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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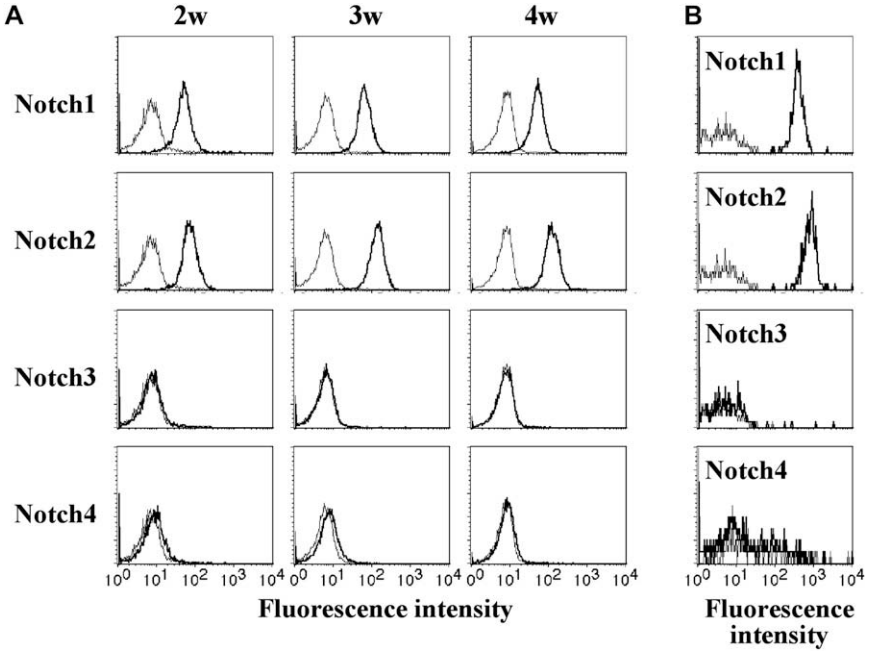

FIG 1. Cell surface expression of Notch receptors on mast cells. The expressions of Notch 1, 2, 3, or 4 *(bold lines)* on c-Kit$^+$FcεRIα$^+$ BMMCs cultured for 2, 3, or 4 weeks **(A)** or fresh c-Kit$^+$FcεRIα$^+$ PMCs **(B)** were analyzed by flow cytometry. *Thin lines* represent isotype-matched control.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

