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Opposite effects of PU.1 on mast cell stimulation

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ABSTRACT

An Ets-family transcription factor PU.1 is involved in the development and specific gene regulation of hematopoietic cells. PU.1 also determines the commitment between several lineages via its expression level. Although enforced expression of PU.1 in mast cells (MC) induced expression of monocyte-specific markers and morphological change from MC to monocytes, especially dendritic cells (DC), in the previous report, intracellular events caused by PU.1 are largely unknown. In the present study, effect of PU.1 on IgE- and LPS-mediated stimulation degrees was analyzed. The amounts of IL-6, IL-13, and TNF-α produced from LPS-stimulated MC were markedly increased by overexpression of PU.1. In contrast, IL-6 and IL-13 production levels in response to IgE were reduced by PU.1, whereas that of TNF-α was up-regulated. β-Hexosaminidase release as a means of degranulation was decreased in PU.1 transfectants. When eicosanoid generation in response to IgE-stimulation was analyzed, overexpression of PU.1 reduced leukotriene C_4 (LTC_4) release, but enhanced PGD_2 production. Microarray analysis suggested that expression of FcεRI signal pathway related molecules were suppressed in PU.1 overexpressing MC as well as DC. These observations indicate that up-regulation of PU.1 suppresses expression of FcεRI signal transduction-related intracellular molecules, but increases the potential of transcription activity of monocyte characters.

Transcription factor PU.1 plays important roles in the development of myeloid- and lymphoid-lineages and specific gene regulation. PU.1 is expressed in lymphoid cells, macrophages, dendritic cells (DC), neutrophils, and mast cells (MC) in a cell type-specific manner [1], and required for the development of these lineages [2–7]. In addition, the expression level of PU.1 determines cell fate between different lineages, including B cells/macrophages [8] and neutrophils/macrophages [9]. In our recent studies, PU.1 exhibited the potential for determination of commitment between MC/monocytes [10–12]. In brief, we found that overexpression of PU.1 in MC and its progenitors induced the expression of monocyte-specific genes and caused the morphological change from MC to DC/macrophages. It is also demonstrated that overexpression of PU.1 in $CD34^+$ human myeloid progenitors triggered development of Langerhans cells (LC) [13].

Although the effect of PU.1 on expression of cell-specific markers and morphological change is drastic, detailed information of the effect for expression of other genes that involve the function of MC is remains unclear. In the present study, we compared the responses against IgE- and LPS-mediated activations between normal MC and PU.1-introduced MC. The production levels of IL-6 and IL-13 in response to stimulation through FcεRI were reduced by overexpression of PU.1; in contrast, that of TNF-α was up-regulated. The amounts of cytokines produced from LPS-stimulated cells were markedly increased by PU.1. Degranulation degree and leukotriene C_4 (LTC_4) production as early event of FcεRI-cross linking were suppressed, whereas PGD_2 production was enhanced. The FcεRI pathway was identified to be significantly down-regulated pathway in PU.1-overexpressing MC as well as DC by microarray analysis, whereas cell surface expression levels of FcεRI and TLR4 were comparable between MC and PU.1-overexpressing MC in FACS analysis. From these observations, we concluded that PU.1 down-regulated expression of intracellular molecules related to the FcεRI signal cascade, but up-regulated potential of cytokine and PG transcription activities.

Materials and methods

Cells and retrovirus transfection. Complementary DNA encoding PU.1 was introduced into bone marrow-derived MC (BMMC),

Abbreviations: BM, bone marrow-derived; DC, dendritic cells; LC, langerhans cells; LT, leukotriene; LPS, lipopolysaccharoide; MC, mast cells; PG, prostagrandine.

