that IL-33 acts directly on pulmonary microvascular endothelial cells and epithelial cells, but not on smooth muscle cells or fibroblasts, via the ST2 receptor. More importantly, we found that Th2 cytokines, such as IL-4 and IL-13, significantly enhanced ST2 expression and function in both endothelial and epithelial cells. These findings suggest that IL-33–mediated inflammatory responses in lung tissue cells may be crucially involved in the chronic allergic inflammation of the asthmatic airway.

Materials and Methods

Reagents

Recombinant human IL-33 was purchased from PeproTech (Rocky Hill, NJ). Recombinant human ST2-Fc chimera was purchased from R&D Systems (Minneapolis, MN). PD98059 and SB202190 were purchased from Calbiochem (La Jolla, CA). Fluticasone propionate was purchased from Sigma (St. Louis, MO).

Primary human cell culture, treatment, and transfection

Normal human bronchial epithelial cells (NHBEs), normal human lung fibroblasts (NHLFs), bronchial smooth muscle cells (BSMCs), human microvascular endothelial cells from lung blood vessels (HMVEC-LBl), neonatal normal human epidermal keratinocytes, normal human dermal fibroblasts, normal HUVECs, and normal human coronary artery endothelial cells (HCAECs) were purchased from Lonza (Walkersville, MD) and maintained exactly as recommended by the manufacturer. NHBEs were cultured in flasks or plates coated with type I collagen (Iwaki, Tokyo, Japan). All the experiments described in this study were performed using second- or third-passage cells in 70–80% confluent monolayers unless otherwise noted.

All the cells were treated with different concentrations of IL-33 for up to 24 h or with 10 ng/ml IL-4 for up to 48 h. In some experiments, NHBEs and HMVEC-LBl were treated with different concentrations of PD98059 or SB202190 for 30 min prior to stimulation with IL-33 (Fig. 7).

Both the SAGM BulletKit and EGM-2MV BulletKit (Lonza), which are optimized for use with NHBEs and HMVEC-LBl, respectively, contain hydrocortisone. Therefore, experiments examining the effects of fluticasone propionate (Fig. 5) were performed after hydrocortisone deprivation for 24 h, as previously described (16). All other experiments described in this study were performed using a complete medium suited for each type of cell (Lonza).

NHBEs and HMVEC-LBl were seeded at 5×10^4 cells/well in 12-well culture plates and cultured until the cells reached 50–60% confluence. Then, the cells were transfected with small interference RNA (siRNA) against ST2 (No. SI00114618; Qiagen, Valencia, CA), STAT6 (No. SI02662905; Qiagen), or nontargeting control siRNA (No. 1027281; Qiagen) at 5 nM (NHBEs) or 10 nM (HMVEC-LBl) using HiPerFect transfection reagent (Qiagen) in accordance with the manufacturer's instructions. The transfected cells were further grown for 48 h and then stimulated with the indicated cytokine(s).

Quantitative real-time PCR

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as previously described (16, 17). Primer sets for six genes were synthesized at Fasmac (Kanagawa, Japan): ST2L (sense, 5′-CTGTCTGGCCCTGAATTTGC-3′; antisense, 5′-AGCAGAGTGGCCTCAATCCA-3′), sST2 (sense, 5′- CTGTCTGGCCCTGAATTTGC-3′; antisense, 5′-TGGAACCACACTCCATTCTGC-3′), IL-8 (sense, 5′-GTCTGCTAGCCAGGATCCACAA-3′; antisense, 5′-GAGAAACCAAGGCACAGTGGAA-3′), IL-6 (sense, 5′- CAATAACCACCCCTgACCCA-3′; antisense, 5′-GCGCAGAATGAGATGAGTTGTC-3′), STAT6 (sense, 5′-TCTGACCGGCTGATCATTGG-3′; antisense, 5′-CCAATCTCTGAGTCGCTGAAGC-3′), and β-actin (sense, CCCAGCCATGTACGTTGCTAT-3′; antisense, 5′-TCACCGGAGTCCATCACGAT-3′). To determine the exact copy numbers of the target genes, quantified concentrations of the purified PCR products of ST2L, soluble ST2 (sST2), IL-8, IL-6, STAT6, and β-actin were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each real-time PCR. The mRNA expression levels were normalized to the β-actin level in each sample.

ELISA

The concentrations of the sST2, IL-8, IL-6, and MCP-1 proteins in cell-free supernatants were measured with specific ELISA kits (R&D Systems) in accordance with the manufacturer's instructions.

Western blotting

Cells were seeded into 6-well plates at 1×10^5 cells/well and cultured until subconfluent (2 or 3 d). The cells were then treated for the indicated time periods with 10 ng/ml IL-4 (for ST2 blotting, see Fig. 3*D*) or 10 ng/ml IL-33 (for phospho-MAPK blotting, see Fig. 6). Whole-cell lysates were extracted with 200 μl NuPAGE sample buffer (Invitrogen, Carlsbad, CA) containing 5% 2-ME and lysed by sonication. Equal amounts of whole-cell lysates were separated by SDS-PAGE (5–15% Ready Gels J; Bio-Rad, Hercules, CA) gel electrophoresis and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks, mini; Invitrogen). Immunoblotting was performed using the following Abs: clone 97203, mouse mAb for ST2/IL-1R4 (R&D Systems); clone D13.14.4E, rabbit mAb for phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204); rabbit polyclonal Ab for phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology, Danvers, MA); and clone AC-15, mouse mAb for β-actin (Sigma), in accordance with the manufacturers' instructions.

Statistical analysis

All data are presented as the mean ± SD. Differences between groups were analyzed using ANOVA with Bonferroni's post hoc test and were considered to be significant when $p < 0.05$.

Results

Preferential expression of ST2 among lung tissue cells

The ST2 gene encodes, by alternative splicing, both membrane-bound ST2L, which is a receptor for IL-33, and sST2, which is a decoy receptor for IL-33 (18). We first examined the expression of ST2 mRNA in lung tissue cells and other human primary cells. We found that both ST2L and sST2 were preferentially expressed in microvascular endothelial cells (HMVEC-LBl) and airway epithelial cells (NHBEs), but not in lung fibroblasts (NHLFs), smooth muscle cells (BSMCs), epidermal keratinocytes (neonatal normal human epidermal keratinocytes), or normal human dermal fibroblasts (Fig. 1*A*, open bars). ST2 mRNA expression was also observed in other human endothelial cells, such as umbilical vein endothelial cells (HUVECs) and coronary artery endothelial cells (HCAECs), suggesting that ST2 is characteristically expressed in human vascular endothelial cells. We further confirmed that the secreted sST2 level in the culture supernatant of each type of cell correlated well with its respective mRNA level (Fig. 1*A*, solid bars).

IL-33–mediated inflammatory responses in lung tissue cells

Because we had elucidated the cell type distribution pattern of ST2L expression, we next examined the biological significance of ST2 expression in lung tissue cells. We examined the ability of IL-33 to induce the production of various cytokines/chemokines by those cells. Consequently, we found that there was good correspondence between the ST2L mRNA distribution and IL-33 responsiveness. More specifically, neither NHLFs nor BSMCs, which did not express ST2L mRNA, responded to IL-33 (Fig. 1*B*, yellow and green bars). In NHBEs, IL-33 induced IL-8 production, detected in the supernatants of 24-h cultures in a dose-dependent manner (Fig. 1*B*, *upper graph*, blue bars). However, IL-33 induced neither IL-6 nor MCP-1 production by NHBEs. Of note, HMVEC-LBl showed dose-dependent, enhanced production of IL-6 and MCP-1 in addition to IL-8 in response to treatment with IL-33 for 24 h (Fig. 1*B*, red bars). Thus, IL-33 induced stronger responses in HMVEC-LBl than in NHBEs in accordance with the levels of ST2 expression in each type of cell. Although we looked for production of other cytokines/chemokines, including IL-4, IL-5, IL-10, IL-12, IL-13, TNF-α, IL-1β, and IP-10, none were found in either HMVEC-LBl or NHBEs.

IL-33 mediates inflammatory responses via the ST2 receptor in lung tissue cells

To elucidate the role of ST2 in IL-33–mediated inflammatory responses in lung tissue cells, we depleted ST2 mRNA by using

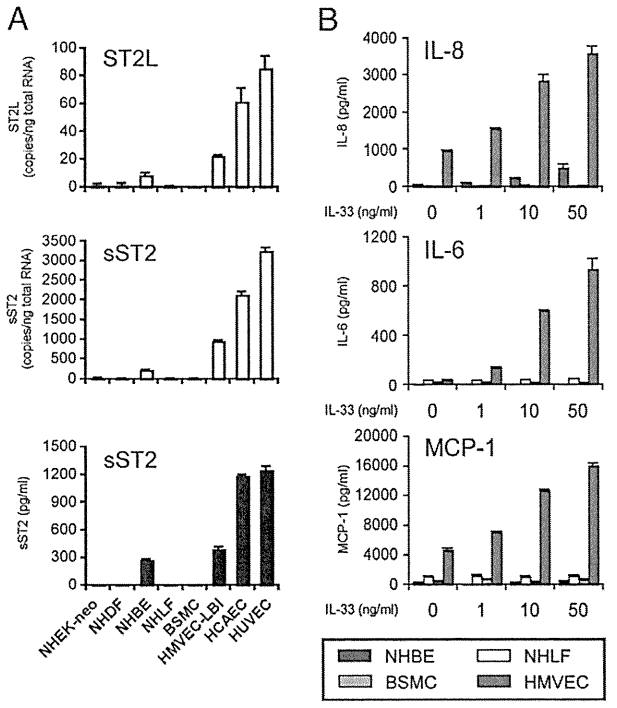

FIGURE 1. *A*, Expression of mRNA for ST2L and sST2 in cultured human primary cells. Total RNA was isolated from various human primary cells in growth phase, and the levels of mRNA for ST2L and sST2 were measured by quantitative real-time PCR (open bars). Concentrations of secreted sST2 protein in the culture supernatants were quantified by ELISA (solid bars). *B*, IL-33–mediated cytokine-chemokine production by lung tissue cells. NHBEs (blue), NHLFs (yellow), BSMCs (green), and HMVEC-LBl (red) were treated with the indicated concentrations of IL-33 for 24 h. Protein concentrations in the culture supernatants are shown. Data are shown as the mean ± SD of triplicate samples and are representative of experiments using at least two different lots from individual donors of NHBEs, NHLFs, BSMCs, and HMVEC-LBl.

siRNA specific for ST2 (No. SI00114618; Qiagen), designed to target a site within the sequence shared by ST2L and sST2. NHBEs (Fig. 2*A*) and HMVEC-LBl (Fig. 2*B*) were transfected with siRNA against nontargeting control siRNA or ST2 and then stimulated with IL-33 for 6 h. Control experiments demonstrated that both ST2L and sST2 mRNA were significantly suppressed by the ST2 siRNA compared with the levels of ST2L and sST2 transcripts, respectively, in nontargeting control siRNA-transfected cells. Induction of IL-8 (NHBEs, HMVEC-LBl) and IL-6 (HMVEC-LBl) by IL-33 was significantly inhibited by the transfection of ST2 siRNA, suggesting that IL-33–mediated responses in these cells occur via an ST2-dependent pathway. Furthermore, we found that IL-33–mediated responses in HMVEC-LBl were IL-33 specific because they were almost completely suppressed by simultaneous treatment with IL-33 and recombinant ST2-Fc chimera (Fig. 2*C*).

Th2 cytokines enhance the expression and function of ST2 in lung tissue cells

IL-33 is a potent inducer of Th2 immunity, and we thus examined the effects of Th2 cytokines such as IL-4 on the expression and function of ST2 in lung tissue cells. As shown in Fig. 3*A*, both ST2L mRNA and sST2 mRNA were significantly upregulated by 10 ng/ml IL-4 treatment in a time-dependent manner. Importantly, this IL-4–mediated upregulation of the ST2 genes was observed in IL-33–responsive cells such as NHBEs and HMVEC-LBl but not in the IL-33–unresponsive cells such as NHLFs and BSMCs. We further confirmed that the sST2 protein levels accumulated in the culture supernatants of NHBEs and HMVEC-LBl in response to IL-4 treatment correlated well with their respective sST2 mRNA levels (Fig. 3*B*, *left graph*).

IL-13 is another Th2 cytokine that plays a prominent role in the pathogenesis of allergic inflammation. IL-13 and IL-4 share many functional properties, stemming from the fact that they share the α subunit of the IL-4R. In fact, we found that IL-13 also induced sST2 production by HMVEC-LBl (Fig. 3*B*, *right graph*). IL-4 or IL-13 stimulation of cells leads to activation of multiple signaling pathways via IL-4R α, one of which involves a transcription factor, STAT6. Therefore, to examine the role of STAT6 on IL-4–induced expression of ST2, we depleted STAT6 mRNA by using siRNA for STAT6 (No. SI02662905; Qiagen). The siRNA for STAT6 or nontargeting control siRNA was transfected into HMVEC-LBl. The transfected cells were further cultured for 48 h and then stimulated with 10 ng/ml IL-4 for 24 h. The efficiency of STAT6 mRNA depletion was more than 70% compared with the level of STAT6 transcripts in control siRNA-transfected cells, which was confirmed by real-time PCR (Fig. 3*C*, *left graph*). Transfection of STAT6 siRNA significantly reduced the IL-4–dependent upregulation of both ST2L mRNA and sST2 mRNA (Fig. 3*C*, *right two graphs*), suggesting that STAT6 is required for IL-4–enhanced expression of ST2 genes.

To confirm the IL-4–enhanced expression of ST2 at the protein level, whole-cell lysates from IL-4–stimulated HMVEC-LBl and NHBEs were subjected to SDS-PAGE followed by immunoblotting with an anti-ST2 Ab or an anti–β-actin Ab as a loading control. We found that IL-4 significantly enhanced ST2L protein in the whole-cell lysates of both HMVEC-LBl and NHBEs (Fig. 3*D*), in parallel with upregulation of ST2L mRNA in these cells (Fig. 3*A*, *upper graph*).

We next examined whether IL-33–mediated responses of lung tissue cells were further enhanced by IL-4 pretreatment. NHBEs and HMVEC-LBl were pretreated with 10 ng/ml IL-4 for 48 h and then stimulated with 10 ng/ml IL-33 for the indicated periods. IL-4–pretreated cells showed significantly enhanced IL-33–mediated responses, including the induction of IL-8 and IL-6 mRNA (Fig. 4). Thus, Th2 cytokines significantly enhanced ST2 expression and function in both lung endothelial and epithelial cells.

