for the downregulation of Ship1 transcription and the enhancement of FceRI-mediated transcription of Il6, Il13, and Tnf induced by priming with Notch ligand

Mouse mast cells highly express Notch receptors, Notch1 and Notch2, on the cell surface (15). To elucidate the role of each receptor in the downregulation of SHP-1 expression and the enhancement of FceRI-mediated cytokine production, BMMCs were lentivirally transduced with shRNA to stably knock down the expression of each Notch receptor. In this study, we used two shRNA constructs each for Notch1 and Notch2 to exclude the possibility of any off-target effects of shRNAs. Both shRNAs against Notch1 (nos. 1 and 2) significantly suppressed the mRNA expression of only Notch1 in BMMCs (Fig. 5A). Alternatively, both shRNAs against Notch2 (nos. 1 and 2) not only markedly suppressed the mRNA expression of Notch2 in BMMCs, but also modestly suppressed that of Notch1 (Fig. 5A). As shown in Fig. 5B, downregulation of Ship1 transcription induced by Dll1 priming was abolished by knockdown of Notch2, whereas that was still observed in Notch1 shRNA no. 2–transduced BMMCs. Additionally, enhancement of FceRI-mediated transcription of Il6, Il13, and Tnf by Dll1 priming was abolished by knockdown of Notch2 (Fig. 5C). In contrast, a slight enhancement of FceRImediated transcription of Il6, Il13, and Tnf by Dll1 priming was observed in Notch1-knockdown BMMCs, although the levels of mRNA for these cytokines were lower in Notch1-knockdown BMMCs than in control BMMCs (Fig. 5C). Interestingly, FceRImediated transcription of Il4 gene was significantly enhanced in both knockdown BMMCs by priming with Dll1, although the level of Il4 mRNA was significantly decreased in both knockdown BMMCs primed with Dll1 compared with control BMMCs primed with Dll1 (Fig. 5C). These results indicate that Notch2-mediated signaling is critical for the downregulation of SHP-1 and the enhancement of FceRI-mediated production of IL-6, IL-13, and TNF-α induced by priming with Notch ligand. Alternatively, it is assumed that FceRI-mediated IL-4 production is enhanced by signaling through either Notch1 or Notch2.

Enhancement of cytokine production by Notch1 and Notch2 ICDs

To test the possibility that the enhanced production of IL-4 and other cytokines induced by priming with Notch ligands is regulated by different mechanisms, we next retrovirally expressed the ICDs of Notch1 (N1ICD) or Notch2 (N2ICD) in BMMCs. Hes-1 is a basic helix-loop-helix transcriptional repressor and a well-known target gene of N1ICD and N2ICD (16, 31). A significant upregulation of Hes1 mRNA expression was detected in both N1ICD- and N2ICD-expressing BMMCs (Fig. 6A), indicating that Notch signaling–mediated gene transcription was activated in both BMMCs. Ship1 mRNA level was significantly decreased in N2ICD-expressing BMMCs, but not in N1ICD-expressing BMMCs, compared with mock vector–transduced BMMCs (Fig. 6B). Additionally, FceRI-mediated production of IL-6 and TNF-α was modestly but significantly increased in N2ICD-expressing BMMCs compared with mock vector–transduced BMMCs, whereas that of IL-6, IL-13, and TNF-α was significantly decreased in N1ICD-expressing BMMCs (Fig. 6C). These results indicate that Notch2-mediated signaling contributes to the downregulation of SHP-1 and the enhancement of cytokine production by mast cells, consistent with the results in Notch2-knockdown BMMCs. In contrast, FceRI-mediated IL-4 production was markedly increased in either N1ICD- or N2ICD-expressing BMMCs compared with mock vector–transduced BMMCs (Fig. 6C). This indicates that IL-4 production was upregulated by a mechanism distinct from that of other cytokines. Briefly, we inferred that IL-4 production is directly enhanced by the Notch intracellular domain, whereas production of various cytokines is commonly enhanced by Notch2-mediated reduction of SHP-1 expression.