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which was developed from bone marrow cells of BALB/c (Japan SLC, Hamamatsu, Japan) after 3 weeks culture in RPMI 1640 (Sigma–Aldrich) supplemented with 10% heat-inactivated FBS (Sigma–Aldrich), 100 μM 2-ME, 10 μM MEM nonessential amino acids solution (Invitrogen), antibiotics, and 10% pokeweed mitogen-stimulated spleen-condition medium [14], using retrovirus vector system as described in our previous report [10]. In brief, a plasmid pMXs-puro-PU.1 was constructed by insertion of BamHI/NotI fragment from pMX-puro-PU.1 [11] to BamHI/NotI-digested pMXs-puro (kindly provided by Dr. T. Kitamura) [15]. Each of pMXs-puro (mock vector) and pMXs-puro-PU.1 (for the expression of PU.1) was transiently introduced into a retrovirus packaging cell, Plat-E [16], with Fugene6 (Roche Diagnostics). BMMC was incubated with harvested culture medium of Plat-E containing infectious viruses for 2 days and cultured for an additional 10–20 days in the presence of puromycin to selectively obtain transfectants [10]. BMDC was prepared from BALB/c bone marrow cells with culture in RPMI 1640 supplemented with 10% FBS, 100 μM 2-ME, 10 μM MEM nonessential amino acids solution, antibiotics, 10 ng/ml mouse rGM-CSF (PeproTech), and 10 ng/ml mouse rIL-4 (PeproTech) based on a previously reported method [17,18].

Flow cytometric analysis. PE-conjugated anti-I-A^d, and FITC-conjugated anti-CD11c Abs were purchased from (BD Pharmingen), and PE/Cy7-conjugated anti-c-kit Ab (BioLegend), FITC-conjugated anti-FcεRIα Ab (eBioscience), and PE-conjugated anti-TLR4 Ab (abcam, Tokyo, Japan) were also used. Cells were stained with Abs in the presence of 2.4G2 for blocking of Fc receptors and analyzed by a FACSCalibur flow cytometer (BD Bioscience).

GeneChip expression analysis. Total RNAs were extracted from three samples; PU.1-overexpresing BMMC, control BMMC, and BMDC were used as templates for the reactions of the One-Cycle Target Labeling method using Affymetrix kits. In brief, double-stranded cDNA was reverse transcribed from 1 μg total RNA with a Poly-A RNA Control Kit and a One-Cycle cDNA Synthesis Kit, and then the biotinylated cRNA was *in vitro* transcribed from cDNA with an IVT Labeling Kit. After cRNA fragmentation, a reaction cocktail prepared with a Hybridization Control Kit was hybridized with an Affymetrix GeneChip microarray, Mouse Genome 430 2.0 Array, according to the manufacturer's instruction. Washing and staining, and scanning of each chip was performed with using the Fluidics Station 400/250, and the GeneChip Scanner 3000, respectively. Transcription levels of whole genes were determined after normalization with each GAPDH value using the Affymetrix GeneChip® Operating Software. Hierarchical clustering analysis was performed for genes, which were selected considering significance ($p < 0.05$) determined by one-way ANOVA statistic analysis with three independent data.

LPS and IgE stimulation. Stimulation via FcεRI was performed according to our previously reported methods [10,19] with a modification of anti-mouse IgE usage instead of Ag. In brief, 0.5–3 × 10^5 cells sensitized with mouse anti-DNP-IgE (BD Pharmingen) for 1 h were stimulated with 0.5 μg/ml anti-mouse IgE (R35-72; BD Pharmingen). For activation via TLR4, LPS (from *Escherichia coli*; Sigma–Aldrich) was used. Each culture medium was harvested after 30 min incubation for determination of concentration of PGD_2 and LTC_4, 3 h for TNF-α, and 6 h for IL-6 and IL-13. The concentration of PGD_2 and LTC_4 was determined by ELISA kit (Cayman Chemical Co., Ann Arbor, MI). ELISA kits of Genzyme Techne (Minneapolis, MN) were also used for measuring TNF-α, IL-6, and IL-13 concentration. Bete-hexosaminidase activity in Tyrode's buffer at 30 min after stimulation with IgE/anti-IgE was also determined as described previously [10,19] to investigate degranulation activity.

Results

Preparation of BMMC-transfectants infected by retrovirus carrying PU.1 cDNA

To generate BMMC possessing enforced expression of PU.1, BMMC at 3 weeks culture from BM was transfected with retrovirus carrying PU.1 cDNA or empty vector. After an additional 2 weeks culture in the presence of puromycine, cell surface expression of lineage-specific molecules was analyzed by FACS (Fig. 1). PU.1 induced marked expression of MHC class II and CD11c on MC (Fig. 1A) and suppressed expression of c-kit (Fig. 1B, top), consistent with the previous study [10]. In contrast, significant difference was not observed in cell surface expression levels of FcεRI and TLR4 between mock and PU.1-overexpressing cells (Fig. 1B). Transfectants were used for the following experiments after confirmation of the cell surface expression.