Effects of corticosteroid on IL-33–mediated responses in epithelial and microvascular endothelial cells

Currently, inhaled corticosteroids are a first-line therapy and known to be one of the most effective therapies available for asthma (19). Therefore, we next examined the effect of corticosteroid on the responses of both NHBEs and HMVEC-LBl to IL-33. Fluticasone propionate (FP) treatment showed significant attenuation of IL-33–mediated IL-8 production by NHBEs even at a low FP concentration (1 nM) (reduction to 28% of the production in the absence of corticosteroid), and the production was almost completely suppressed by 100 nM FP treatment (Fig. 5*A*). In contrast, FP treatment showed only partial attenuation of IL-33–mediated IL-6, IL-8, and MCP-1 production by HMVEC-LBl (reduction to 80, 63, and 74% of the respective production in the absence of corticosteroid) even at a high concentration of FP (100 nM) (Fig. 5*B*). The higher levels of IL-8 production by NHBEs compared with the results observed in Fig. 1*B* may be due to the hydrocortisone deprivation before IL-33 stimulation. Because IL-33–mediated IL-8 production by NHBEs was sensitively inhibited by corticosteroid treatment, we presume that

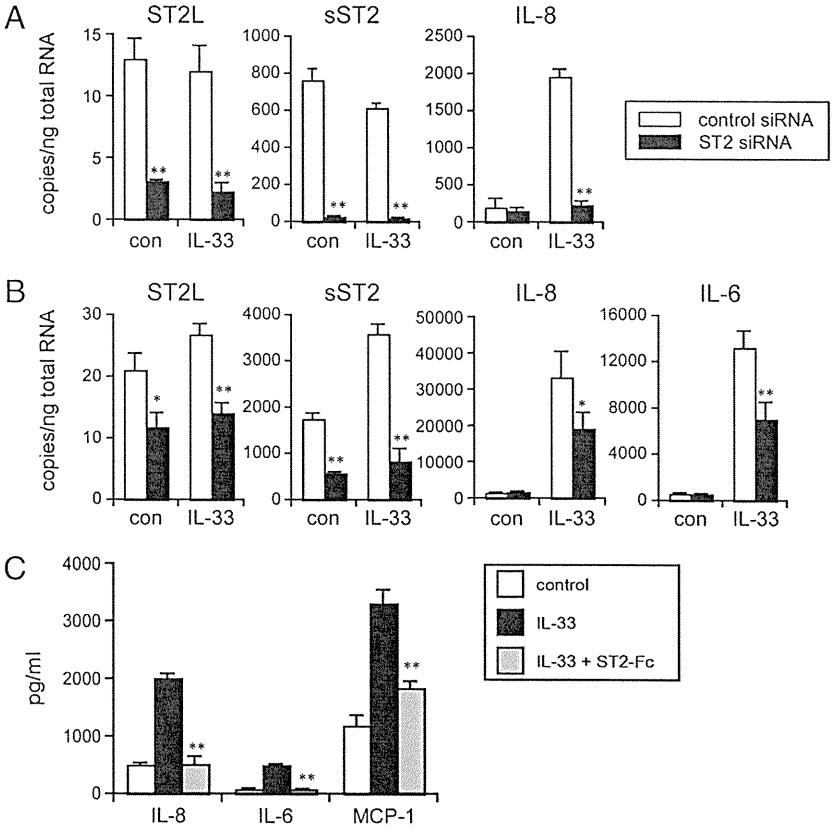

FIGURE 2. IL-33 mediates inflammatory responses via the ST2 receptor on HMVEC-LBl and NHBEs. *A* and *B*, Cultured NHBEs (*A*) and HMVEC-LBl (*B*) were transfected with siRNA against ST2 (solid bars) or nontargeting control siRNA (open bars) at 5 nM (NHBEs) or 10 nM (HMVEC-LBl). The transfected cells were further cultured for 48 h and then stimulated with 10 ng/ml IL-33 for 6 h. The levels of mRNA for ST2L, sST2, IL-8, and IL-6 were determined by real-time PCR. Data are shown as the mean ± SD of triplicate samples and are representative of two individual experiments. $*p < 0.05$; $**p < 0.01$ compared with nontargeting control siRNA. *C*, IL-33–mediated responses in cultured HMVEC-LBl were IL-33 specific. HMVEC-LBl were stimulated in the presence of 10 ng/ml IL-33 and 10 μg/ml neutralizing ST2-Fc chimera for 24 h. Protein concentrations in the culture supernatants are shown. Data are shown as the mean ± SD of triplicate samples and are representative of two individual experiments. $**p < 0.01$ compared with 10 ng/ml IL-33.

IL-33 robustly enhanced IL-8 production in the absence of corticosteroid.

IL-33–induced phosphorylation of MAPK in epithelial and microvascular endothelial cells

We next sought to evaluate the signaling pathways involved in the IL-33 responses in both NHBEs and HMVEC-LBl. Although the signaling pathways activated by IL-33 remain poorly understood, it was reported that IL-33–mediated IL-8 production by human mast cells is mediated by a signaling pathway involving p38 MAPK (8). Therefore, we investigated whether IL-33 induces phosphorylation of MAPK, including ERK and p38, in NHBEs and HMVEC-LBl. In HMVEC-LBl, transient phosphorylation of both ERK and p38 was observed after 5 to 15 min treatment with IL-33 (Fig. 6). In contrast, in NHBEs, phosphorylation of ERK was observed for up to 60 min of treatment with IL-33, whereas constitutive phosphorylation of p38 was unaffected.

Effects of ERK and p38 MAPK inhibitors on IL-33–mediated responses in microvascular endothelial cells and epithelial cells

To verify which MAPK was involved in the IL-33–mediated responses in HMVEC-LBl, the cells were treated with various concentrations of ERK inhibitor PD98059 or p38 inhibitor SB202190 for 30 min prior to treatment with IL-33. IL-33–mediated productions of IL-8, IL-6, and MCP-1 were dramatically and dose-dependently reduced by the addition of p38 inhibitor SB202190 but not by ERK inhibitor PD98059 (Fig. 7*A*). These results clearly indicate that p38 MAPK is required for IL-33–mediated responses in HMVEC-LBl.

In contrast, IL-33–mediated production of IL-8 by NHBEs was significantly reduced by the addition of ERK inhibitor PD98059, but not by p38 inhibitor SB202190 (Fig. 7*B*). This indicates that, conversely from HMVEC-LBl, ERK is required for IL-33–mediated IL-8 production by NHBEs.

Discussion

In this study, we found that IL-33, a pro-Th2 cytokine, acts directly on pulmonary microvascular endothelial cells and epithelial cells and mediates inflammatory responses.

First, our investigation of lung tissue cells found that both ST2L and sST2 were preferentially expressed in microvascular endothelial cells (HMVEC-LBl) and airway epithelial cells (NHBEs) but not in either lung fibroblasts (NHLFs) or smooth muscle cells (BSMCs) (Fig. 1*A*). Furthermore, there was good correspondence between the ST2 distribution (Fig. 1*A*) and IL-33 responsiveness (Fig. 1*B*) among these lung tissue cells. This suggests that IL-33 mediates its actions via the ST2 receptor on both HMVEC-LBl and NHBEs. Indeed, depletion of ST2 mRNA significantly reduced the IL-33–mediated responses of these cells (Fig. 2).

Neither NHLFs nor BSMCs showed any expression of ST2 (Fig. 1*A*) or responsiveness to IL-33 (Fig. 1*B*), suggesting that

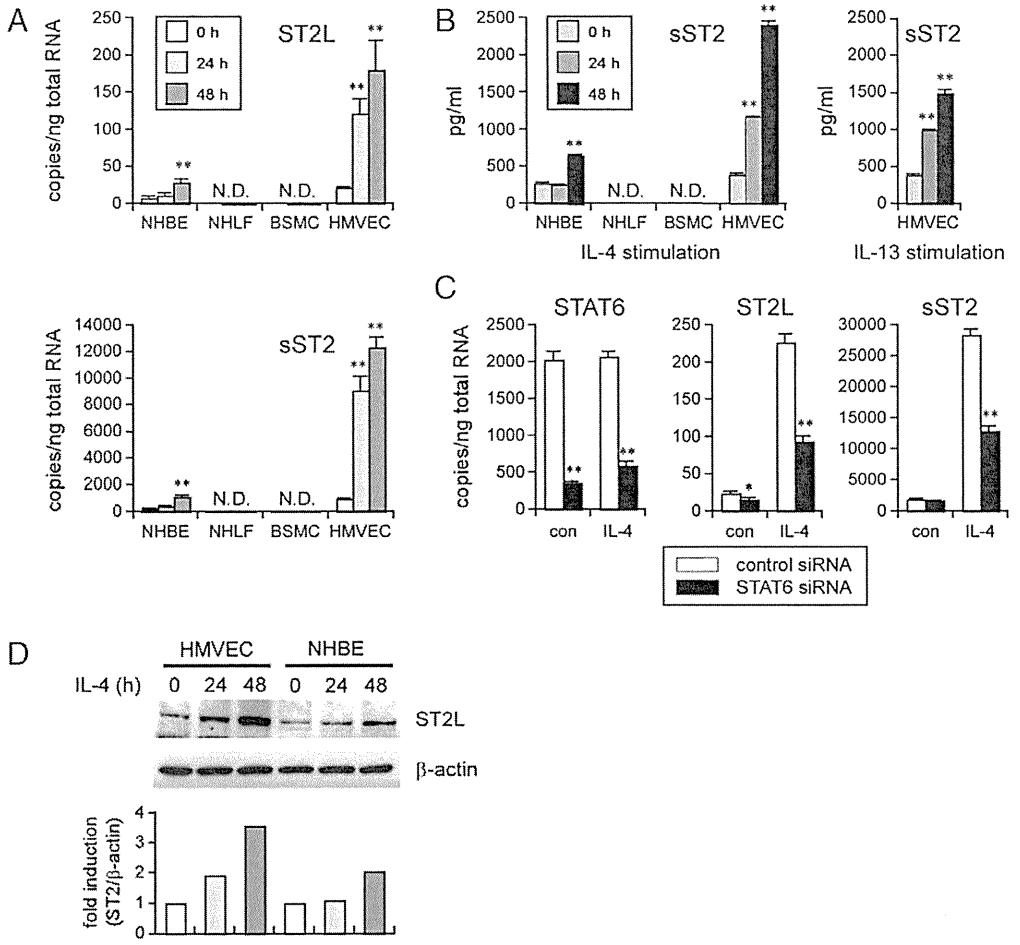

FIGURE 3. Effects of Th2 cytokines on the expression of ST2 in cultured lung tissue cells. Cells were treated with 10 ng/ml IL-4 for the indicated periods. *A*, The levels of mRNA for ST2L and sST2 are shown. *B*, The accumulated sST2 protein levels after 10 ng/ml IL-4 treatment (*left graph*) or 10 ng/ml IL-13 treatment (*right graph*) for the indicated periods are shown. $^{**}p < 0.01$ compared with no cytokine treatment (0 h). *C*, STAT6 is required for IL-4–enhanced expression of ST2 in HMVEC-LBl. Cultured HMVEC-LBl were transfected with siRNA against STAT6 (solid bars) or nontargeting control siRNA (open bars). The transfected cells were further cultured for 48 h and then stimulated with 10 ng/ml IL-4 for 24 h. The levels of mRNA for STAT6, ST2L, and sST2 were determined by real-time PCR. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with nontargeting control siRNA. *D*, Whole-cell lysates from IL-4–stimulated HMVEC-LBl and NHBEs were harvested, and ST2L and β-actin were analyzed by Western blotting. The fold induction of ST2L protein was determined by densitometry and normalized to the respective β-actin level (*lower graph*). Data are shown as the mean ± SD of triplicate samples (*A–C*) and are representative of three (*A*, *B*) or two (*C*, *D*) individual experiments.

IL-33 does not act directly on these cells in the asthmatic airway. Of note, ST2 was preferentially expressed in vascular endothelial cells, including HUVECs and HCAECs (Fig. 1*A*). These observations are consistent with recent reports of sST2 secretion by human venous and arterial endothelial cells (20, 21).

IL-33 drives production of Th2-associated cytokines, including IL-4, IL-5, and IL-13, by various hematopoietic cells (1, 3, 4, 8–10). Unlike in those hematopoietic cells, IL-33–mediated cytokine-chemokine production by the lung tissue cells was rather limited (Fig. 1*B*), and we found no production of Th2-associated cytokines (data not shown). It was recently reported that the ST2/IL-33 pathway is necessary not only for the development of an allergic inflammatory response but also for its maintenance (22). Thus, the actions of IL-33 on lung tissue cells may not contribute to the development of allergic inflammation but rather to the maintenance of chronic inflammation. It should be noted that Th2 cytokines, such as IL-4, significantly enhanced ST2 expression (Fig. 3) and function (Fig. 4) in both lung endothelial and epithelial cells. These findings are important when considering chronic inflammation in the lung and suggest that allergic individuals may be more susceptible to IL-33–mediated inflammatory responses of lung tissue cells than nonallergic individuals. Aoki et al. (21) recently reported that IL-33 stimulated secretion of IL-6 and IL-8 by HUVECs. Notably, they showed that ST2 gene expression in HUVECs was growth-dependent and was downregulated when the cells were differentiated to form vascular structures on an extracellular membrane matrix in vitro, whereas vascular endothelial growth factor gene expression was not downregulated. These results suggest that blood vessels normally would not respond to IL-33. In contrast, Th2-inflamed lung blood vessels and/or epithelium seem to be potential targets for the actions of IL-33.

Although the results of this study were limited to in vitro experiments, several reports by others have shed light on the in vivo roles of IL-33 by exogenous administration of recombinant IL-33 to mice (1, 23–25) or by transgenic overexpression of IL-33 in mice (26). Those studies have independently provided evidence that excessive expression of IL-33 in vivo might lead to an increase in the number of inflammatory cells in the airway via release of endogenous Th2 cytokines and chemokines. Notably, Zhiguang et al. (26) showed that pulmonary inflammation with infiltration of inflammatory cells was observed around the blood vessels in the airway of IL-33–transgenic mice, supporting our conclusion from this study that pulmonary endothelial cells can be direct targets of IL-33. Furthermore, both administration and

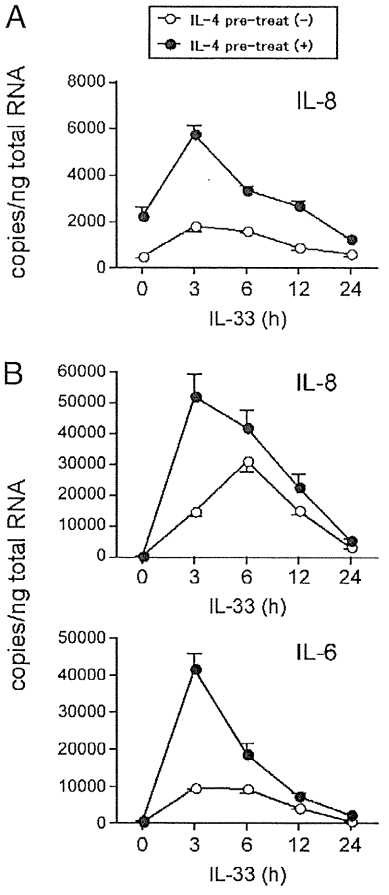

FIGURE 4. IL-33–mediated responses were further enhanced by IL-4 pretreatment of NHBEs and HMVEC-LBl. Cultured NHBEs (*A*) or HMVEC-LBl (*B*) were treated with 10 ng/ml IL-4 for 48 h, and then the cells were washed twice with HBSS and replaced with fresh medium containing 10 ng/ml IL-33 for the indicated periods. The levels of mRNA for IL-8 and IL-6 were determined by real-time PCR. Data are shown as the mean ± SD of triplicate samples and are representative of three individual experiments.