CNS2 enhancer is critical for the enhancement of FceRI-mediated Il4 gene transcription by Notch signaling

In previous reports, several regions have been identified as the enhancer for Il4 gene transcription. For example, the HS2 region located in the second intron of the Il4 locus is a critical enhancer for GATA-3–mediated Il4 transcription in Th2 cells (21). Additionally, CNS2 is a distal 3′ enhancer important for Il4 transcription in memory-type CD4+ T cells, NKT cells, follicular helper T cells, and mast cells (10, 32, 33). Importantly, the CNS2 enhancer has Notch effector RBP-J binding sites and is activated by Notch and RBP-J signaling in CD4+ T cells and NKT cells (32, 34).

To test the hypothesis that Notch signaling directly enhances FceRI-mediated Il4 transcription in mast cells, we generated BMMCs from mice lacking the HS2 or CNS2 regions in the Il4 locus (Fig. 7A). These mast cells were cocultured with Dll1-expressing or control CHO cells for 6 d, and then IL-4 mRNA expression induced by FceRI cross-linking was measured. Expression of IL-4 mRNA was weakly detected in control BMMCs generated from wild-type mice and CNS2-deficient mice, whereas it was hardly detectable in control HS2-deficient BMMCs (Fig. 7B). Importantly, the FceRI-mediated IL-4 mRNA expression in CNS2-deficient BMMCs was increased only 12.6-fold by Dll1 priming, whereas that in wild-type or HS2-deficient BMMCs was increased 25.4- and 23.5-fold, respectively. Although a statistically significant increase in IL-4 mRNA expression by Dll1 priming was detected in BMMCs from all mouse lines, the mRNA expression level was markedly lower in HS2-deficient and significantly lower in CNS2-deficient BMMCs than in wild-type BMMCs (Fig. 7B). In contrast, there were no significant differences in the transcription of Il13 and Tnf between CNS2-deficient BMMCs and wild-type BMMCs (Supplemental Fig. 4). The transcription of Il6 in Dll1-primed CNS2-deficient BMMCs was higher than Dll1-primed wild-type BMMCs (Supplemental Fig. 4). These data indicate that the HS2 region is essential for FceRI-mediated Il4 transcription in mast cells, and that the CNS2 region is critical for the enhancement of FceRI-mediated Il4 transcription by Notch signaling.

Discussion

Mast cell functions are influenced by various microenvironmental factors. In this study, we showed that Notch signaling enhances FceRI-mediated cytokine production by mast cells through direct and indirect mechanisms. Notch1 and Notch2 are constitutively expressed on mast cells, and Notch ligands are expressed in various tissues, including intestinal epithelial cells, epidermis keratinocytes, and vascular endothelial cells (17–20). Connective tissue–type mast cells generally localize around the vessels in connective tissues, and mucosal mast cells localize in the intra-epithelium and subepithelium in mucosal tissues (35). Therefore,
Notch ligands are inferred to act as a microenvironmental factor for tissue-resident mast cells.

Priming with Notch ligands Jag2, Dll1, and Dll4 significantly enhanced FcεRI-mediated production of IL-4, IL-6, IL-13, and TNF-α by BMCCs, whereas Jag1 only modestly enhanced FcεRI-mediated IL-4 production (Fig. 1). The results of knockdown of Notch receptors and overexpression of Notch intracellular domains in BMCCs indicate that Notch2 is critical for the enhancement of IL-6, IL-13, and TNF-α production by BMCCs induced by priming with Notch ligands (Figs. 5C, 6C). Thus, the binding affinity of Jag1 for Notch2 may be weak compared with that of other ligands for Notch2. This difference may reflect the differential affinity of Notch receptors for ligands caused by glycosylation of the Notch extracellular domain (36). Shimizu et al. (23) showed that Jag1 and Jag2 are similar ligands for Notch2 on the mouse pro-B cell line Ba/F3. Therefore, the differential affinity of Jag1 for Notch2 may be unique to BMCCs.