PU.1 down-regulates FcεRI-mediated production of IL-6 and IL-13, but up-regulates that of TNF-α as well as LPS-stimulation signal

To evaluate the effect of PU.1 on MC activation, the amounts of cytokines produced from IgE- or LPS-stimulated MC were determined (Fig. 2). Production levels of IL-6 and IL-13 from IgE-stimulated MC were significantly suppressed by expression of exogenous PU.1. In contrast, surprisingly, the amount of TNF-α released from PU.1-overexpressing MC in response to IgE-stimulation was markedly higher compared with that of control MC. In addition, overexpression of PU.1 up-regulated all cytokine levels generated from MC in response to LPS-stimulation. These results suggest that PU.1 amplified stimulation activity via TLR4 but exhibited different effects on expression of cytokines through FcεRI signal cascades in a target-dependent manner.

Effects of PU.1 on eicosanoids generation and degranulation in response to IgE-stimulation

Stimulation of MC by cross-linking of FcεRI with IgE and antigens (or anti-IgE Ab) elicits not only transcription of cytokine genes, but also secretory granule exocytosis and immediate eicosanoid generation. To examine the effect of PU.1 on these early events, β-hexosaminidase activity and amounts PGD_2 and LTC_4 in culture supernatant of IgE-stimulated MC 30 min following cross-linking were determined (Fig. 3). β-Hexosaminidase activity that reflects degranulation degree was suppressed by overexpression of PU.1 (Fig. 3A) as in the previous study [10]. The concentration of PGD_2 in supernatant was markedly increased by enforced expression of PU.1 (Fig. 3B), whereas the amount of LTC_4 was decreased in supernatant of PU.1 transfectants (Fig. 3C). These results suggest that PU.1 exhibited an opposite effect on early events in IgE-stimulated MC; in brief, PU.1 suppressed degranulation and LTC_4 production, but enhanced PGD_2 generation.

Identification of a set of genes that were suppressed by PU.1

The effect of PU.1 on FcεRI-mediated stimulation was suppressor for degranulation, and IL-6, IL-13, and LTC_4 production but was a positive amplifier for TNF-α and PGD_2 production. To elucidate the complex effect of PU.1, we evaluated whole genes by comparing expression levels among control MC, PU.1-overexpressing MC, and BMDC using DNA microarray technology. From approximately 45,000 probes on mouse whole genome GeneChip, 2411 probes showing significantly different expression levels were obtained by one-way ANOVA analysis on probes passed through detection call filtering. Hierarchical clustering analysis indicated that 542