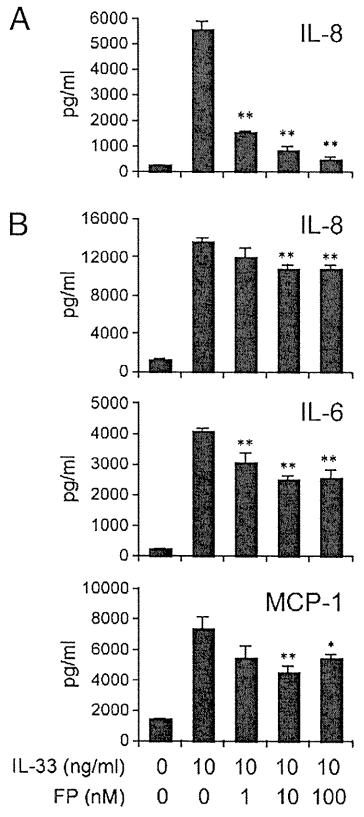

FIGURE 5. Effects of corticosteroid on IL-33–mediated responses in NHBEs and HMVEC-LBl. Cultured NHBEs (*A*) and HMVEC-LBl (*B*) were simultaneously treated with IL-33 and FP for 24 h at the indicated concentrations. Concentrations of IL-8, IL-6, and MCP-1 in the culture supernatants are shown. Data are shown as the mean ± SD of triplicate samples and are representative of at least three individual experiments. $*p < 0.05$; $**p < 0.01$ compared with 10 ng/ml IL-33.

transgenic overexpression of IL-33 in mice led to increased numbers of neutrophils as well as eosinophils in the airway (23, 25, 26). Neutrophils are not regarded as direct target cells of IL-33 because they have few ST2 receptors on their surface. Therefore, we surmise that IL-33 can promote neutrophil infiltration in the airway through IL-33–induced release of neutrophil chemoattractants, including IL-8 family members, by lung tissue cells.

Today, inhaled corticosteroids are a first-line anti-inflammatory treatment and known to be one of the most effective therapies available for asthma (19). Indeed, FP treatment showed significant attenuation of IL-33–mediated IL-8 production by NHBEs even at a low FP concentration (1 nM), and that production was almost completely abrogated by 100 nM FP treatment (Fig. 5*A*), suggesting that corticosteroids are capable of effectively reducing IL-33–mediated airway epithelial inflammation. In contrast, FP treatment was only partially effective against IL-33's actions on microvascular endothelial cells (Fig. 5*B*), which were found to be the main IL-33–targeted cells among the lung tissue cells (Fig. 1*B*). We recently showed that corticosteroid treatment was also only weakly effective on TNF-α–mediated microvascular inflammation, including chemokine production (16). In addition, corticosteroid enhanced TNF-α–mediated leukocyte adhesion to pulmonary microvascular endothelial cells via upregulation of cell-surface expression of ICAM-1 and VCAM-1 (16). Taken together, those various findings suggest that the poor effect of corticosteroid on TNF-α/IL-33–mediated inflammatory responses is not only a specific feature of airway microvessels but is also crucially involved in the refractoriness seen in the asthmatics. Notably, Préfontaine et al. (13) recently demonstrated that dexamethasone fails to abolish TNF-α–induced IL-33 upregulation in primary human airway smooth muscle cells, further suggesting a contribution of IL-33 as well as TNF-α to the refractory phenotype of certain asthmatics treated with corticosteroids.

Persistent chronic inflammation in the lung leads to structural alterations in the airway wall (i.e., airway remodeling), which is thought to cause irreversible airflow obstruction and exacerbation of asthma (15). Recent compelling evidence has demonstrated that

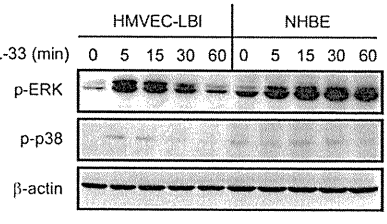

FIGURE 6. IL-33–induced phosphorylation of MAPK in cultured HMVEC-LBl and NHBEs. Whole-cell lysates were examined at the indicated time points after stimulation with 10 ng/ml IL-33 for the expression of phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), and β-actin (as a loading control).

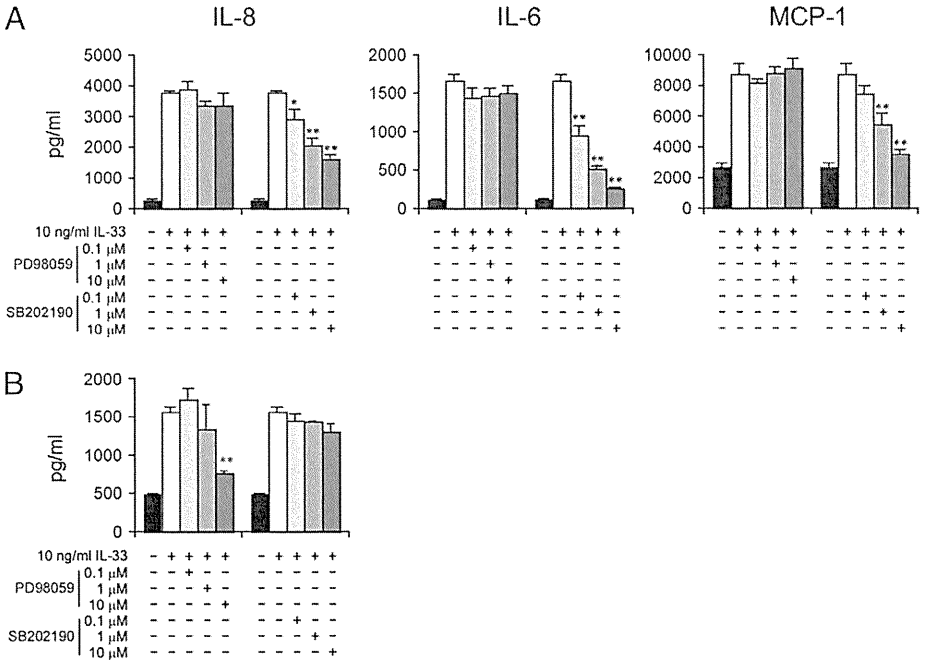

FIGURE 7. Divergent effects of ERK inhibitor (PD98059) and p38 MAPK inhibitor (SB202190) on IL-33–mediated responses in HMVEC-LBl and NHBEs. Cultured HMVEC-LBl (*A*) and NHBEs (*B*) were treated with the indicated concentrations of PD98059 or SB202190 for 30 min prior to stimulation with 10 ng/ml IL-33 for 24 h. Data are shown as the mean ± SD of triplicate samples and are representative of three individual experiments. $*p < 0.05$; $**p < 0.01$ compared with 10 ng/ml IL-33.

airway hypervascularity in severe asthma, an element of airway remodeling resulting from accelerated angiogenesis, responds poorly to corticosteroid treatment and is clinically involved in reduced lung function (27–29). We previously showed that autocrine CXCR2 chemokines, such as IL-8, are indispensable for lung angiogenesis in a corticosteroid-insensitive manner (16, 17, 30). As shown in our current study, IL-33 can induce IL-8 production by pulmonary endothelial and epithelial cells (Fig. 1*B*), suggesting that IL-33 is involved in lung angiogenesis and the resultant airway hypervascularity. As a matter of fact, Choi et al. (31) recently demonstrated that IL-33 promotes angiogenesis and vascular permeability by stimulating endothelial NO production via the ST2 receptor.

As shown in Fig. 1*B*, both IL-6 and MCP-1, which were also secreted by IL-33–stimulated pulmonary microvascular endothelial cells, are known to be critically involved in allergic inflammation (32–34). Therefore, these proinflammatory mediators originating from IL-33–stimulated pulmonary microvessels may also play roles in the maintenance of chronic allergic inflammation of the asthmatic airway.

Although IL-33/ST2 signaling pathways remain poorly understood, it could be expected that the signaling molecules are similar to those for other IL-1 family cytokines. Indeed, it was reported that IL-33, as well as IL-1β, can enhance MAPK (ERK and p38) phosphorylation in both murine (1) and human (8) mast cells. We confirmed that IL-33 can activate MAPK (ERK and p38) phosphorylation in HMVEC-LBl (Fig. 6). Moreover, as was reported for IL-33–induced IL-8 production by human mast cells (8), IL-33–induced production of each of IL-8, IL-6, and MCP-1 by HMVEC-LBl was dramatically and dose-dependently reduced by treatment with a p38 inhibitor, SB202190, but not with an ERK inhibitor, PD98059 (Fig. 7*A*). This suggests that the IL-33–mediated signaling pathway in human microvascular endothelial cells is similar to that in human mast cells. In contrast, ERK, but not p38, is required for IL-33–mediated IL-8 production by NHBEs (Fig. 7*B*). Because ST2L mRNA remained at a lower level in NHBEs than in HMVEC-LBl (Fig. 1*A*), we initially considered that NHBEs respond only partially to IL-33 and produce only IL-8 (not IL-6 or MCP-1) (Fig. 1*B*) simply due to a smaller number of ST2 protein molecules on the surface of NHBEs compared with that of HMVEC-LBl. However, as described above, we found a distinct difference between these cells in their requirements for MAPK in the IL-33–mediated signaling pathway. These mechanistic differences between HMVEC-LBl and NHBEs in their IL-33–mediated signaling pathways should be further elucidated. Nevertheless, these observations suggest that, contrary to our initial expectation, IL-33–mediated responses in NHBEs use signal transduction pathways that are distinct from the pathways in HMVEC-LBl and human mast cells.

The main sources of IL-33 involved in the pathogenesis of asthma remain controversial. IL-33 was originally identified as NF-high endothelial venules, which is an NF preferentially expressed in high endothelial venules (35). The same group also reported that endothelial cells constitute a major source of IL-33 mRNA in chronically inflamed tissues from patients with rheumatoid arthritis and Crohn's disease (36). Furthermore, they showed abundant nuclear expression of IL-33 in endothelial cells from both large and small blood vessels in most normal human tissues (37), suggesting that endothelial cells constitute major sources of IL-33 in vivo. Indeed, we also confirmed expression of IL-33 mRNA and protein in whole-cell lysates, but not culture supernatants, of HMVEC-LBl by real-time PCR and ELISA, respectively (data not shown). Although further studies are clearly needed, we speculate that endogenous IL-33 released from inflamed and/or injured blood vessels acts on neighboring vessels as an endogenous "danger signal" (37), leading to chronic inflammatory responses. Notably, release of the IL-33 "danger signal" by damaged/injured endothelial cells has recently been demonstrated (38), lending further support to the endogenous "danger signal" hypothesis.

In conclusion, IL-33, a pro-Th2 cytokine, acts directly on pulmonary microvascular endothelial cells and epithelial cells (among lung tissue cells), which express its ST2 receptor. Importantly, Th2 cytokines significantly enhanced ST2 expression and function in both endothelial and epithelial cells. Furthermore, the responses of those cells, especially microvascular endothelial cells, to IL-33 are almost refractory to corticosteroid treatment, and we thus anticipate that IL-33 and/or its receptor, ST2, may be able to be exploited as a novel target for development of curative drugs for refractory asthma.

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Disclosures

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Notch signaling confers antigen-presenting cell functions on mast cells

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Background: Notch signaling is involved in cell fate determination along with the development of the immune system. However, very little is known about the role for Notch signaling in mast cells.

Objective: We investigated the role of Notch signaling in mast cell functions.

Methods: After mouse bone marrow–derived mast cells (BMMCs) or peritoneal mast cells (PMCs) were cocultured with mouse Notch ligand-expressing chinese hamster ovary cells for 5 days, we examined the mast cell surface expressions of MHC-II molecules and OX40 ligand (OX40L), FcεRI-mediated cytokine production, and the effects of the mast cells on proliferation and differentiation of naive $CD4^+$ T cells *in vitro*.

Results: We showed that BMMCs and PMCs constitutively expressed Notch1 and Notch2 proteins on the cell surface. We also found that Delta-like 1 (Dll1)/Notch signaling induced the expression of MHC-II and upregulated the expression level of OX40L on the surface of the mast cells. Dll1/Notch signaling augmented FcεRI-mediated IL-4, IL-6, IL-13, and TNF production by BMMCs. Dll1-stimulated MHC-II^+ $OX40L^{high}$ BMMCs promoted proliferation of naive $CD4^+$ T cells and their differentiation into T_H2 cells producing IL-4, IL-5, IL-10, and IL-13.

Conclusion: Dll1/Notch signaling confers the functions as an antigen-presenting cell on mast cells, which preferentially induce the differentiation of T_H2. (J Allergy Clin Immunol 2009;123:74-81.)

Key words: *Mast cells, Notch, MHC class II, OX40 ligand, antigen presentation*

Abbreviations used
APC: Antigen-presenting cell
BMDC: Bone marrow–derived cultured dendritic cell
BMMC: Bone marrow–derived cultured mast cell
cysLT: Cysteinyl leukotriene
DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester
Dll: Delta-like
Jag: Jagged
LT: Leukotriene
OX40L: OX40 ligand
PMC: Peritoneal mast cell

Mast cells are widely recognized as critical effector cells in allergic disorders and other IgE-dependent immune responses. Recently, numerous studies revealed that mast cells play a protective role in host defense against bacteria through the production of some cytokines as a result of Toll-like receptors-mediated activation.[1-3] Therefore, mast cells are also important as initiators and effectors of innate immunity. In addition, some studies indicate that mast cells contribute to the sensitization phase of acquired immune responses and directly or indirectly regulate T-cell differentiation and function.[4,5] Mast cells are involved in the development of Tcell–mediated hypersensitivity disorders, such as delayed-type contact hypersensitivity,[6] asthma,[7] rheumatoid arthritis,[8] inflammatory bowel disease,[9] and multiple sclerosis.[10,11] Mast cell–derived factor, including TNF-α, contributes to the microenvironment that primes naive T cells for polarized differentiation,[12,13] indicating that mast cells indirectly influence T-cell responses through cytokine production. In contrast, the direct mechanisms of mast cell–T cell interactions have not been fully resolved. Mast cells migrate to the spleen and lymph nodes under inflammatory conditions[14,15] and express some costimulatory or inhibitory molecules on the cell surface.[16,17] Although these results suggest that mast cells stimulate T cells through direct interaction, inconsistent observations concerning the expression of MHC-II on mast cells have been reported. In brief, recent studies reported that MHC-II molecules are not detected on mast cells,[17,18] whereas constitutive expression of MHC-II intracellularly[19] or on the cell surface[20-22] was reported in previous studies. Therefore, it is still controversial whether mast cells are able to regulate T-cell differentiation and function directly as antigen-presenting cells (APCs). We have previously reported that mast cells acquire the expression of MHC-II by overexpression of a transcription factor PU.1.[23,24] This result indicates that mast cells express MHC-II on the cell surface under some circumstances. Considering a report that Notch signaling upregulates PU.1 expression in immature hematopoietic progenitor cells,[25] the Notch ligand is one of the candidates responsible for induction of APC-related gene expression in mast cells.