BMCCs generated by culturing bone marrow cells with IL-3 and SCF resemble immature mucosal mast cells (37). In contrast, BMCCs cultured in the presence of IL-9 and TGF-β, which are known to promote the maturation of mucosal mast cells, modestly secreted IL-4 in response to FcεRI cross-linking (Supplemental Fig. 1A). Interestingly, FcεRI-mediated IL-4 production was markedly enhanced in BMCCs by priming with Dll1 in an IL-9/TGF-β-independent manner. Furthermore, Notch signaling acted synergistically with IL-9 and TGF-β to enhance FcεRI-mediated production of IL-4, IL-6, and IL-13 (Supplemental Fig. 1A). These findings suggest that Notch ligands may be a novel inducer of IL-4-producing mast cells in the mucosal environment.

A fundamental mechanism for the upregulation of mast cell cytokine production by Notch is indirect action through the modulation of mast cell activation signals. Our results indicate that Dll1 priming suppressed the expression of SHIP-1, a master negative regulator of the FcεRI signaling pathway (Fig. 3A, 3B), leading to the augmentation and prolongation of FcεRI-mediated phosphorylation of MAPKs in BMCCs primed with Dll1 (Figs. 2, 4). FcεRI-mediated cytokine production by Dll1-BMCCs was suppressed by treatment with pharmacological inhibitors of JNK or p38 but not ERK (Supplemental Fig. 3C). Thus, the augmentation of JNK or p38 is suspected to be important for the enhancement of cytokine production induced by Dll1 priming.
Mast cells constitutively express Notch1 and Notch2 on the cell surface. Although the levels of FcεRI-mediated production of IL-4 is upregulated by both Notch1 and Notch2, whereas those of IL-6 and TNF-α were upregulated by Notch2 but not by Notch1. BMMCs were retrovirally transduced with N1ICD/pMXs-puro, N2ICD/pMXs-puro, or mock vector and then selected in the presence of puromycin for 14 d. Total RNA was isolated from these transduced cells, and then real-time PCR analysis of mRNA expression of Hes-1 (A), a common target gene of both N1ICD and N2ICD, or SHIP-1 (B). Data were normalized to the expression of GAPDH mRNA and are shown as means ± SD. Similar results were obtained in three independent experiments. *p < 0.05, **p < 0.005 compared with the result for mock, one-way ANOVA. (C) FcεRI-mediated cytokine production. The transduced cells were sensitized with IgE and stimulated with 1 µg/ml anti-IgE mAb for 6 h. Cytokine concentrations in culture supernatants were measured by ELISA. Data are shown as means ± SD. Similar results were obtained in three independent experiments. *p < 0.05, **p < 0.005 compared with the result for the corresponding mock, one-way ANOVA. ND, not detected.

Because previous reports have revealed that the level of histamine

**FIGURE 6.** FcεRI-mediated production of IL-4 is upregulated by both Notch1 and Notch2, whereas those of IL-6 and TNF-α were upregulated by Notch2 but not by Notch1. BMMCs were retrovirally transduced with N1ICD/pMXs-puro, N2ICD/pMXs-puro, or mock vector and then selected in the presence of puromycin for 14 d. Total RNA was isolated from these transduced cells, and then real-time PCR analysis of mRNA expression of Hes-1 (A), a common target gene of both N1ICD and N2ICD, or SHIP-1 (B). Data were normalized to the expression of GAPDH mRNA and are shown as means ± SD. Similar results were obtained in three independent experiments. *p < 0.05, **p < 0.005 compared with the result for mock, one-way ANOVA. (C) FcεRI-mediated cytokine production. The transduced cells were sensitized with IgE and stimulated with 1 µg/ml anti-IgE mAb for 6 h. Cytokine concentrations in culture supernatants were measured by ELISA. Data are shown as means ± SD. Similar results were obtained in three independent experiments. *p < 0.05, **p < 0.005 compared with the result for the corresponding mock, one-way ANOVA. ND, not detected.