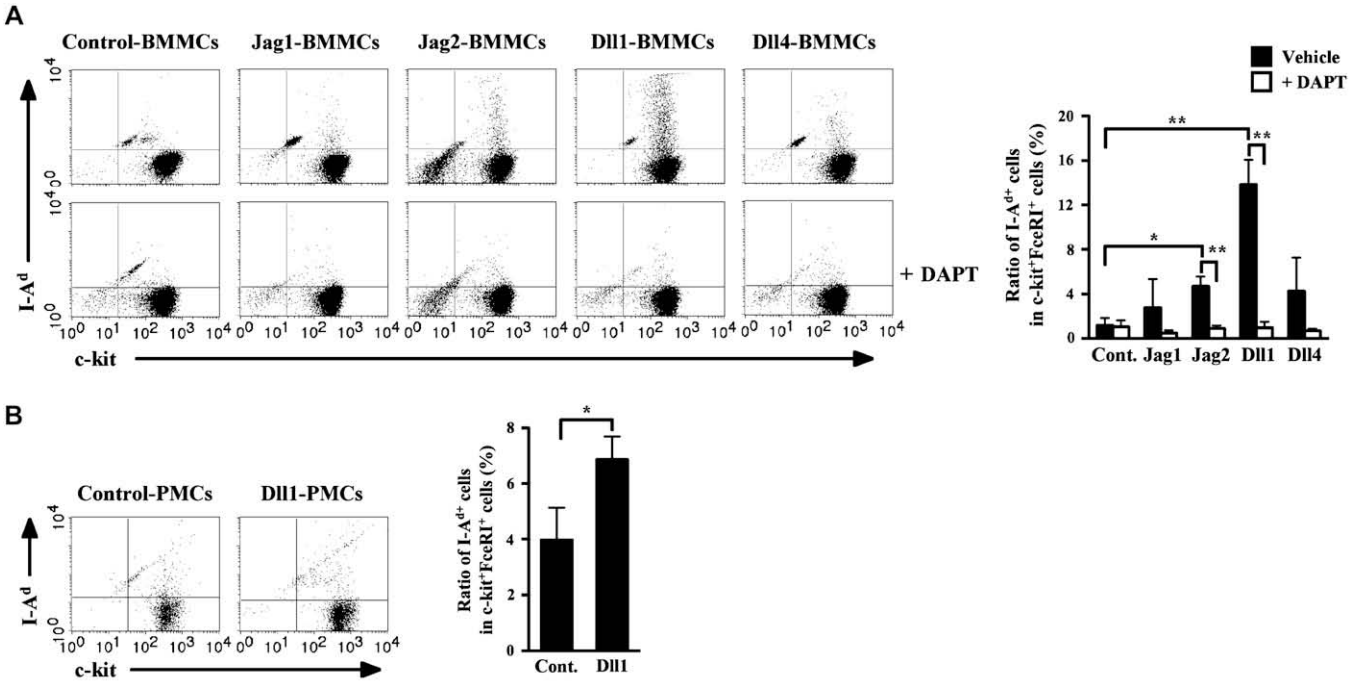

FIG 2. Notch signaling induces MHC-II expression on mast cell surface. BMMCs **(A)** or PMCs **(B)** were cocultured with the indicated CHO–Notch ligand—expressing cell lines or CHO control cells in the presence or absence of DAPT. *Cont.,* Control. *Dot blots* show the expressions of MHC-II (I-A) and 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

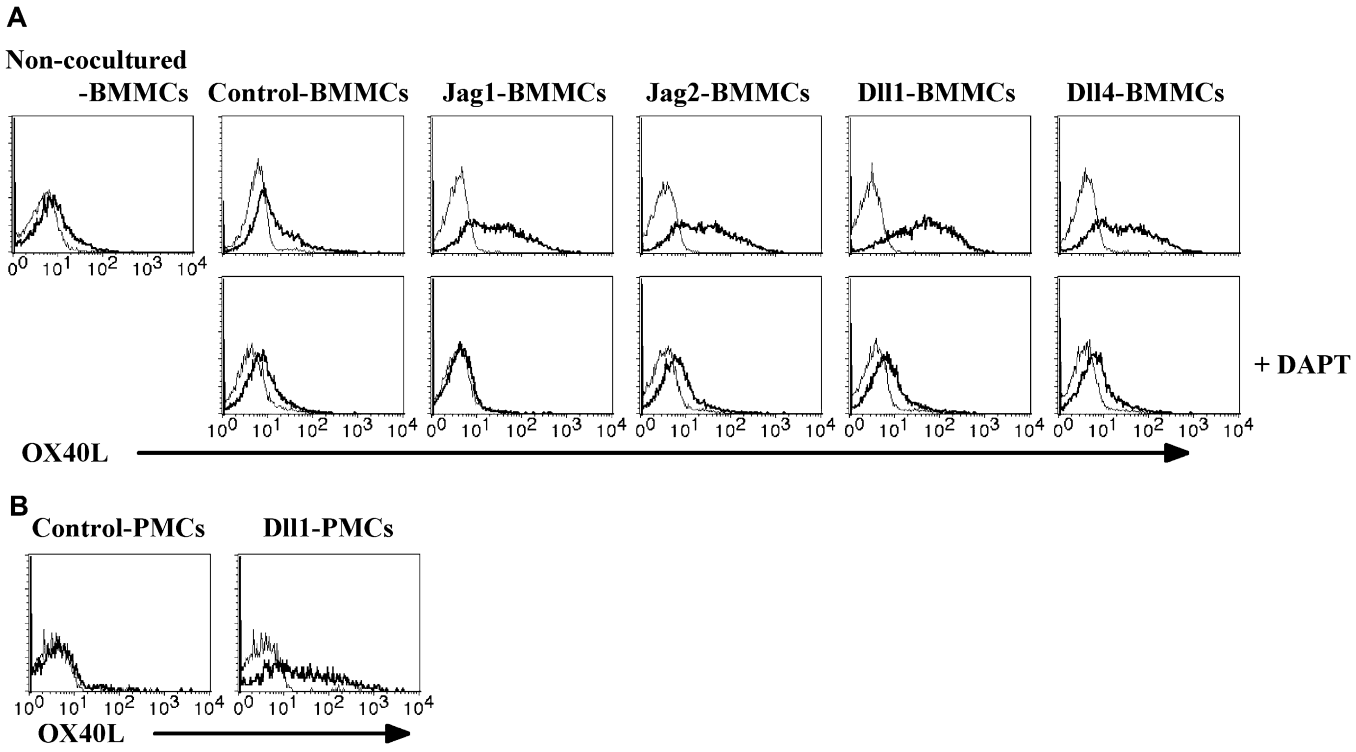

FIG 3. Notch signaling upregulates OX40L expression level on mast cell surface. BMMCs **(A)** or PMCs **(B)** were cocultured with the indicated CHO–Notch ligand—expressing cell lines or CHO control cells in the presence or absence of DAPT. The expression of OX40L *(bold lines)* on c-Kit$^+$FcεRIα$^+$ 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