Notch proteins are epidermal growth factor–like transmembrane receptors. In mammals, 4 Notch genes, *Notch 1, 2, 3,* and *4*, and 5 ligands, Jagged (Jag)–1 (Jag1), Jag2, Delta-like (Dll)–1, Dll3, and Dll4, have been identified. Dll3 protein is predominantly detected in the intracellular region, including in the Golgi network, although the other ligand proteins are detected on the cell surface.[26] Notch signaling regulates various cell fate determinations, such as myogenesis,[27] neurogenesis,[28] gliogenesis,[29] and lymphocyte development.[30,31] Although Jönsson et al[32] previously reported that

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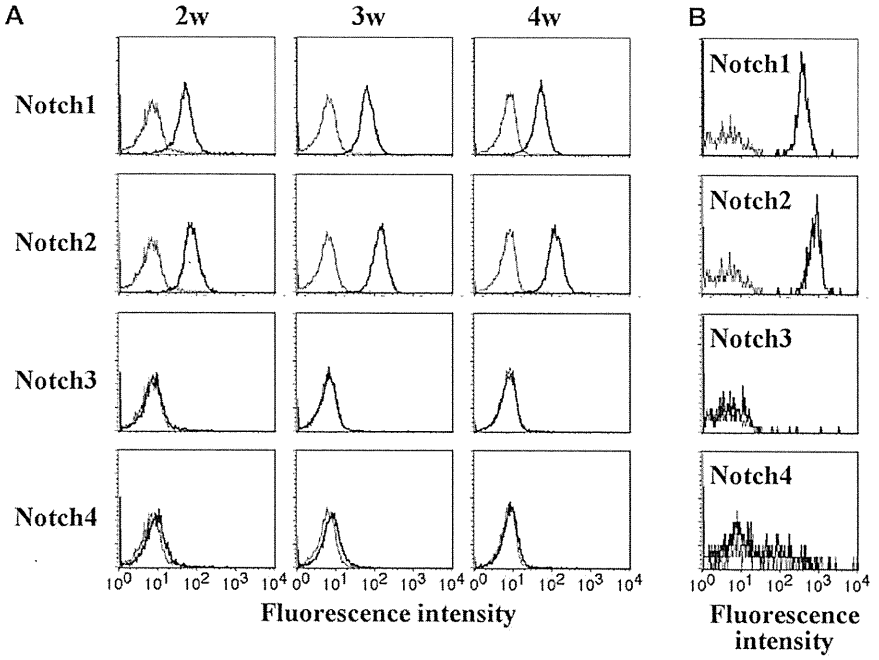

FIG 1. Cell surface expression of Notch receptors on mast cells. The expressions of Notch 1, 2, 3, or 4 *(bold lines)* on c-Kit$^+$FcεRIα$^+$ BMMCs cultured for 2, 3, or 4 weeks **(A)** or fresh c-Kit$^+$FcεRIα$^+$ PMCs **(B)** were analyzed by flow cytometry. *Thin lines* represent isotype-matched control. A representative of 3 repeated experiments with similar results is shown.

murine mast cells express mRNAs for *Notch1* and *Notch2*, the expression of Notch proteins on mast cell surfaces and the role for Notch signaling in mast cells have not been determined.

In this study, we showed that mouse bone marrow-derived mast cells (BMMCs) and peritoneal mast cells (PMCs) constitutively expressed Notch1 and Notch2 proteins on the cell surface and that the BMMCs acquired the APC function by Dll1/Notch signaling. These findings suggest that mast cells play an important role in adaptive immunity through direct cross-talk with T cells.

METHODS

Mice

Wild-type BALB/c mice and ovalbumin-specific T-cell receptor transgenic mice on the BALB/c background, clone DO11.10, which recognizes the 323–339 peptide fragment of ovalbumin,[33] were purchased from Japan SLC (Hamamatsu, Japan) and The Jackson Laboratories (Bar Harbor, Me), respectively, and maintained in the specific pathogen-free animal facility at Juntendo University. All animal experiments were performed according to the approved manual of the Institutional Review Board of Juntendo University, Tokyo, Japan.

Preparation of mast cells

A protocol is described in the Methods section in the Online Repository at www.jacionline.org.

Coculture of BMMCs with CHO cell lines expressing Notch ligands

The generation of mouse Notch ligand-expressing CHO cell lines (CHO-Jag1, CHO-Jag2, CHO-Dll1, and CHO-Dll4) was described previously.[34,35] The CHO cells were seeded at a density of 6×10^3 cells/cm^2 in plates and cultured for 1 hour, and then bone marrow cells cultured for 2 weeks were placed at a density of 1.2×10^5 cells/cm^2 into the plates and cultured for 5 days in MEM Alpha (Invitrogen, Carlsbad, Calif) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μmol/L 2-mercaptoethanol, 10 mmol/L sodium pyruvate, 10 μmol/L minimal essential medium nonessential amino acid solution, 100 U/mL recombinant murine IL-3, and 0.5 U/mL recombinant murine stem cell factor. A γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT; Sigma-Aldrich, St Louis, Mo), was used at 10 μmol/L.

Flow-cytometric analysis

A protocol is described in the Methods section in the Online Repository.

Analyses of eicosanoid and cytokine production and degranulation of mast cells

A protocol is described in the Methods section in the Online Repository.

Preparation of bone marrow–derived cultured dendritic cells

A protocol is described in the Methods section in the Online Repository.

Mast cell or dendritic cell–CD4$^+$ T-cell coculture

Spleen cells of DO11.10 mice were prepared in single-cell suspensions, and CD4$^+$ T cells (purity >96%) were isolated by magnetic cell sorting using a CD4$^+$ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. As described, after 5 days of coculture with CHO control cells or CHO-Dll1 cells, mast cells were purified by magnetic cell sorting using a magnetic microbead-conjugated antimouse CD117/c-Kit mAb (Miltenyi Biotec), and then the cells were treated with medium alone or sensitized with IgE for 1 hour at 4°C. After IgE sensitization, BMMCs and bone marrow–derived cultured dendritic cells (BMDCs; 1×10^6 cells/mL) were treated with 50 μg/mL mitomycin C (MP Biomedicals, Solon, Ohio) for 30 minutes at 37°C. Mitomycin C treated-BMMCs or BMDCs (1×10^5 cells) were cocultured with 1×10^5 CD4$^+$ T cells in the BMMC culture medium with or without 2 μmol/L ovalbumin$_{323\text{-}339}$ peptide (Abgent, San Diego, Calif) in the presence or absence of 1 μg/mL antimouse IgE in round-bottomed 96-well culture plates. In some coculture experiments, 96-well culture plates equipped with a Transwell insert (Corning, Acton,

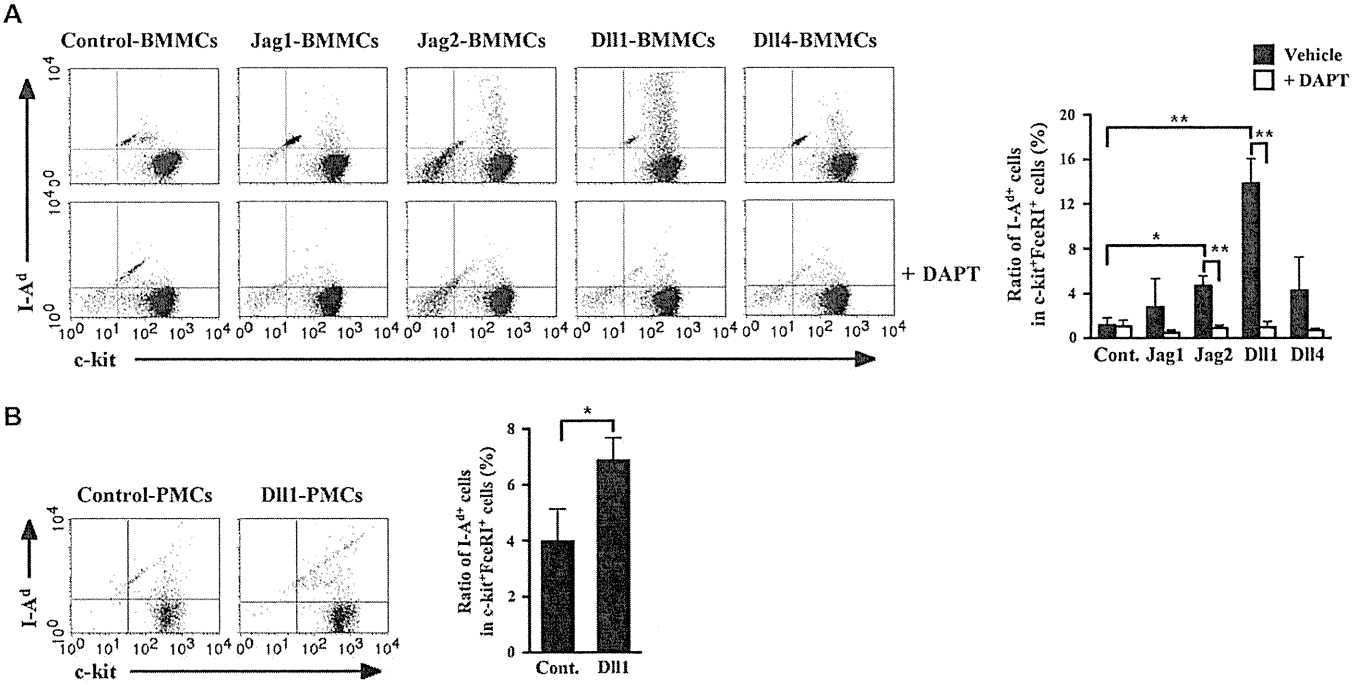

FIG 2. Notch signaling induces MHC-II expression on mast cell surface. BMMCs **(A)** or PMCs **(B)** were cocultured with the indicated CHO–Notch ligand–expressing cell lines or CHO control cells in the presence or absence of DAPT. *Cont.,* Control. *Dot blots* show the expressions of MHC-II (I-A) and c-Kit on FcεRIα$^+$ cells. Data in the graphs show the ratio of I-A^{d+} cells in c-Kit$^+$FcεRIα$^+$ cells, and are indicated as means ± SDs of quadruplicate samples. $*P < .05$; $**P < .005$, as determined by the Student *t* test.

Mass) were used to prevent direct contact of CD4$^+$ T cells and BMMCs or BMDCs. CD4$^+$ T cells were plated in the lower wells, and BMMCs or BMDCs were added to the upper wells in the same condition as described. After 48 hours of coculture, the concentration of IL-2 in the culture supernatants was determined using an ELISA kit (R&D Systems, Minneapolis, Minn). In some experiments, anti–OX40 ligand (OX40L) mAb (RM134L) at the indicated concentrations was added into the medium. T-cell proliferation was measured by pulsing for 16 hours with 1 μCi/well [^{3}H]thymidine (Amersham Bioscience, Uppsala, Sweden) and collected on day 3.

Analyses of cytokine production by T cells

A protocol is described in the Methods section in the Online Repository.

RESULTS

Mast cells express Notch receptors on cell surface

To determine whether Notch signaling is functional in mast cells, we first examined the expression of Notch receptors on mouse BMMCs and PMCs. Flow-cytometric analysis showed a high expression of both Notch1 and Notch2, but not Notch3, on 2-week-culture, 3-week-culture, and 4-week-culture BMMCs (Fig 1, *A*) and freshly prepared PMCs (Fig 1, *B*). Notch4 was expressed on 2-week-old and 3-week-old BMMCs at a low level (Fig 1, *A*), but not on 4-week-culture BMMCs and PMCs (Fig 1, *A and B*). These observations indicate that mouse mast cells constitutively express Notch1 and Notch2 on the cell surface throughout the developmental process.

Notch signaling induces MHC-II expression on mast cells

Because mast cells expressed Notch1 and Notch2, we then investigated the effect of Notch signaling on mast cells. Two-week-culture bone marrow cells or fresh PMCs were cocultured with Notch ligand–expressing CHO cells (CHO-Jag1, CHO-Jag2, CHO-Dll1, or CHO-Dll4) or control CHO cells for 5 days in the presence or absence of the γ-secretase inhibitor DAPT. Because Notch signaling is activated by nuclear localization of the intracellular domain of Notch, which is induced by γ-secretase cleavage, the γ-secretase inhibitor DAPT is able to block canonical Notch signaling.[36,37] The expression level of the Notch ligand on each CHO transfectant was confirmed to be comparable by flow-cytometric analysis (data not shown). As shown in Fig 2, a substantial population of I-A^d–expressing mast cells appeared after coculturing with Notch ligand–expressing CHO cells. In particular, the ratio of I-A^d–expressing cells was significantly increased in BMMCs cocultured with CHO-Jag2 (Jag2-BMMCs) and BMMCs cocultured with CHO-Dll1 (Dll1-BMMCs) compared with BMMCs cocultured with the control CHO cells (control-BMMCs), which was completely inhibited by the DAPT treatment (Fig 2, *A*). These observations indicate that the MHC-II expression on BMMCs was induced by activation of Notch signaling. When PMCs were cocultured with CHO-Dll1 (Dll1-PMCs), the ratio of I-A^d–expressing cells was also significantly increased compared with that of control-PMCs (Fig 2, *B*), indicating that Dll1/Notch signaling induces MHC-II expression on mature mast cells.