**FIGURE 7.** CNS2 is required for the upregulation of FcεRI-mediated IL-4 transcription by Notch signaling in BMMCs. (A) Schematic diagram of the Il4 locus showing three defined regulatory elements. (B) Real-time PCR analysis of IL-4 mRNA expression. BMMCs generated from wild-type and Hs2- or CNS2-deleted mice were cocultured with Dll1-expressing CHO cells or control CHO cells for 6 d, and then purified as c-Kit+ cells by MACS. Purified BMMCs were sensitized with IgE and stimulated with 1 µg/ml anti-IgE mAb for 3 h, and then total RNA was isolated. Data were normalized to the expression of GAPDH mRNA and are shown as means ± SD. Similar results were obtained in two independent experiments. *p < 0.005 compared with the result for the corresponding BMMCs primed with control (Ctrl), two-way ANOVA with Sidak’s method. **p < 0.005 compared with the result for the corresponding WT-BMMCs, two-way ANOVA with Dunnett’s method. ND, not detected.
content is lower in mature intestinal mucosal mast cells than in connective-tissue mast cells (40, 41), the decreased cellular content of histamine may represent the maturation of BMMCs by Notch2 signaling. Therefore, Notch2 signaling–induced enhancement of multiple cytokine production by BMMCs appears to be caused not only by suppression of SHIP-1 expression and augmentation of MAPK activation but also by alteration in the intracellular environment associated with mast cell differentiation and maturation, such as the epigenetic modifications of DNA and histones and the alteration of expression levels of other signaling molecules. N2ICD transduction led to a significant decrease in SHIP-1 expression in BMMCs (Fig. 6B) and only a modest increase in FceRI-mediated IL-6 and TNF-α production by BMMCs (Fig. 6C). This result indicates that downregulation of SHIP-1 is insufficient to enhance multiple cytokine production by BMMCs. Other factors that act cooperatively with downregulation of SHIP-1 may be necessary for the enhancement of multiple cytokine production. Thus, the enhancing effect of N2ICD on cytokine production was modest in BMMCs, probably due to the adverse effect of the excessive signaling caused by overexpression of N2ICD. Although Da’as et al. (42) have reported that zebrafish notch1b regulates mast cell development through gata2, previous reports by Sakata-Yanagimoto et al. (16, 17) and our results indicate that mouse Notch2 but not Notch1 is involved in mast cell development. Notch2 signaling–induced downregulation of SHIP-1 may contribute to the differentiation of mast cells and the enhancement of multiple cytokine production in mice. However, we previously demonstrated that mouse Notch1 but not Notch2 induces MHC class II expression on the cell surface of BMMCs through downregulation of GATA-1 and GATA-2 (43). Thus, Notch1 signaling may have a role in regulating cell functions through GATAs. Alternatively, the enhancing effect of Dll1 priming on FceRI-mediated IL-4 production was not abolished by knockdown of either Notch1 or Notch2 (Fig. 5C). FceRI-mediated IL-4 production was dramatically increased by both N1ICD and N2ICD, suggesting that IL-4 production by BMMCs is upregulated by signaling through Notch1 and/or Notch2. These findings indicate that IL-4 and other cytokines are controlled by distinct mechanisms in BMMCs. Because FceRI-mediated IL-4 production by N2ICD-expressing BMMCs was 2-fold higher than that by N1ICD-expressing BMMCs (Fig. 6C), Notch2 signaling may act indirectly to upregulate IL-4 production through the suppression of SHIP-1 expression and the alteration of other factors in addition to a common mechanism shared by Notch1 and Notch2. The transcription of the Il4 gene is regulated by some cis-regulatory elements, such as HS2 and CNS2, on the Il4 locus. Tanaka et al. (32) previously demonstrated that Notch-mediated binding of RBP-J to the CNS2 enhancer located downstream of the Il4 locus upregulates IL-4 production by NKT cells and memory-type CD4+ T cells. Although FceRI-mediated Il4 gene transcription was markedly enhanced by priming with Dll1 in wild-type BMMCs, the enhancement of Il4 transcription by priming with Dll1 was modest in CNS2-deficient BMMCs (Fig. 7B). Thus, Notch signaling can directly upregulate FceRI-mediated IL-4 expression in mast cells through the CNS2 enhancer of the Il4 gene as in lymphoid cells. Additionally, the level of FceRI-mediated Il13 transcription was significantly lower in CNS2-deficient BMMCs primed with Dll1 than in wild-type BMMCs (Supplemental Fig. 4). Because the Il13 gene is located adjacent to the Il4 gene, Il13 transcription may be affected by Notch signaling through the CNS2 enhancer. In this study, we showed that Notch signaling indirectly enhanced FceRI-mediated proinflammatory cytokine production through alterations of the intracellular environment, such as the modulation of MAPKs by reduced SHIP-1. Additionally, Notch signaling directly enhanced FceRI-mediated IL-4 production through the CNS2 enhancer of the Il4 gene locus. In allergic diseases, dramatic increases in the numbers of mucosal mast cells are observed in the mucosal epithelia of the nose, bronchi, and gastrointestinal tract (44). In this situation, it is likely that Notch ligands expressed on epithelial cells augment the production of proinflammatory and Th2-type cytokines by mucosal mast cells. Therefore, the blockade of Notch signaling in inflamed tissues may be a novel strategy for the treatment of allergic diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