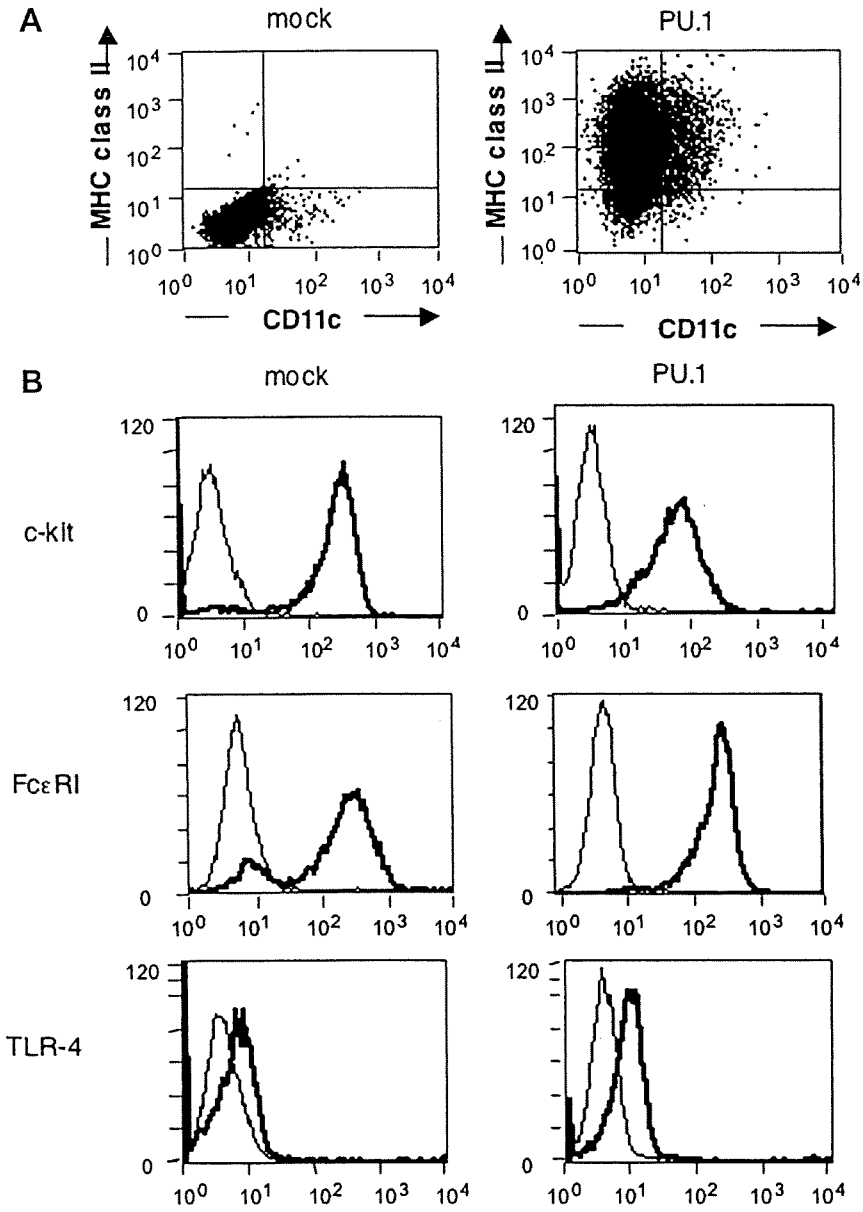


Fig. 1. Cell surface expression levels of lineage-specific markers and receptors for IgE and LPS. (A) PU.1-mediated induction of DC-specific cell surface markers expression. Transfectants with pMXs-puro or pMXs-puro-PU.1 were selected through 14 days culture in the presence of puromycin, and were double stained with PE-labeled anti-I-A^d Ab and FITC-labeled anti-CD11c Ab. (B) Expression levels of c-kit, FcεRI, and TLR4. Thick-line histogram represent cells with each Ab. Thin-line histogram indicates control with 2.4G2 alone. Mock (left panel), control BMMC transfected with empty vector pMXs-puro; PU.1 (right panel), BMMC transfected with pMXs-puro-PU.1. A representative result of five independent experiments is shown in A and B.

members were localized in a cluster characterized at a high level in MC, low level in DC, and down-regulated by PU.1 overexpression (Fig. 4). When KEGG pathway analysis was performed, FcεRI signaling pathway was found ($p < 0.05$; http://www.genome.jp/kegg/pathway/has/hsa04664.html). Gene symbols significantly found in the FcεRI signaling pathway and their signal intensities were shown to the right of the expression profiles in Fig. 4. This result suggests that expression of intracellular molecules relating to FcεRI-signal transduction was down-regulated by PU.1.

Discussion

PU.1 plays important roles in hematopoietic cell development and determines cell fate. We found that enforced expression of PU.1 in MC and MC-progenitors caused several changes toward monocytes, including induction of specific gene expression and morphological change in the previous studies [10–12]. In the present study, DNA microarray analysis was performed to evaluate the effect of PU.1 on expression levels of whole genes. FcεRI pathway was identified to be down-regulated by PU.1 according to pathway analysis using one-way ANOVA-treated microarray data. Degranulation, LTC_4 release, and production of IL-6 and IL-13 in response to IgE-stimulation were suppressed by enforced expression of PU.1 in MC, suggesting that FcεRI signal transduction was weakened due to the reduced expression of intracellular molecules relating to this signal pathway. These observations indicate that up-regulation of PU.1 suppresses intracellular MC-characters.