Notch signaling upregulates OX40L expression on mast cells

For T-cell activation by mast cells, OX40L plays an important role as a costimulatory molecule expressed on mast cells.[16,17] We therefore examined the effect of Notch signaling on OX40L expression in mast cells. As shown in Fig 3, *A*, OX40L was expressed on BMMCs cultured without CHO cells (noncocultured BMMCs) and control-BMMCs at a low level as previously

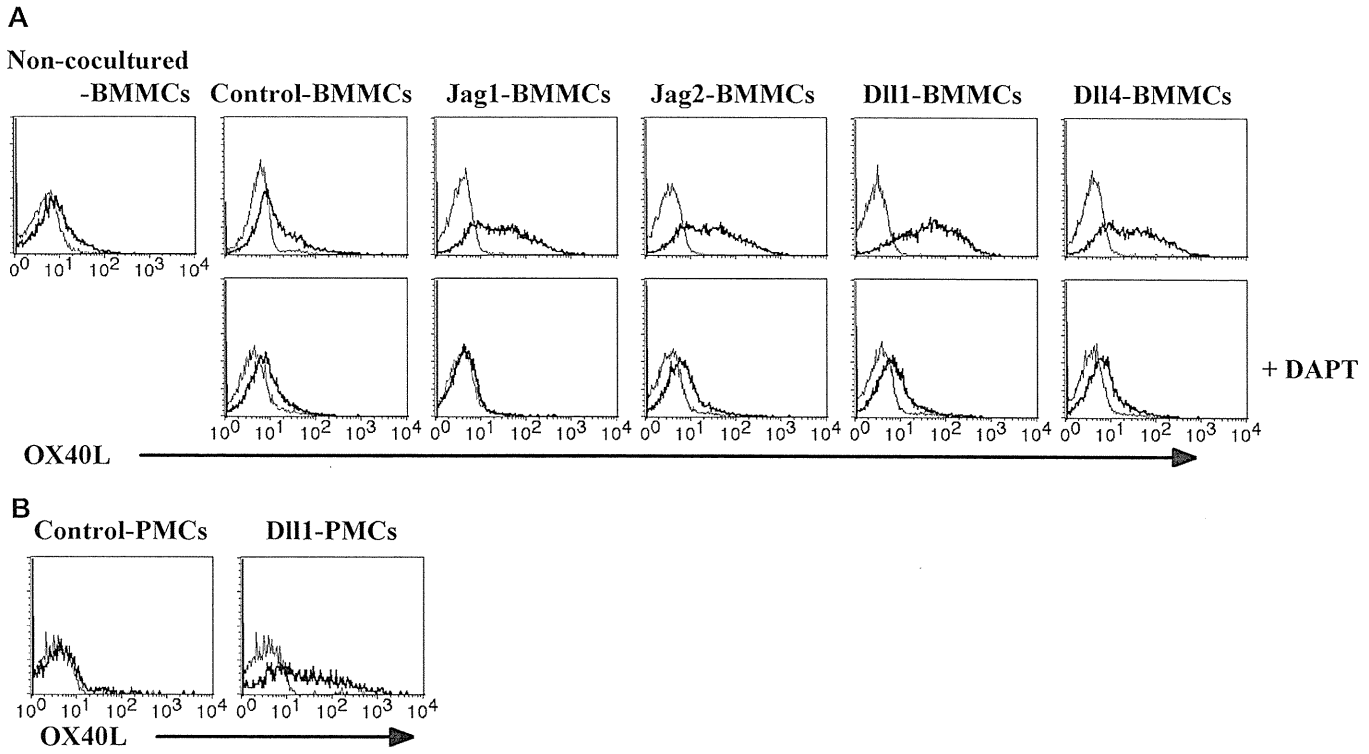

FIG 3. Notch signaling upregulates OX40L expression level on mast cell surface. BMMCs **(A)** or PMCs **(B)** were cocultured with the indicated CHO–Notch ligand—expressing cell lines or CHO control cells in the presence or absence of DAPT. The expression of OX40L *(bold lines)* on c-Kit$^+$FcεRIα$^+$ cells was analyzed by flow cytometry. *Thin lines* represent isotype-matched control. A representative of 3 repeated experiments with similar results is shown.

reported.[17] The OX40L expression on BMMCs was markedly upregulated by coculturing with Notch ligand–expressing CHO cells, and this upregulation was completely inhibited by the DAPT treatment. In particular, Dll1 most strongly increased the expression of OX40L on BMMCs. A similar upregulation of OX40L expression by Dll1 was also observed on PMCs (Fig 3, *B*). Expression of CD80 or CD86, which are major costimulatory molecules on DCs, was not detected on BMMCs under any conditions tested in this study (data not shown). These findings indicate that Notch ligands, particularly Dll1, upregulate OX40L expression on mast cells.

Dll1/Notch priming augments FcεRI-mediated cytokine production by BMMCs

Degranulation and production of cytokines and arachidonic acid–derived eicosanoids induced by cross-linking of FcεRI are important for mast cell–mediated allergic reactions. Thus, we investigated the response of Dll1-primed mast cells to FcεRI stimuli. The expression level of FcεRI on Dll1-BMMCs was almost comparable to that of control-BMMCs (Fig 4, *A*). As shown in Fig 4, *B*, the FcεRI cross-linking did not affect MHC-II expression on either Dll1-BMMCs or control-BMMCs. OX40L expression on control-BMMCs was enhanced by FcεRI cross-linking (Fig 4, *B*), as observed in a previous report.[17] The increased OX40L expression level on Dll1-BMMCs was further upregulated by FcεRI cross-linking (Fig 4, *B*). β-Hexosaminidase release and the production of cysteinyl leukotrienes (cysLTs), composed of leukotriene (LT) C_4, LTD_4, and LTE_4, were enhanced approximately 65% and 41% by priming BMMCs with Dll1, respectively, whereas the production of prostaglandin D_2 and LTB_4 were unchanged (Fig 4, *C, left columns*). The production of TNF-α, IL-4, IL-6, and IL-13 was markedly enhanced in Dll1-BMMCs compared with control-BMMCs (Fig 4, *C, right columns*). In particular, the production of IL-4, which was not detected in control-BMMCs, was significantly induced in Dll1-BMMCs. These observations indicate that Dll1/Notch priming augments the FcεRI-mediated cytokine production by BMMCs.

Dll1-BMMCs directly activate CD4$^+$ T cells

We found that Dll1/Notch signaling induced MHC-II expression (Fig 2) and upregulated OX40L expression on BMMCs (Fig 3). Therefore, to ascertain whether Dll1-BMMCs induce the activation of CD4$^+$ T cells, proliferation and IL-2 production by CD4$^+$ T cells were analyzed by *in vitro* coculture systems. As shown in Fig 5, *A*, ovalbumin$_{323–339}$ peptide-dependent T-cell proliferation was slightly enhanced by coculture with control-BMMCs. The proliferation was enhanced approximately 4.7-fold by priming BMMCs with Dll1, and was approximately 62% of the proliferation induced by LPS-treated BMDCs. T-cell proliferation induced by control-BMMCs was significantly enhanced by FcεRI cross-linking, whereas that induced by Dll1-BMMCs was not affected by the sensitization or FcεRI cross-linking. The enhancement of T-cell proliferation induced by Dll1-BMMCs or LPS-treated BMDCs was not observed when BMMCs or BMDCs were separated from T cells by a Transwell membrane (Fig 5, *B*), suggesting that the T-cell proliferation effect of BMMCs requires the interaction with T cells. IL-2 concentrations in the supernatants increased approximately 2-fold by Dll1 priming in an IgE-independent manner, but the concentrations were remarkably low compared with that induced by LPS-treated BMDCs (Fig 5, *C*). The addition of a neutralizing anti-OX40L mAb to the coculture significantly suppressed the

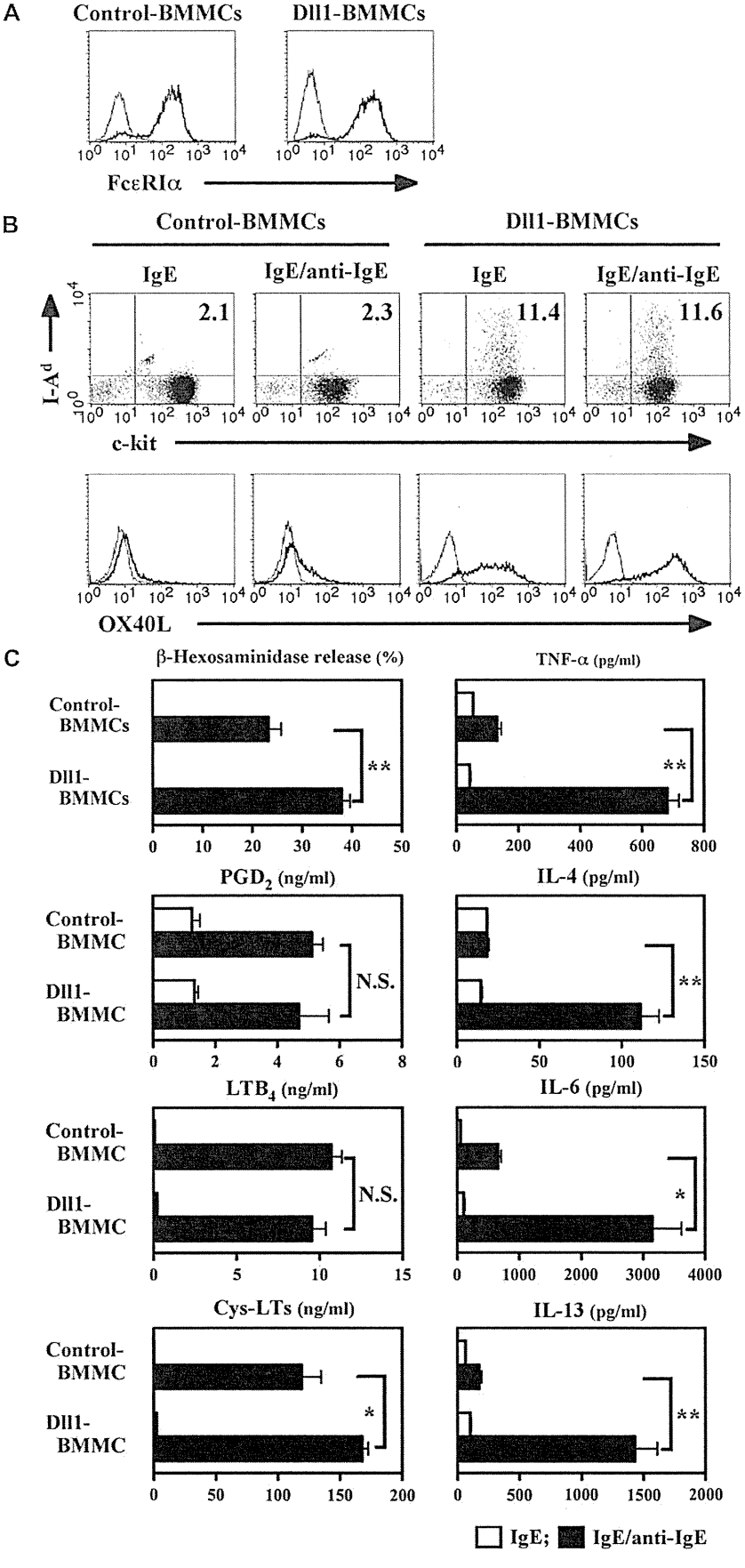

FIG 4. *(See next page for figure legend).*

T-cell proliferation induced by Dll1-BMMCs, whereas it did not affect the proliferation induced by control-BMMCs or LPS-treated BMDCs (Fig 5, *D*). The addition of isotype-matched control IgG (rat IgG_{2b}) did not affect these proliferations at any concentration (data not shown). These results indicate that Dll1-BMMCs induce $CD4^+$ T-cell proliferation in an IL-2–independent manner, which partly depends on costimulation by OX40L.

Dll1-BMMCs enhance IL-4, IL-10, and IL-13 production and suppress IFN-γ production by $CD4^+$ T cells

The OX40-OX40L interaction has been implicated in the triggering of T_H2 immune responses.[38,39] Therefore, we investigated whether MHC-II^+ Dll1-BMMCs induce T_H2 cells from naive $CD4^+$ T cells in the *in vitro* coculture systems. As shown in Fig 6, $CD4^+$ T cells primed by control-BMMCs produced higher levels of IFN-γ, IL-4, IL-13, and IL-17 ($P < .005$) and a similar level of TNF-α compared with those primed by BMDCs. The production of IL-5 and IL-10 was almost comparable to that from naive $CD4^+$ T cells. In response to Dll1-BMMCs, production of IL-4, IL-10, and IL-13 significantly increased, whereas IFN-γ production significantly decreased. These results suggest that MHC-II^+ Dll1-BMMCs induced the generation of T_H2 cells. FcεRI cross-linking resulted in significant decreases in the production of IFN-γ from $CD4^+$ T cells primed by control-BMMCs, and significant decreases in the production of IL-5 and IL-10 ($P < .05$) and an increase in IL-17 production ($P < .005$) from those primed by Dll1-BMMCs. These observations demonstrate that IgE-mediated activation of mast cells modulates the differentiation of $CD4^+$ T cells when they act as APCs.

DISCUSSION

In this study, we showed that Notch1 and Notch2 proteins were constitutively expressed on mouse mast cell surface, and Notch signaling induced the expression of MHC-II and OX40L on the mast cells, resulting in activation of $CD4^+$ T cells. This is the first report showing the expression and functions of Notch receptors in mast cells, although it has been reported that the expression of mRNAs for *Notch1* and *Notch2* was detected in murine mast cells.[32] Dll1 most remarkably increased the expression of MHC-II and OX40L on BMMCs, and all ligands markedly upregulated the OX40L expression level. Although Monsalve et al[40] have reported that the IFN-γ–induced expression of MHC-II proteins was increased by transfection of constitutively active intracellular domain of Notch1 ($Notch1^{IC}$) in mouse monocyte cell line RAW 264.7 cells, the detailed mechanisms by which the MHC-II expression increased by Notch signaling are uncertain. We observed that the cell surface expression of MHC-II and an increase in the PU.1 expression level were detected in BMMCs by transfection of $Notch1^{IC}$, but not $Notch2^{IC}$ (data not shown). These observations are consistent with our previous results that MHC-II expression was induced by overexpression of PU.1 in mast cells[23,24] and the report by Schroeder et al[25] that the transfection of $Notch1^{IC}$ led to increased PU.1 expression in immature hematopoietic progenitor cells. In the current study, the mRNA level of *Pu.1* in Dll1-BMMCs was similar to that in control-BMMCs, but the mRNA levels of *Gata-1* and *Gata-2* in Dll1-BMMCs were significantly lower than those in control-BMMCs (data not shown). Considering that GATAs inhibit the function of PU.1 and vice versa by forming an inactive PU.1/GATA-1 or GATA-2 complex,[41] MHC-II expression on Dll1-BMMCs may result from an increase in the active form of PU.1 brought about by decreases in GATAs. In this study, although the expressions of Notch1 and 2 on PMCs were almost the same level as that on BMMCs, PMCs primed with Dll1 was only a modest shift in MHC-II expression was observed in PMCs primed with Dll1-BMMCs (Figs 1 and 2). Considering the results in our previous study that overproduction of PU.1 induced MHC-II expression in BMMCs but not PMCs,[24] BMMCs may possess the higher capacity to express MHC-II compared with PMCs. From these observations, we assume that these discrepancies may reflect the difference in the expression profile of other transcription factors between BMMCs and PMCs. Further detailed analyses on downstream signaling of Notch are required for revealing the role for Notch signaling in mast cells.

On mast cells, Notch signaling affected not only the cell surface expression of MHC-II and OX40L, but also the cytokine production induced by FcεRI stimuli. Although the FcεRI expression level on the BMMC surface did not change by the Dll1-priming (Fig 4, *A*), the degranulation and production of cysLTs, which are eicosanoids, as early events in response to FcεRI cross-linking were modestly enhanced, and the production of TNF-α, IL-4, IL-6, and IL-13 as later events in response to FcεRI cross-linking was markedly enhanced (Fig 4, *C*). Considering that these cytokines and eicosanoids are involved in the initiation and maintenance of inflammatory reactions and the promotion of T_H2 cell differentiation, Dll1-mediated enhancement may accelerate priming of naive T cells for polarized T_H2 differentiation by affecting microenvironment conditions.