10 NOTCH ENHANCES CYTOKINE PRODUCTION BY MAST CELLS
Supplemental Figure S1. Nakano, et al.

Notch priming acts synergistically with IL-9 and TGF-β to enhance FcεRI-mediated production of IL-4, IL-6, and IL-13 by BMMCs. BMMCs were cocultured with Dll1-expressing CHO cells or control CHO cells for 6 days in coculture medium containing 10 ng/ml IL-3 and 10 ng/ml SCF or coculture medium containing 1 ng/ml IL-3, 20 ng/ml SCF, 5 ng/ml recombinant mouse IL-9 (Wako Pure Chemical Industries), and 1 ng/ml recombinant human TGF-β1 (Wako Pure Chemical Industries), and then purified as c-Kit⁺ cells by MACS. (A) Purified BMMCs were sensitized with IgE and stimulated with 1 µg/ml anti-IgE mAb for 6 h. Cytokine concentrations in culture supernatants were measured by ELISA. Data are shown as means ± SD. *P < 0.05, **P < 0.005 (one-way ANOVA) compared with the result for Ctrl in the presence of IL-3 + SCF; #P < 0.005 (one-way ANOVA) compared with the result for Dll1 in the presence of IL-3 + SCF; †P < 0.005 (one-way ANOVA) compared with the result for Ctrl in the presence of IL-3 + SCF + IL-9 + TGF-β. ND, Not detected. Similar results were obtained in two independent experiments. (B) Total RNA were isolated from purified BMMCs. Quantitative real-time PCR analysis was performed for SHIP1 mRNA. Data were normalized to the expression of GAPDH mRNA and are shown as means ± SD. *P < 0.05, **P < 0.005 (one-way ANOVA) compared with the result for Ctrl in the presence of IL-3 + SCF.

Supplemental Figure S1. Nakano, et al.
Supplemental Figure S2. Priming with Notch ligands enhances FcεRI-mediated degranulation of BMMCs but decreases histamine production in BMMCs.

BMMCs were cocultured with CHO cells expressing the indicated Notch ligands or control CHO cells for 6 days. After coculture with CHO cells, cells were purified as c-Kit+ cells by MACS, and then were sensitized with IgE. IgE-sensitized cells were resuspended in Tyrode’s buffer (10 mM HEPES [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, and 0.1% BSA) and stimulated with 1 µg/ml anti-IgE mAb for 40 min at 37°C. (A, B) The level of β-hexosaminidase release was measured as a marker of mast cell degranulation. β-Hexosaminidase activity in the supernatant was measured as follows. Culture supernatants were incubated with 1.3 mg/ml 4-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich) for 90 min at 37°C. After developing the reaction with 0.2 M glycine (pH 11), absorbance was measured at 405 nm. The total β-hexosaminidase content in the cells was determined after cell lysis with 1% Triton X-100 (A). Release was calculated as a percentage of the total β-hexosaminidase content and was determined by subtracting the value of samples treated with IgE alone without anti-IgE mAb (B). Data are shown as means ± SD, **P < 0.005 (one-way ANOVA) compared with the result for Ctrl. Similar results were obtained in three independent experiments. 