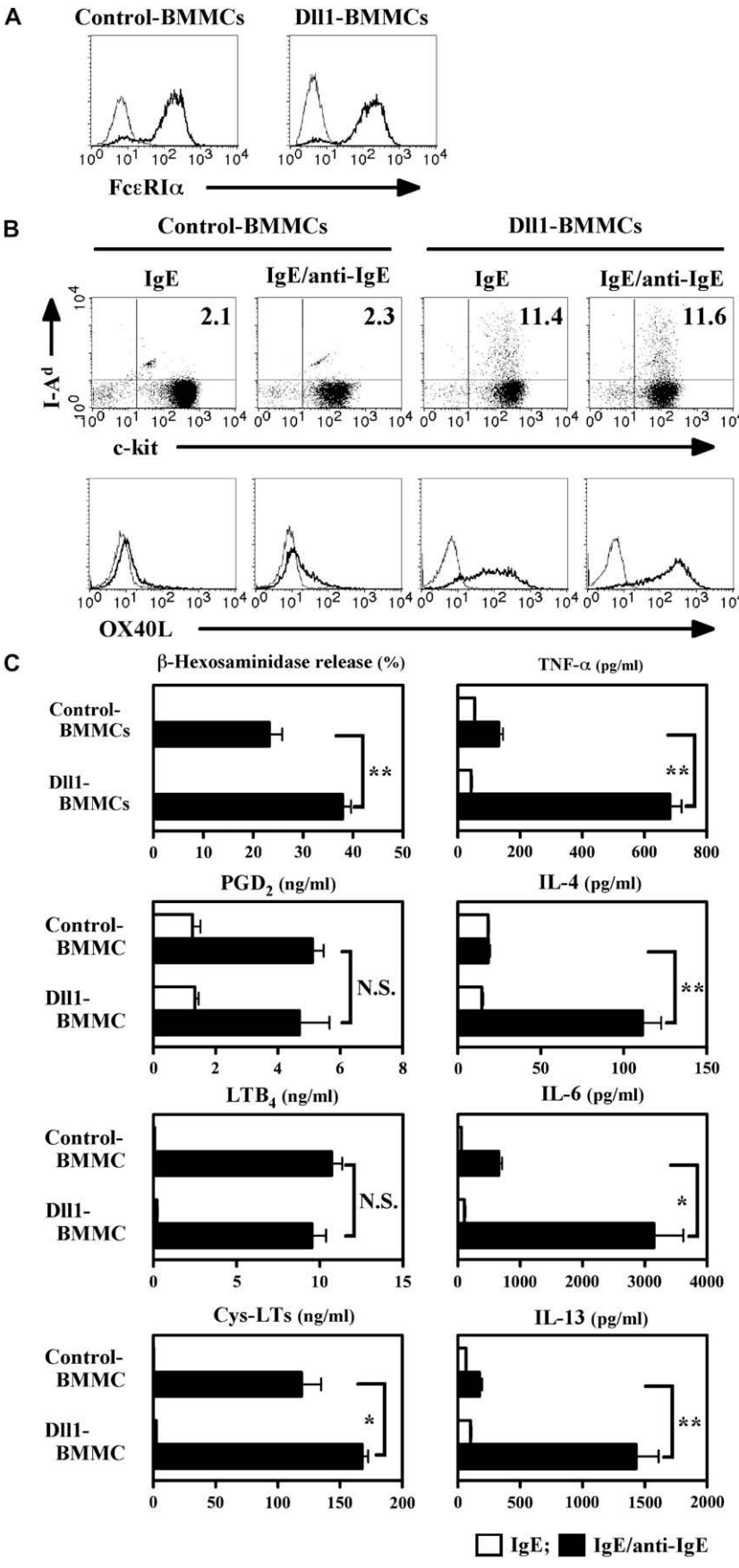

FIG 4. *(See next page for figure legend).*

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-$Kit^+FcεRIα^+$ 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.