The mast cells primed with Dll1 exhibited MHC-II expression and higher OX40L expression, implying that the mast cells acquired the characters of APCs by Dll1/Notch signaling. Dll1-BMMCs induced the proliferation of naive $CD4^+$ T cells from DO11.10 mice in the presence of ovalbumin peptide (Fig 5, *A*). However, the mechanism of the $CD4^+$ T cells' activation by Dll1-BMMCs may be different from that by DCs, because Dll1-BMMCs expressed OX40L but not CD80 or CD86 (data not shown), and IL-2 production by $CD4^+$ T cells primed with Dll1-BMMCs was markedly lower than that by $CD4^+$ T cells primed with DCs (Fig 5, *B*). Neutralization by anti-OX40L mAb significantly but not completely inhibited the $CD4^+$ T-cell proliferation induced by Dll1-BMMCs, suggesting the presence of other factors regulating $CD4^+$ T-cell proliferation. Despite the lack of MHC-II on the cell surface, control-BMMCs induced slight proliferation of $CD4^+$ T cells (Fig 5, *A*). Therefore, a part of the $CD4^+$ T-cell proliferation might be a result of an indirect effect of the cytokines derived from BMMCs. The control-BMMCs promoted $CD4^+$ T cells to produce the T_H1 cytokine IFN-γ. In

FIG 4. Effects of Dll1/Notch signaling on FcεRI-mediated mast cell responses. **A,** FcεRIα expression *(bold lines)* on control-BMMCs or Dll1-BMMCs. **B,** The expressions of MHC-II and OX40L on control-BMMCs or Dll1-BMMCs in response to FcεRI cross-linking. *Dot blots* show the expressions of MHC-II (I-A) and c-Kit on $FcεRIα^+$ cells. Histograms show OX40L expression *(bold lines)* on $c\text{-}Kit^+FcεRIα^+$ cells. *Thin lines* represent isotype-matched control. A representative of 3 repeated experiments with similar results is shown. **C,** β-Hexosaminidase activity and production of eicosanoids (prostaglandin D_2, LTB_4, and cysLTs) and cytokines in control-BMMCs or Dll1-BMMCs in response to FcεRI cross-linking. Data are indicated as means ± SDs of triplicate samples. $*P < .05$; $**P < .005$; *N.S.*, not significant, as determined by the Student *t* test.

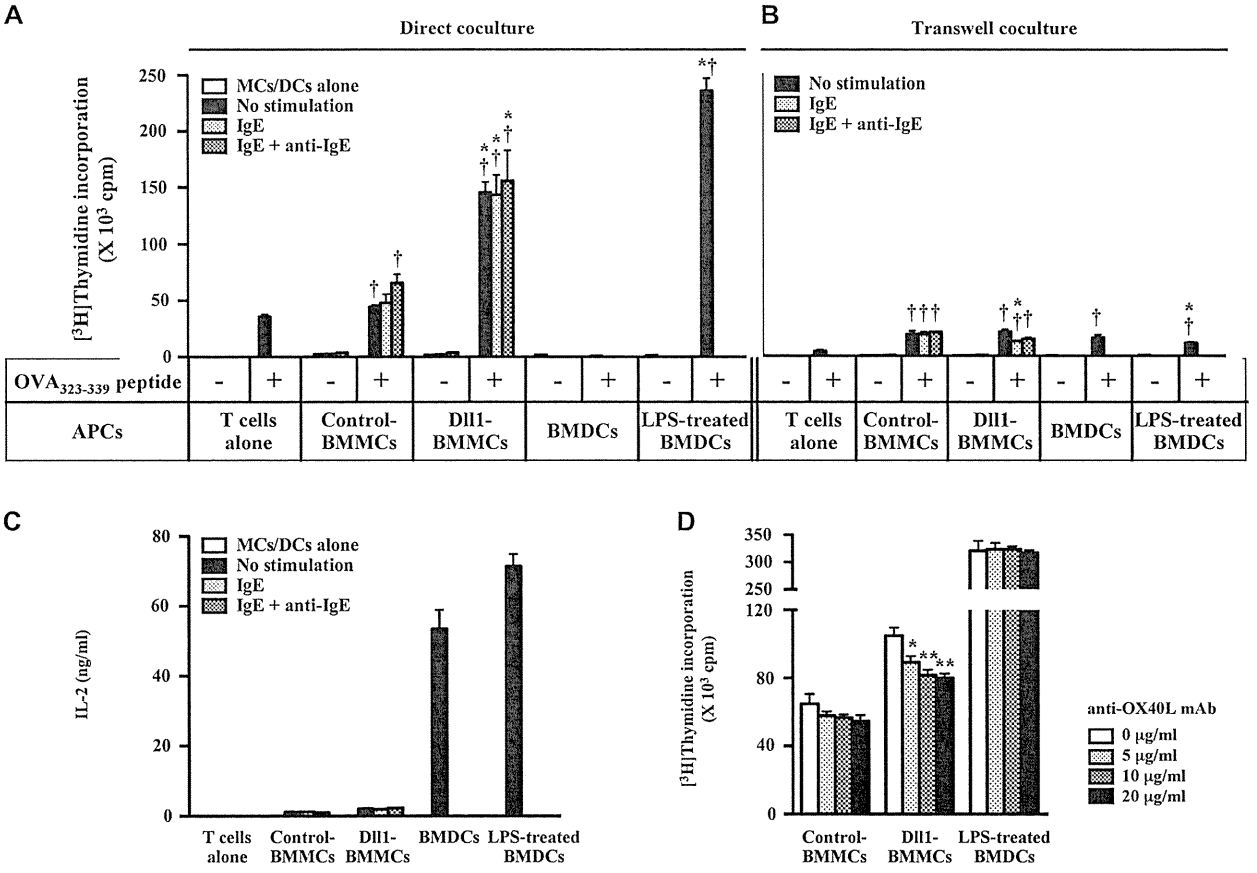

FIG 5. $CD4^+$ T-cell activation mediated by Dll1-BMMCs. [^{3}H]Thymidine incorporation into the $CD4^+$ T cells from DO11.10 mice cocultured together with BMMCs or BMDCs **(A)** or separated from them by a Transwell membrane **(B)** in the presence or absence of ovalbumin *(OVA)* peptide. Data are indicated as means ± SDs of triplicate samples. †$P < .05$ vs T cells alone in the presence of OVA peptide; *$P < .05$ vs corresponding values for T cells plus control-BMMCs. *MC,* Mast cell; *DC,* dendritic cell. **C,** IL-2 level in the supernatants when $CD4^+$ T cells from DO11.10 mice were direct cocultured with BMMCs or BMDCs at 48 hours in the presence of OVA peptide. Data are indicated as means ± SDs of triplicate samples. **D,** [^{3}H]Thymidine incorporation into the $CD4^+$ T cells from DO11.10 mice direct cocultured with BMMCs or BMDCs in the presence of OVA peptide and anti-OX40L neutralizing mAb at indicated concentrations. Data are indicated as means ± SDs of triplicate samples. *$P < .05$; **$P < .005$, significantly different as determined by the Student *t* test compared with the corresponding control.

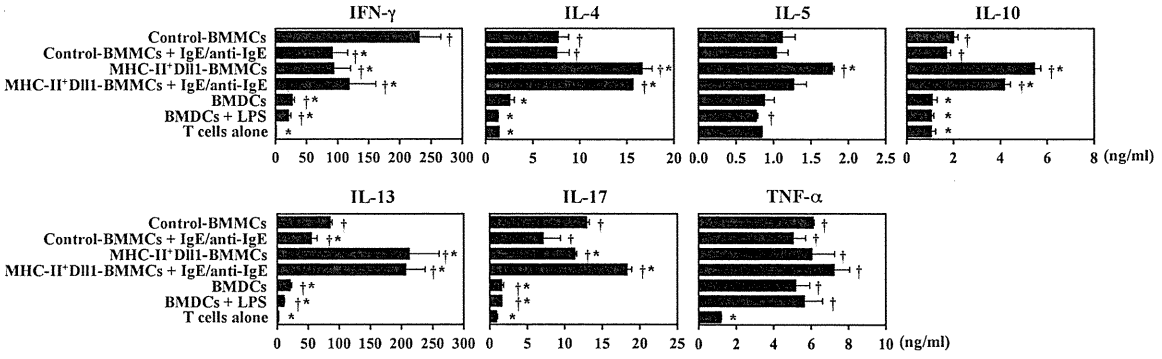

FIG 6. Dll1-BMMCs enhance IL-4, IL-10, and IL-13 production and suppress IFN-γ production by $CD4^+$ T cells. Purified $CD4^+$ T cells from DO11.10 mice were direct cocultured with BMMCs or BMDCs in the presence of ovalbumin peptide. After 4 days of coculture, the cocultured cells were restimulated with anti-CD3 mAb and anti-CD28 mAb for 24 hours, and then the cytokine concentrations in the supernatants were measured. Data are indicated as means ± SDs of triplicate samples. †$P < .05$ vs T cells alone; *$P < .05$ vs T cells plus control-BMMCs.

contrast, the direct interaction with MHC-II^+ Dll1-BMMCs promoted $CD4^+$ T cells to produce the T_H2 cytokines IL-4, IL-5, IL-10, and IL-13. The $CD4^+$ T cells primed by MHC-II^+ Dll1-BMMCs seem to have the characteristics of conventional T_H2 cells.[42] It has been reported that keratinocytes, dendritic cells, bone marrow stroma, and thymic epithelium express Notch ligands including Dll1.[43,44] Therefore, tissue-resident mast cells may be primed by Notch ligands expressed in their microenvironment. Mast cells then migrate to the spleen and lymph nodes under inflammatory conditions and influence the development and character of the immune response.[14,15] The proximity of mast cells and T cells in these organs allows mast cells to influence the priming of

naive T cells directly. Regardless, further *in vivo* analysis is required to clarify the roles of mast cells primed by Notch ligands.

Our findings in the current study suggest that Dll1/Notch signaling induces the commitment of mast cells to an APC population, which induce the differentiation of naive $CD4^+$ T cells toward conventional T_H2 cells. Further studies on the role of mast cells in the sensitization phase of acquired immune responses will contribute to the prevention and/or treatment of infectious diseases, autoimmune disorders, and allergic disorders.

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Clinical implications: The findings that mast cells acquire APC functions by Notch signaling and induce the differentiation of T_H2 will contribute to the elucidation of the pathogenic mechanism of allergic disorders.

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METHODS

Preparation of mast cells

Bone marrow–derived cultured mast cells were generated from the femoral bone marrow cells of BALB/c mice as described previously.[E1] Cells were incubated for 2 to 4 weeks in RPMI 1640 (Sigma-Aldrich, St Louis, Mo) supplemented with 10% heat-inactivated FCS (Invitrogen, Carlsbad, Calif), 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μmol/L 2-mercaptoethanol, 10 mmol/L sodium pyruvate, 10 μmol/L MEM nonessential amino acid solution (Invitrogen), 100 U/mL recombinant murine IL-3 (PeproTech EC, London, United Kingdom), and 0.5 U/mL recombinant murine stem cell factor (PeproTech EC). Peritoneal mast cells were isolated from whole peritoneal cells by density-gradient centrifugation techniques using metrizamide (Sigma-Aldrich) with >98% purity[E2] and were maintained in the BMMC-culture medium as described. Both mast cells were identified by flow-cytometric analysis of cell surface expression of c-Kit and high-affinity IgE receptor (FcεRI) α-chain.

Flow-cytometric analysis

Phycoerythrin-conjugated antimouse I-A^d mAb (AMS-32.1; BD Pharmingen, San Diego, Calif), OX40L mAb (RM134L; eBioscience, San Diego, Calif), FITC antimouse FcεRIα mAb (MAR-1; eBioscience), and phycoerythrin/Cy7 antimouse c-Kit mAb (2B8; BioLegend, San Diego, Calif) were used to stain each cell surface molecule after blocking Fc receptors with antimouse CD16/CD32 mAb (2.4G2; BD Pharmingen). To stain Notch receptors, biotin-conjugated mAbs against mouse Notch1 (HMN1-12), Notch2 (HMN2-35), Notch3 (HMN3-133), and Notch4 (HMN4-14) originally generated in our laboratory[E3] were used as the first antibody after blocking Fc receptors, and phycoerythrin-conjugated streptavidin (BD Pharmingen) was used in the second step of the staining. The expression of cell surface markers was analyzed on a FACSCalibur (BD Biosciences, San Jose, Calif).

Analyses of eicosanoid and cytokine production and degranulation of mast cells

After 5 days of coculture with CHO or CHO-Dll1 cells, mast cells (purity >98%) were purified by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) using a magnetic microbead-conjugated antimouse CD117/c-Kit mAb (Miltenyi Biotec) according to the manufacturer's instructions. The separated BMMCs were sensitized with 1 μg/mL mouse IgE (BD Pharmingen) for 1 hour at 4°C and then washed. IgE-sensitized cells were resuspended at a concentration of 1×10^6 cells/mL and then incubated in the presence or absence of 1 μg/mL antimouse IgE mAb (R35-72; BD Pharmingen) for 30 minutes in serum-free RPMI 1640 to determine eicosanoid production, and for 6 hours in the BMMC-culture medium to determine cytokine production, respectively. The levels of eicosanoids and cytokines in the culture supernatants were determined by using a corresponding enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, Mich) and ELISA kit (R&D Systems, Minneapolis, Minn), respectively. The degranulation levels of the separated BMMCs were measured by using the β-hexosaminidase release assay as previously described.[E4]

Preparation of BMDCs

Bone marrow–derived cultured dendritic cells were generated from the femoral bone marrow cells of BALB/c mice as described previously.[E5] Cells were incubated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μmol/L 2-mercaptoethanol, 10 mmol/L sodium pyruvate, 10 μmol/L MEM nonessential amino acid solution, and 200 U/mL recombinant murine granulocyte-macrophage colony-stimulating factor (PeproTech EC) in bacterial Petri dishes for 10 days. After 10 days of culture, nonadherent cells were collected, centrifuged, and resuspended in fresh medium containing 100 U/mL recombinant murine granulocyte-macrophage colony-stimulating factor into tissue culture plastic dishes. Cells were then cultured for an additional day in the presence or absence of 1 μg/mL LPS (Sigma-Aldrich). The cells were >92% I-$A^{d+}$$CD11c^+$ by flow-cytometric analysis (data not shown).