Interestingly, in contrast to IL-6 and IL-13, the amount of TNF-α released from IgE-stimulated MCs was up-regulated by PU.1. One possible explanation for this opposite appearance is the difference of gene expression mechanism that TNF-α, which accumulated within granules, is also rapidly released by degranulation in addition to production of TNF-α induced by stimulation-dependent transactivation, whereas the promoters of IL-6 and IL-13 are

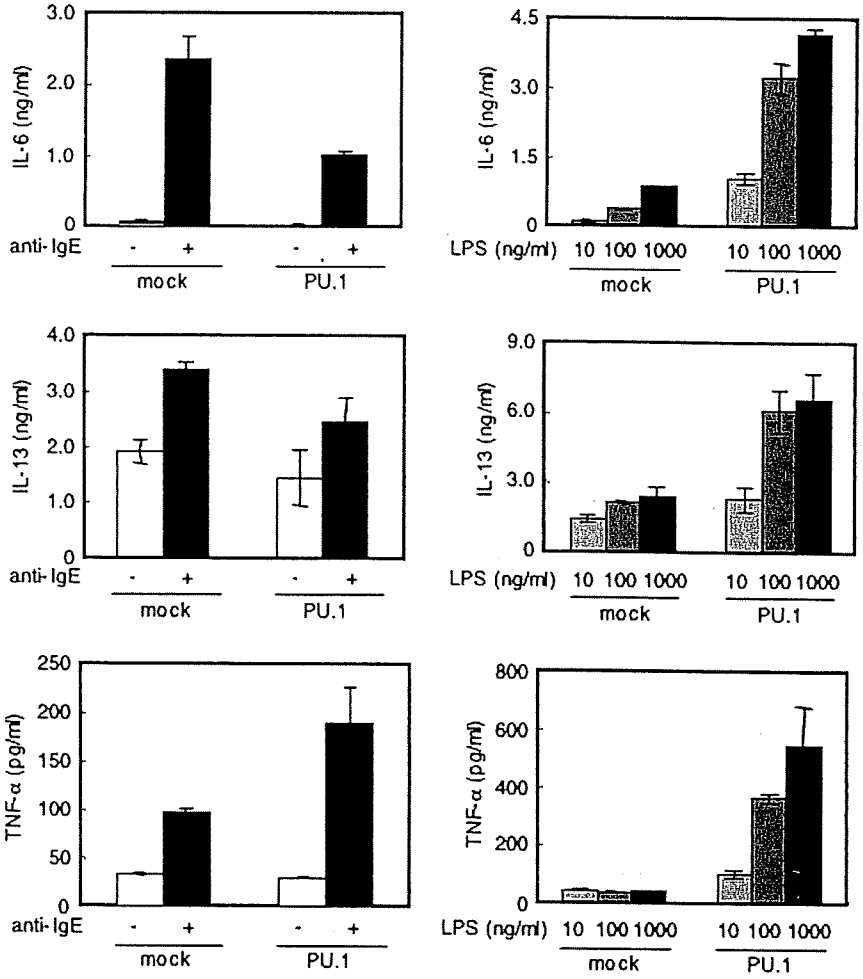


Fig. 2. Effect of PU.1 on production levels of cytokines from MC in response to stimulations by IgE and LPS stimulation. IL-6 and IL-13 concentrations in culture medium after 6 h of stimulation and TNF-α after 3 h were measured by ELISA. Each representative result performed with quadrant was shown as means ± SD. Similar results were observed in the other three independent experiments.

strictly dependent on IgE-mediated induction. This hypothesis coincides with the TNF-α mRNA level in microarray data, a 6-fold increase by overexpression of PU.1 in MC even in a resting state (data not shown). Previously, it was reported that PU.1 bound to *cis*-enhancing element in TNF-α promoter, which was essential for transactivation by LPS-stimulation in monocytes [20]. Therefore, enforced expression of PU.1 may directly amplify TNF-α promoter activity by binding to the critical element in the promoter. Regardless, further detailed analysis to evaluate the role of PU.1 on TNF-α promoter function will be required to clarify the opposite effects.