(C) The levels of histamine in the cell lysates and the supernatants were measured using a competitive ELISA (Oxford Biomedical Research, Rochester Hills, MI). Data are shown as means ± SD, **P < 0.005, ##P < 0.005 (one-way ANOVA) compared with the result for the corresponding Ctrl. Similar results were obtained in two independent experiments. (D) Release was calculated as a percentage of the total histamine content and was determined by subtracting the value of samples treated with IgE alone without anti-IgE mAb. Data are shown as means ± SD, **P < 0.005 (one-way ANOVA) compared with the result for Ctrl. Similar results were obtained in two independent experiments.
Supplemental Figure S3. FcεRI-mediated phosphorylation of MAPKs and cytokine production in BMMCs primed with Notch ligands.

BMMCs were cocultured with the indicated Notch ligand-expressing CHO cells or control CHO cells for 6 days, and then purified as c-Kit+ cells by MACS. (A, B) To examine the phosphorylation of MAPKs in response to FcεRI cross-linking, purified BMMCs were sensitized with IgE and stimulated with 1 µg/ml anti-IgE mAb for 0 or 15 min. SDS-lysed total cell lysates were subjected to western blot analysis as indicated (A). Densitometric analysis was performed on total and phosphorylated MAPKs and represented as the ratio of phosphorylated MAPKs/total MAPKs (B). Data were shown as mean ± SEM from three independent experiments. *P < 0.05 (one-way ANOVA) compared with the result for Ctrl at 0 min; **P < 0.005 (one-way ANOVA) compared with the result for Ctrl at 15 min. Similar results were obtained in three independent experiments. (C) Effects of MAPK inhibitors on FcεRI-mediated cytokine production by BMMCs primed with Dll1. SP600125, which is a JNK inhibitor, SB203580, which is a p38 inhibitor, U0126, which is an ERK inhibitor, were purchased from Sigma-Aldrich. Dll1-primed BMMCs were sensitized with IgE. After preincubation for 10 min in the presence of 10 µM the indicated inhibitor or vehicle (DMSO), cells were stimulated with 1 µg/ml anti-IgE mAb for 6 h in the presence of 10 µM the indicated inhibitor or vehicle. Cytokine concentrations in culture supernatants were measured by ELISA. Data were shown as mean ± SD from three independent experiments. *P < 0.05, **P < 0.005 (one-way ANOVA) compared with the result for vehicle. Similar results were obtained in two independent experiments.
**Supplemental Figure S4.** FcεRI-mediated transcription of *Il6*, *Il13*, and *Tnf* in BMMCs generated from wild-type and HS2- or CNS2-deleted mice.

BMMCs generated from wild-type and HS2- or CNS2-deleted mice were cocultured with Dll1-expressing CHO cells or control CHO cells for 6 days, and then purified as c-Kit⁺ cells by MACS. Purified BMMCs were sensitized with IgE and stimulated with 1 μg/ml anti-IgE mAb for 3 h, and then total RNA was isolated. Quantitative real-time PCR analysis was performed for IL-6, IL-13, and TNF-α mRNA. Data were normalized to the expression of GAPDH mRNA and are shown as means ± SD. #*P < 0.05, **P < 0.005 (2-way ANOVA with Sidak’s method) compared with the result for the corresponding BMMCs primed with Ctrl; ***P < 0.005 (2-way ANOVA with Dunnett’s method) compared with the result for the corresponding WT-BMMCs. Similar results were obtained in two independent experiments.