Analyses of cytokine production by T cells

After 5 days of coculture with CHO control cells or CHO-Dll1 cells, c-Kit^+ BMMCs (purity >98%) from CHO-cocultured cells and MHC-II^+ BMMCs (purity >98%) from CHO-Dll1-cocultured cells were purified by magnetic cell sorting using a magnetic microbead-conjugated mAb against mouse CD117/c-Kit and I-A (Miltenyi Biotec) according to the manufacturer's instructions, respectively, and then treated with mitomycin C. $CD4^+$ T cells (1×10^5) from DO11.10 mice were cocultured with 5×10^4 cells of the mitomycin C–treated BMMCs or BMDCs for 4 days, and then the mixture of primed $CD4^+$ T cells and BMMCs or BMDCs was collected. The collected cells at a concentration of 7.5×10^5 T cells/mL were stimulated with 2 μg/mL antimouse CD28 mAb (PV-1; Beckman Coulter, Fullerton, Calif) in antimouse CD3 mAb-coated 96-well culture plates (BD Biosciences) for 24 hours. The cytokine levels in the supernatants were measured by using a corresponding ELISA kit (all kits from R&D Systems).

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GATA2 and Sp1 Positively Regulate the c-*kit* Promoter in Mast Cells

Keiko Maeda, Chiharu Nishiyama, Hideoki Ogawa, and Ko Okumura

The c-*kit* gene is expressed in hematopoietic stem cells and lineage progenitor cells but is downregulated during cell development in most lineages, except for mast cells. In mast cells, high expression of c-*kit* is maintained during development, and c-Kit signaling is essential for mast cell development. To analyze the mechanisms by which c-*kit* gene expression are regulated in mast cells, we examined mast cell type-specific regulation of the c-*kit* promoter region. We observed that a GC-box in the c-*kit* promoter was critical for transcriptional activity and was bound to the transcription factor Sp1 as assessed using reporter assay and electrophoretic mobility assay. Chromatin immunoprecipitation assay and coexpression analyses showed that the transcription factor GATA2, which was recruited to the c-*kit* promoter in a mast cell-specific manner, in addition to Sp1, transactivated the c-*kit* promoter via the GC-box. Electrophoretic mobility assay and rechromatin immunoprecipitation assay indicated that GATA2 binds to the GC-box by forming a complex with Sp1. Introduction of Sp1 small interfering RNA significantly reduced the amount not only of Sp1 but also of GATA2 binding to the c-*kit* promoter in mast cells, resulting in suppression of c-*kit* transcription. Knockdown of GATA2 suppressed the recruitment of GATA2 toward the c-*kit* promoter, subsequently suppressing cell surface expression of c-Kit. These findings indicate that GATA2 and Sp1 play crucial roles in expression of the c-*kit* gene in mast cells. *The Journal of Immunology*, 2010, 185: 4252–4260.

The c-*kit* gene encodes a receptor tyrosine kinase that is essential for hematopoiesis and is known as stem cell factor (SCF) receptor and is one of the specific markers of mast cells. In the hematopoietic system, c-*kit* is expressed in stem cells and linage progenitor cells, but is downregulated upon terminal erythrocyte and monocyte/macrophage development. In mast cells, c-*kit* is expressed throughout the developmental pathway to mature mast cells from hematopoietic stem cells, including mast cell progenitors (Lin^-, c-Kit^+, $Sca1^-$, $Ly6c^-$, $Fc\varepsilon RI\alpha^-$, $CD27\text{-}b7^+$, $T1/ST2^+$) in mouse adult bone marrow (1), and basophil and mast cell bipotent progenitors (Lin^-, c-Kit^+, $Fc\gamma RII/III^+$, $\beta7^+$) in mouse spleen (2). The c-Kit/SCF signaling is essential for mast cell development in both mice and humans as follows. *W* and *SI* mice, which have mutations in the chromosomal loci encoding c-Kit and SCF, respectively, have severely decreased numbers of mast cells (3, 4), and numerous *W* mutations affecting c-Kit structure and function have been identified and characterized. In the *W* mutant *Wsh*, c-*kit* expression is diminished in hematopoietic progenitors in the bone marrow (BM) and lost in BM-derived mast cells (BMMCs) (5). Mastocytosis is characterized by accumulation of mast cells in various organs and release of mast cell mediators, caused by active mutations in the c-*kit* gene (6, 7). The D816V-mutated active variant of c-Kit, which is most frequently detected in patients with systemic mastocytosis, induces cluster formation and expression of several molecules involved in mast cell differentiation and adhesion, including microphthalmia transcription factor, IL-4 receptor, histamine, CD63, and ICAM-1 (8), demonstrating that c-Kit–mediated signaling is critical for differentiation and stimulation of mast cells. Therefore, determination of the mechanism of c-*kit* expression in mast cells will be useful for prevention and/or treatment of c-*kit*–mediated mast cell disorders.

The role of MITF as a transcription factor that regulates c-*kit* expression in mast cells has been extensively studied by Kitamura's group using *mi/mi* mice that carry a serious mutation in *Mitf* (9, 10). Their reports demonstrate that MITF is critical for the expression of c-*kit* in mast cells but not in erythroid precursors, testicular germ cells, or neurons. Their studies also suggest the presence of other unknown transcription factors that regulate c-*kit* expression in mast cells based on a number of observations including an increase in the proportion of c-Kit positive mast cells in *mi/mi* mice after birth.

The GATA family is comprised of six zinc-finger transcription factors, GATA1–6. Among them, GATA1, 2, and 3 are indispensable for hematopoiesis. GATA3 is considered to be essential for T cell differentiation from the earliest stages of development, and is a master regulator of Th2 differentiation (11, 12). In addition, GATA3 is upregulated by Notch2 signaling in myeloid progenitors, which facilitates development toward mast cell-producing progenitors (13). GATA1 is expressed in erythroid cells, megakaryocytes, eosinophils, and mast cells, and is involved in the development of these lineages (14–16). Furthermore, GATA1 regulates the expression of mast cell-specific molecules, including FcεRI α-, β-chains (17–20), and carboxypeptidase A genes (21). GATA2 is expressed at particularly high levels in progenitors of the several hematopoietic lineages, and is required for mast cell development but is dispensable for the terminal differentiation of erythroid cells and macrophages (22). *Gata2* knockout mice exhibit panhemapoietic defects and embryonic lethality (23), whereas enforced expression of GATA2 in mouse embryonic stem cells enhances proliferation of hematopoietic progenitors (24). In a

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Abbreviations used in this paper: BM, bone marrow; BMDC, BM-derived dendritic cell; BMMC, BM-derived mast cell; ChIP, chromatin immnoprecipitation; Ct, threshold cycle number; SCF, stem cell factor; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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mouse embryonic cells and OP9 stroma cell system, GATA2 not only inhibits macrophage differentiation, but also induces lineage distortion to megakaryocyte or erythroid cell differentiation by suppressing the expression and function of the transcription factor PU.1, depending on differentiation status (25). Both GATA1 and GATA2 are expressed in mast cells. Although GATA2 is dominantly expressed in immature cells (16), GATA1 is mainly expressed in mature cells. The repression of GATA1/2 leads to the impairment of cell survival, degranulation and cytokine production, due to defective signal transduction in mast cells (26, 27). These observations suggest that GATA1 and 2 play important roles in gene expression in mast cells. However, the role of GATA1 and 2 in c-*kit* expression in mast cells is largely unknown.

In the current study, we analyzed the regulation mechanisms of c-*kit* expression in mast cells and found that c-*kit* expression of mast cells is maintained by GATA2 and Sp1 recruitment to the promoter region.

Materials and Methods

Cell culture

For BMMCs, linage negative BM cells from BALB/c mice were prepared using the MACS linage negative cell preparation system (Miltenyi Biotec, Tubingen, Germany), and cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO), supplemented with FBS (10%, Sigma-Aldrich), nonessential amino acid solution (1%, Invitrogen, Carlsbad, CA), SCF (100 ng/ml, Wako Junyaku, Osaka, Japan), and IL-3 (10 ng/ml, Wako Junyaku) for at least 4 wk based on previously established methods (28, 29).

For BM-derived dendritic cells (BMDCs), BM cells from BALB/c mice were cultured in RPMI 1640, supplemented with FCS (10%), nonessential amino acid solution (1%), and 10 ng/ml GM-CSF (Peprotech, London, U.K.). After at least 10 d, we harvested the loosely adherent cells.

The PT18 mouse mast cell line was cultured in RPMI 1640, supplemented with FCS (10%) and nonessential amino acid solution (1%), and the CV1 simian kidney cell line was cultured in DMEM (Sigma-Aldrich), supplemented with FCS (10%) and nonessential amino acid solution (1%).

Plasmid constructs

The mouse c-*kit* promoter (−622/+22) was subcloned into the reporter plasmid pGL3-Basic (Promega, Madison, WI) at the XhoI/HindIII sites and deletion mutants were generated using an endonuclease/exonuclease deletion kit (Takara Bio, Otsu, Japan) in a reporter assay.

Plasmids in which several bases were replaced were generated by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All mutations were verified by sequencing analysis.

GATA2 open reading frame cDNA was obtained from PT18 mRNA by RT-PCR using SuperScript First-Strand synthesis system (Invitrogen) and Advantage 2 DNA polymerase (Clontech, Mountain View, CA), and Sp1 and GATA1, described in a previous study (17, 30), GATA2 was subcloned into pCR3.1 expression plasmids (Invitrogen), and the generated GATA1/pCR, GATA2/pCR, and Sp1/pCR were used to produce GATA1, GATA2, and Sp1, respectively.

EMSAs

PT18 nuclear extracts were prepared as described in our previous report (19). Binding reactions were allowed to proceed at room temperature for 15 min in 10 mM HEPES, 100 ng poly(dIdC) (Amersham Pharmacia Biotech, Piscataway, NJ), FITC-labeled probes (Invitrogen), and 5 μg nuclear extract. Abs and competitor DNA were added to the reaction mixtures before the probes. Samples were analyzed by Fluoroimager (Amersham Pharmacia Biotech) after 4% PAGE. The oligonucleotides used were mouse c-*kit* −108/−77, 5′-CCC GGG CGG GAG AAG GGA GGG GCG TGG CCA CG-3′, c-*kit* GC-box competitor of c-*kit*, −108/−77 (oligo mGC) 5′-CCC GGG CGG GAG AAG GGA GGG GAC GCG TCA CG-3′, −180/−148, 5′-GGC ACA GCG CCC CCG GGA TCA GCT TAT TGC AGC-3′, and −232/−197, 5′-CTA GGA GGA AGA GGA TCC AGG GTG AAG GGC CTG TGG-3′. The underlined nucleotides indicate the position of the GC-box motif in the oligonucleotide probe and nonlabeled oligonucleotides were used as self-competitors.

Abs against Sp1 (clone 1C6), GATA1 (clone N6), and GATA2 (clone H116) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and isotype control mouse IgG, rabbit IgG, rat IgG, respectively, were purchased from BD Biosciences (San Diego, CA).

For in vitro transcription and translation, Sp1/pCR, GATA1/pCR, and GATA2/pCR were used with the TnT T7 Quick coupled transcription/translation system (Promega).

RNA isolation and quantitative PCR analysis

Total RNA was prepared using the RNeasy kit (Qiagen, Hilden, Germany). For quantitative analysis, cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and analyzed with a gene specific TaqMan probe (Applied Biosystems), GATA2 (Mm 00492300_m1), Sp1 (Mm 00489039_m1), and c-*kit* (Mm 00445212_m1), using the 7500 real-time PCR system (Applied Biosystems). Quantitative values were obtained from the threshold cycle number (Ct), by subtracting the average Ct of the gene from that of GAPDH (Mm9999915_g1, Applied Biosystems) and expressed as 2-ΔCt (20).

Luciferase assay

BMMCs and BMDCs (1–0.5 × 10^6 cells) were transfected with 5 μg reporter plasmid, 3 μg expression plasmid (for coexpression analysis), and 50 ng pRL-null (Promega) by electroporation using Nucleofector II (Lonza, Basel, Switzerland) using a Macrophage Kit (Lonza) and a dendritic cell Kit (Lonza) according to the manufacturer's instructions, respectively. CV1 cells (1 × 10^5) were transfected with 1 μg reporter plasmid, 200 ng expression plasmid, and 1 ng pRL-null by FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions.

After 20 h, cells were harvested and the Dual-luciferase assay kit (Promega) and Micro Lumat Plus (Berthord Technologies, Bad Wildbad, Germany) were used to assess luciferase activity. Firefly luciferase activity derived from the reporter plasmid was normalized to Renilla luciferase activity derived from pRL-null.

Chromatin immunoprecipitation and rechromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (20) according to manufacturer's instructions (Upstate, Lake Placid, NY) using 1–2 × 10^6 BMMCs and PT18 cells per sample. The amounts of target DNA bound to GATA1, GATA2 or Sp1 were quantified using the 7500 real-time PCR system. The ratio of a specific DNA fragment in each immunoprecipitate to the fragment in the DNA before immunoprecipitation (input DNA) was calculated from each Ct value.

Anti-GATA1 Ab, anti-GATA2 Ab, anti-Sp1 Ab, and isotype control Abs were used the same Abs for EMSA analysis. The primer and MGB probe sequences used for this analysis were as follows: for the promoter region, c-*kit*_F (5′-CCGGGATCAGCTTATTGCA-3′), c-*kit*_R (5′-GCTACAGCTC-TCGCCCAAGT-3′), and MGB probe c-*kit*_T (5′-FAM-CAGCTCGTGGC-CAC-3′); for the cis control region, c-*kit* -*cis*C_F (5′-TCAGCTGCA-TAGTGAAGTCAACTTAG-3′), -*cis*C_R (5′-TCCAGCCTGGTCTACATA-GGAATTA-3′), and BGM probe -*cis*C_T (5′-FAM-CTACATAGCAAGA-AACTT-3′).

The re-ChIP assay was performed as described previously (31). The primary Ab-treatment was performed as for the ChIP assay, except that the conditions were scaled up. Briefly, 1 × 10^7 BMMCs were fixed in formaldehyde, lysed in cell lysis buffer, and sonicated to generate DNA fragments, to which 10 μg of the primary Ab, anti-GATA2 Ab, or isotype control rabbit IgG, was added. The immunocomplex was precipitated with protein G-agarose, was washed with various washing buffers, and was then released from the beads by incubation in the elution buffers at 37°C for 1h. The eluant of the primary immunocomplex obtained using this first Ab was diluted 10-fold with dilution buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100. A second Ab, anti-Sp1 Ab, or isotype control IgG, (10 μg each) was added to the diluted solution to obtain the secondary immunocomplex. After extraction of the immunocomplex, which was precipitated with protein G-agarose, in the same way as that for the first complex, the cross-linking between chromosome DNA and nuclear proteins was reversed. The chromosome DNA was then purified by phenol and chloroform extraction and was subjected to real-time PCR using the same primers and probe sets as used in the ChIP assay.

Flow cytometry analysis

For detection of cell surface expression of c-Kit, cells were incubated with FITC-labeled anti-Fc ε RI α-chain Ab and PE-labeled anti-mouse c-Kit Ab purchased from eBioscience (San Diego, CA) and BD Bioscience (Franklin Lakes, NJ), respectively, and analyzed using flow cytometry (FACS Calibur, BD Biosciences).