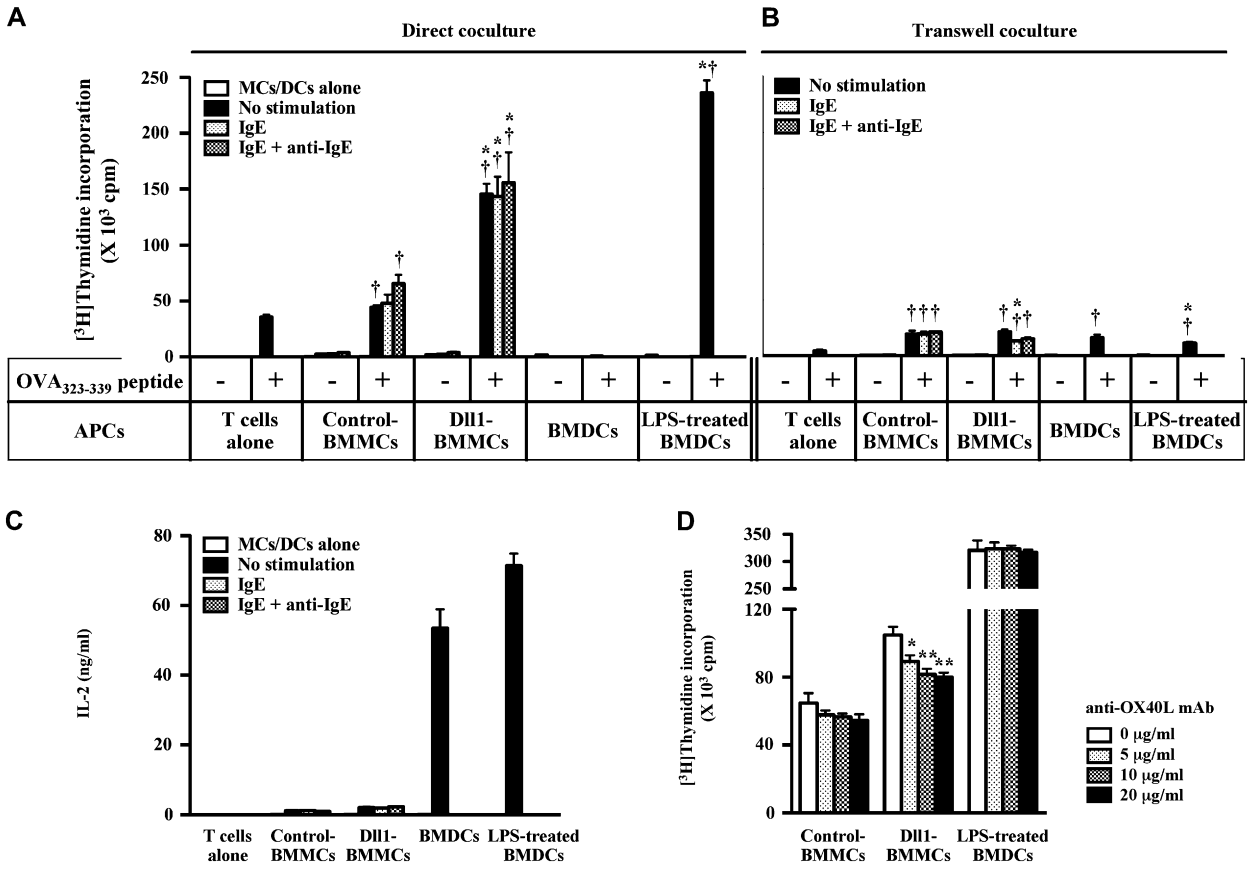

FIG 5. $CD4^+$ T-cell activation mediated by Dll1-BMMCs. [^{3}H]Thymidine incorporation into the $CD4^+$ T cells from DO11.10 mice cocultured together with BMMCs or BMDCs **(A)** or separated from them by a Transwell membrane **(B)** in the presence or absence of ovalbumin *(OVA)* peptide. Data are indicated 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.

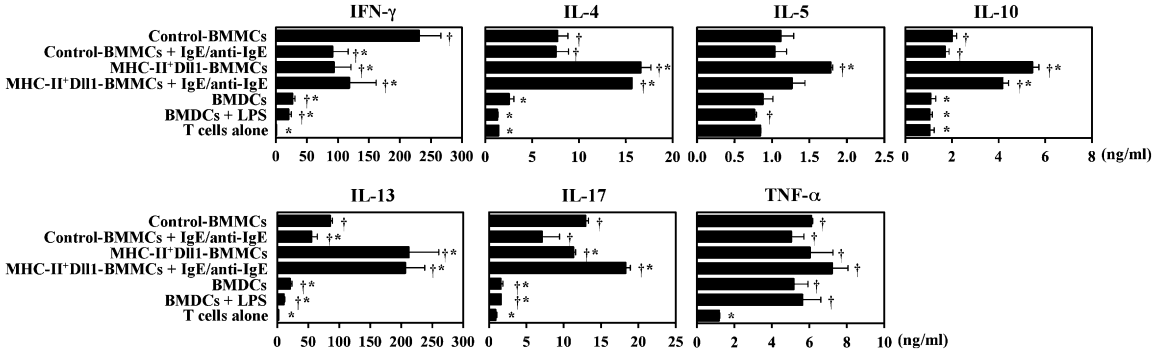

FIG 6. Dll1-BMMCs enhance IL-4, IL-10, and IL-13 production and suppress IFN-γ production by $CD4^+$ T cells. Purified $CD4^+$ T cells from DO11.10 mice were direct cocultured with BMMCs or BMDCs in the presence of ovalbumin peptide. After 4 days of coculture, the cocultured cells were restimulated with 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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