BMMC generate mainly PGD_2 and LTC_4 in response to IgE-stimulation immediately via different *de novo* synthesis pathways: PGD_2 via the cyclooxygenase pathway and LTC_4 via the 5-lipoxygenase pathway. Increase of PGD_2 generation in PU.1-overexpressing MC suggests that PU.1 is involved in the cyclooxygenase pathway. The immediate synthesis of PGD_2 is mediated by PG endoperoxidase H synthase (PGDH)-1 and hematopoietic PGD_2 synthase (hPGDS). Transcription level of hPGDS was increased 8-fold by enforced expression of PU.1 in microarray data (not shown). This up-regulation of hPGDS may explain the reason for increased production of PGD_2 in PU.1-overexpressing MC. A transcription factor MITF was essential for transactivation of mouse hPGDS promoter in MC in a previous report [21]. Considering that PU.1 regulates gene expression not only alone, but also with MITF in synergistic manner through interaction [22,23], PU.1 may up-regulate hPGDS promoter activity via affecting MITF function.

In the previous report, PU.1 was identified to bind a *cis*-enhancing element, which is highly conserved between human and mouse, and essential for TLR4 gene promoter activity [24]. However, expression level of TLR4 was not increased by exogenous PU.1 in this study, suggesting that other transcription factors are still required for up-regulation of TLR4 at the level of DC. One of candidates is IRF-8/ICSBP, which binds a target sequence with forming heterodimer with PU.1 and is also identified to involve in TLR4 gene promoter function [24]. Transcription level of IRF-8/ICSBP was slightly increased by PU.1-overexpression (2.5-fold), but was quietly lower than that of DC (35-fold compared with normal MC) (data not shown). Co-expression of PU.1 and IRF-8/ICSBP may be required to clarify the necessity of these two transcription factors for full TLR4 promoter activity. Even though TLR4 expression level was comparable, LPS-induced cytokine production was up-regulated by enforced expression of PU.1. These results suggest that function and/or expression of intracellular molecules relating LPS-stimulation signal were enhanced by PU.1, resulting in obtaining monocyte-like characters. However, TLR4-signaling pathway was not found by pathway analysis, suggesting that one or a few key molecules in TLR4-signaling cascade was a target of PU.1 in contrast to the case of FcεRI-signaling pathway. So far, we cannot identify the putative molecules by searching the microarray data. We will continue further detailed analysis to clarify the target of PU.1, which regulates magnitude of LPS-stimulation signaling.

Effect of enforced expression of PU.1 is mainly suppressive for mast cell characters and an amplifier for monocyte characters. In