Retrovirus infection

An oligonucleotide designed to function as a short hairpin RNA (shRNA) against GATA2, (5′-GATCCCCCAAGTGCATGCAAGAGAATTCAAGAGATTCTCTTGCATGCACTTGGTTTTTA-3′), was inserted into the retroviral vector for expression of shRNA, pSUPER retro-puro (OligoEngine, Seattle, WA), and retroviral supernatants were prepared using transfection of PLAT/E packaging cells. The underlined nucleotides were required as spacers. PT18 cells were cultured in growth medium with 1 μg/ml puromycin for 10 d to obtain transfectants prior to culture in normal medium.

Western blot analysis

Cell nuclear extracts (from 1×10^6 cells) were electrophoresed through a 10% SDS polyacrylamide gel and transferred to a membrane (Immobilon-P, Millipore, Bedford, MA). The proteins on the transferred membrane were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) and the density of bands analyzed. Anti-YY1 (clone H10) and anti-GATA2 Abs were purchased from Santa Cruz Biotechnology. Alexa680-conjugated anti-rabbit IgG (Invitrogen), and IRDye800-conjugated anti-mouse IgG (Rockland, Philadelphia, PA) were used as the secondary Abs.

Transfection of small interfering RNA

GATA2 small interfering RNA (siRNA) (SI01009764), Sp1 siRNA (SI1429869), and nonsilencing control siRNA (107280) were purchased from Qiagen. A 5 μl aliquot of 20 μM siRNA was added to 5×10^5 BMMCs with Nucleofector II. After 20 h of culture, GATA2, Sp1, and c-*kit* mRNA was analyzed by quantitative PCR.

Statistical analysis

We used Student unpaired two-tailed *t* test for all statistical analyses. Differences between groups were considered significant when $p < 0.05$, and indicated with a single asterisk ($p < 0.05$), double asterisks ($p < 0.01$), or triple asterisks ($p < 0.001$). All statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

The GC-box is critical for c-kit *promoter activity*

To examine cell type-specific promoter regulation of the c-*kit* gene, we generated the reporter plasmid −622/pGL3-Basic (−622/B), by introducing the 5′-upstream region of the putative translational initiation codon of the mouse c-*kit* gene, including the 622 bp promoter and 22 bp 5′-UT (−622/+22), upstream of the luciferase gene of pGL3-Basic (Basic). Then, to screen *cis*-acting elements within the c-*kit* promoter region, three 5′-deletion constructs carrying various lengths of 5′-flanking region were generated from −622/B. The luciferase activity of BMMCs that had been transiently transfected with −232/pGL3-basic (−232/B) was ~12-fold higher than that of BMMCs transfected with Basic (Fig. 1*A*). The promoter activity was significantly reduced by the deletion of −232/−62, although the deletion of −622/−233 markedly increased the promoter activity. In contrast, c-*kit* promoter activity driven from −232/B, −61/B, and −5/B was not significantly different from that of Basic in BMDCs, in which c-Kit cell surface expression was not detected (Fig. 1*B*). These results suggest that the −232/−62 region contains the elements necessary for expression of the c-*kit* promoter, which is active only in c-Kit positive cells.

To identify the critical *cis*-enhancing elements in the −232/−62 region, we constructed various mutant promoters based on the −232/B plasmid with introduction of nucleotide replacements by site-directed mutagenesis. As shown in Fig. 2, several transcription factor binding motifs; two GATA motifs, the PU.1 motif, the GC-box, and the E-box, were found in the −232/+22 region based on motif analysis. The introduction of a GC-box mutation (mGC/B) markedly reduced the luciferase activity of −232/B, whereas mutation of PU.1 or the E-box (mPU/B or mE/B, respectively) did not affect the promoter activity of −232/B. In contrast, lacking either the GATA motif of −232GA/B or −165GA/B enhanced c-*kit* promoter activity. These results suggest that the GC-box is crucial for functioning of the c-*kit* promoter in BMMCs.

Specific binding of Sp1 to the c-kit *promoter via the GC-box*

To identify the transcription factor binding to the GC-box, EMSA was performed by using FITC-labeled double-stranded oligonucleotides as the probe (−108/−77 region) with PT18 nuclear extract in the presence or absence of the competitive oligonucleotides or Abs. The intensity of the band marked with the arrow (Fig. 3*A*, *lane 1*) was decreased by addition of anti-Sp1 Ab (Fig. 3*A*, *lane 3*), whereas isotype control IgG had no effect on this band (Fig. 3*A*, *lane 2*). When Sp1 produced by an in vitro transcription/translation system was used for EMSA, the band that showed mobility identical with that observed with the nuclear protein from PT18 was detected (Fig. 3*A*, *lane 4*) and was supershifted by addition of anti-Sp1 Ab as shown by the asterisk (Fig. 3*A*, *lane 5*). Unlabeled competitive oligonucleotides were also used to confirm the binding of Sp1 to the GC-box. The band that appeared in the mixture of probe and PT18 nuclear extract disappeared by addition of the self-competitor (Fig. 3*A*, *lane 6*). In contrast, mutant competitor lacking the GC-box (oligo mGC) had no effect on this band, (Fig. 3*A*, *lane 7*). Similarly, the band caused by addition of in vitro Sp1 disappeared in the presence of self-competitor (Fig. 3*A*, *lane 8*), but remained in the presence of the oligo mGC competitor (Fig. 3*A*, *lane 9*). These results indicate that the transcription factor Sp1 binds to the GC-box of the c-*kit* promoter.

To examine the possibility that GATA1 and/or GATA2 bind to two GATA motifs in the −232/B reporter plasmid, we performed EMSA as shown in Fig. 3*B*. Both GATA1 and GATA2 proteins, which were produced using an in vitro transcription/translation system, bound to the −232/−197 and −180/−148 probes, and bands with GATA proteins and probes disappeared in the presence of anti-GATA1 or anti-GATA2 Ab. These findings suggest that both GATA1 and GATA2 may be recruited to GATA motifs.

To further investigate whether GATA factors and Sp1 bind to the c-*kit* promoter in vivo, a ChIP assay was performed using BMMCs and BMDCs (Fig. 4). A markedly higher amount of chromatin-containing c-*kit* promoter region (−168/+22) was immunoprecipitated by anti-GATA2 Ab (Fig. 4*A*) compared with control IgG in BMMCs, whereas significant binding of GATA2 was not detected in BMDCs (Fig. 4*C*). In addition, a significantly higher amount of c-*kit* promoter region was detected in the immunoprecipitant produced by anti-Sp1 Ab compared with isotype control IgG in BMMCs (Fig. 4*A*), but not in BMDCs (Fig. 4*C*). In contrast, a significant difference was not detected between anti-GATA1 and isotype control IgG in either BMMCs or BMDCs. Anti-GATA1 Ab, anti-GATA2 Ab, and anti-Sp1 Ab in both BMMCs and BMDCs did not immunoprecipitate the *cis*-control region of the c-*kit* promoter (Fig. 4*B*, 4*D*). These results suggest that GATA2 and Sp1, but not GATA1, are recruited to the c-*kit* promoter in a BMMC-specific manner.

The GC-box is essential for GATA2-mediated transactivation of the c-kit *promoter*

Furthermore, to elucidate the function of GATA1, GATA2, and Sp1 in the c-*kit* promoter, we introduced the GATA1/pCR, GATA2/pCR , or Sp1/pCR expression plasmid with various mutant plasmids based on −232/B into CV1 cells, and determined luciferase activity. As shown in Fig. 5*A*, each of the mutant reporter plasmids, lacked the GATA and/or the GC-box motifs by nucleotides replacement, was used for coexpression analysis. Enforced expression of GATA2 additionally enhanced the luciferase activities of −232/B and *m*-222,165GA/B to ~4-fold that of empty

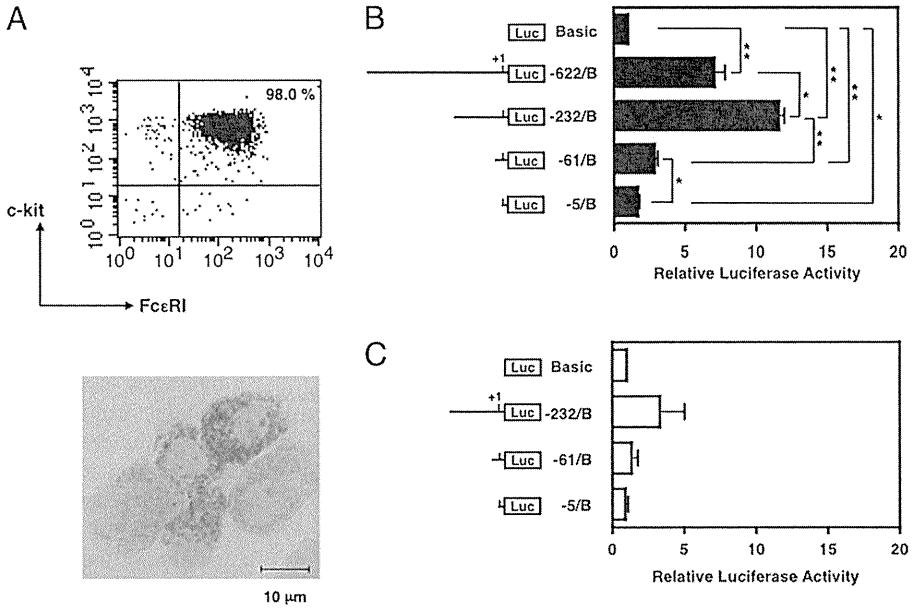

FIGURE 1. c-*kit* promoter activity was reduced by deletion between −232/−62 in BMMCs. *A*, Surface expression of c-Kit and FcεRI, and staining with Alcian blue (original magnification ×630) of BMMCs used in the current study. *B* and *C*, The 5 μg amount of each reporter plasmid was introduced into BMMCs (*B*) or BMDCs (*C*) by electroporation. The relative luciferase activity driven by −622/pGL3-Basic (−622/B), −232/pGL3-Basic (−232/B), −108/pGL3-Basic (−108/B), −61/pGL3-Basic (−61/B), and −5/pGL3-Basic (−5/B) are represented as the ratio to the activity driven by pGL3-Basic (Basic). Luciferase activities were normalized to the activity of pRL-null. Each experiment was conducted in duplicate for each sample, and the results expressed as the mean ± SEM for more than three independent experiments in Figs. 1, 2, and 5.

expression plasmid, but not that of mGC/B and mGA,GC/B, whereas enforced expression of GATA1 did not affect the luciferase activities derived from CV1 cells transfected with any reporter plasmids. Interestingly, overexpression of Sp1, which was identified to bind the GC-box, did not affect any luciferase activities. In addition, we introduced GATA2/pCR expression plasmid with the mutant plasmid into BMMCs by electroporation, and the luciferase activity was determined to evaluate the effect of GATA2 on the c-*kit* promoter in mast cells (Fig. 5*B*). The luciferase activities derived from BMMCs transfected by the plasmids, −232/B and −222,165GA/B, were increased to ~2-fold by GATA2 coexpression, whereas the promoter lacking GC-box (mGC/B and mGA, GC/B) was not significantly activated. To clarify whether GATA2 interact directly to c-*kit* promoter via Sp1 of GC-box, we performed EMSA using FITC-labeled probe containing GC-box motif (−108/−77) and in vitro translated Sp1 and GATA2 (Fig. 5*C*). When Sp1 and GATA2 were added to the probe after incubation at room temperature, the band marked with an asterisk appeared (*lane 2*) with lower mobility shift than that of the band containing Sp1 and the probe shown with the arrow (*lane 1*), suggesting that the m.w. of the complex marked with an asterisk is higher compared with that of Sp1 and probe. In addition, this band with lower mobility was supershiftred by addition of anti-Sp1 as shown with double asterisks (*lane 5*) with showing higher m.w. than that of probe/Sp1/anti-Sp1 Ab complex as marked with an arrowhead with an asterisk (*lane 4*). In contrast, the band by addition of GATA2 alone to the probe mixture did not cause the specific band shift (*lane 3*). These results suggest that GATA2 transactivates the c-*kit* promoter via GC-box *cis*-element, and that GATA2 binds the GC-box in the presence of Sp1.

To further confirm the interaction between GATA2 and Sp1 on the c-*kit* promoter in the BMMC chromosome, we performed a re-ChIP assay. The results of this assay indicate that GATA2 and Sp1 are simultaneously recruited to a region, including GC-box of the c-*kit* promoter, but not to a control region (Fig. 5*D*).

c-kit *expression was reduced by knockdown of GATA2 and/or Sp1*

Although a transactivating function of GATA2 was found in the coexpressing reporter assay, no effect of exogenous Sp1 was observed in the assay, which may have been due to the endogenous Sp1. Therefore, to further confirm the necessity of GATA2 and Sp1 for c-*kit* expression in BMMCs, GATA2, and/or Sp1 siRNA were introduced by electroporation. The c-*kit* mRNA levels in the resultant transfectants were determined using quantitative real-time PCR after 20 h cultivation (Fig. 6*A–C*), and binding of GATA2 and/or Sp1 to the c-*kit* promoter was analyzed by ChIP analysis (Fig. 6*D–G*). The GATA2 mRNA level in the GATA2 siRNA transfectants siGATA2/BMMC (GATA2 in Fig. 6*A*), and the Sp1 mRNA level in the Sp1 siRNA transfectants Sp1 siRNA (siSp1)/BMMC (Sp1 in Fig. 6*B*), were reduced by ~20 and 15%, respectively, whereas transfection of GATA2 siRNA and Sp1 siRNA had little effect on the mRNA levels of the other. The mRNA levels of c-*kit* in BMMCs were decreased to 40 or 60% by knockdown of GATA2 or Sp1, respectively (c-*kit* in Fig. 6*A*, 6*B*). These data indicate that a decrease in either GATA2 or Sp1

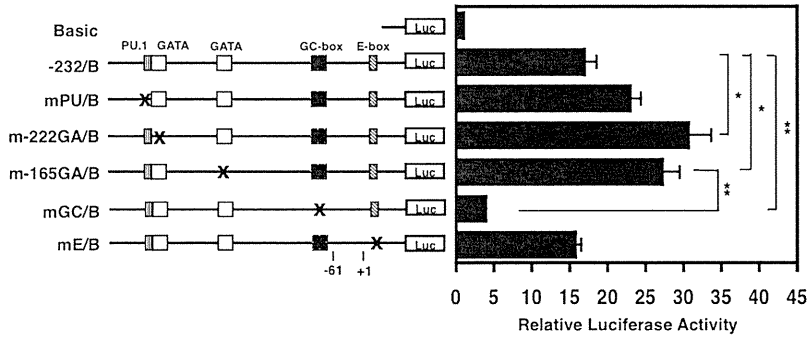

FIGURE 2. Mutation of the GC-box impaired c-*kit* promoter activity. BMMCs were transfected with 5 μg of each reporter plasmid, Basic, −232/B, or the mutated −232/B constructs produced by nucleotide replacement (shown as × here and in Fig. 5). The relative luciferase activity is represented as the ratio to the activity driven by pGL3-Basic (Basic).