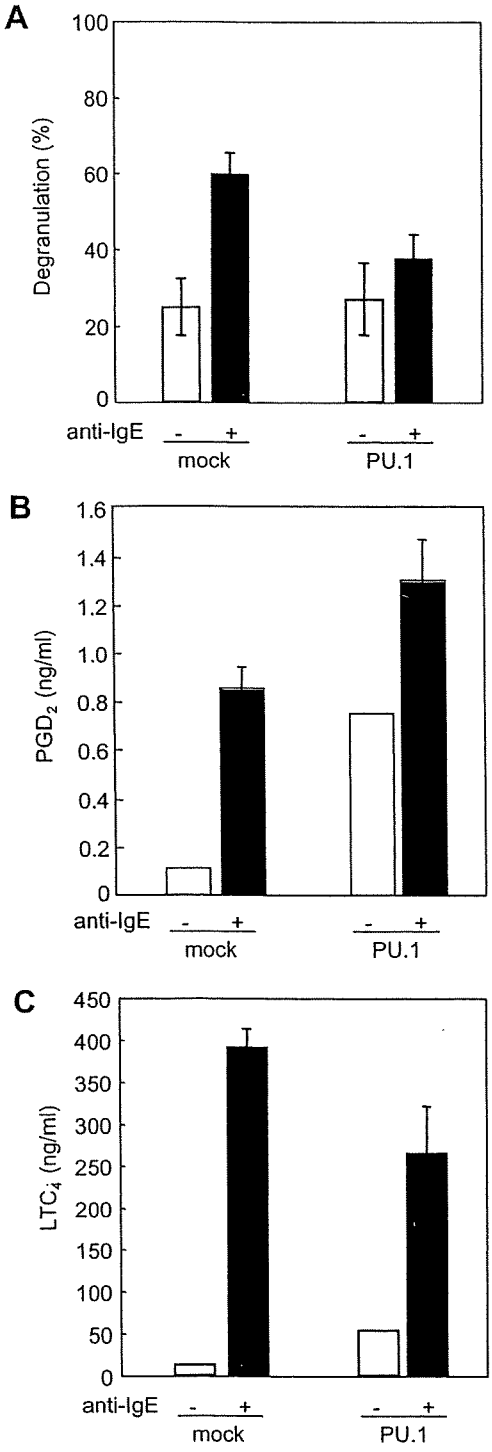
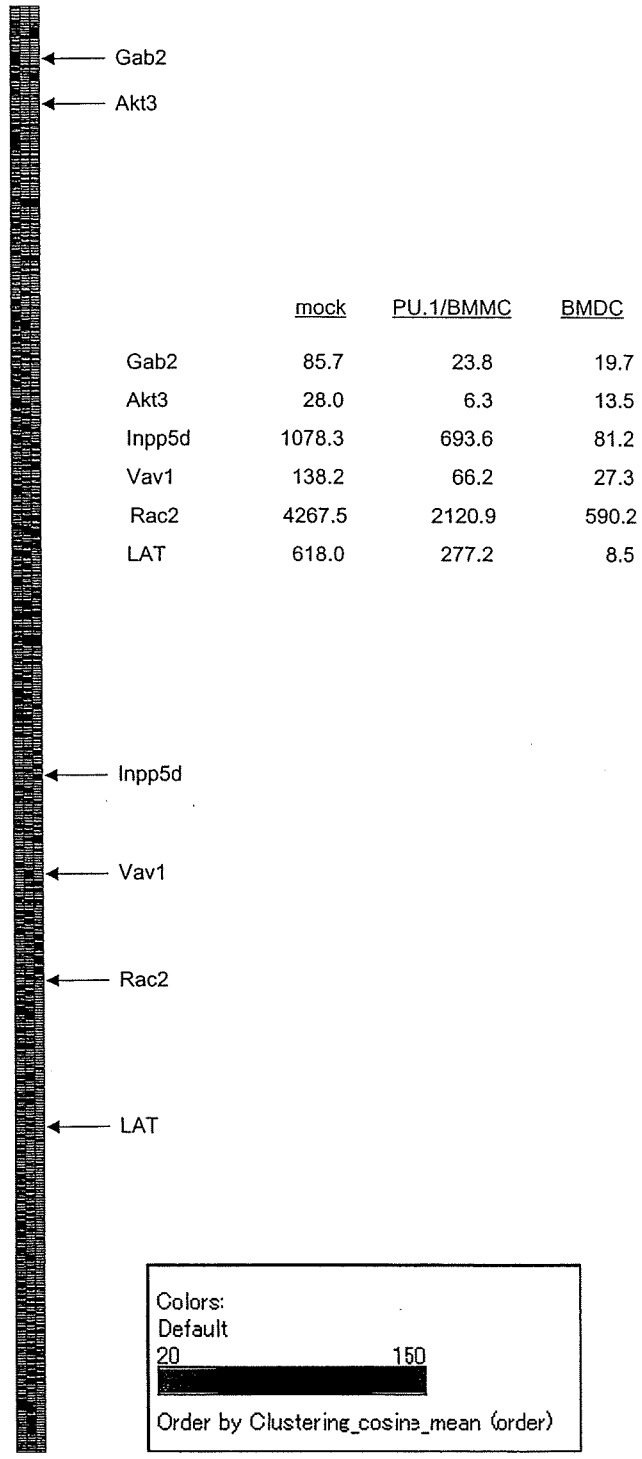


Fig. 3. Generation of eicosanoids and degranulation degree. (A) β-Hexosaminidase release. β-Hexosaminidase activity in supernatant was measured after 30 min incubation from stimulation by IgE/anti-IgE (+) or IgE alone (−). PGE_2 (B) LTC_4 (C) generation levels. Each concentration in culture medium after 30 min of stimulation by IgE/anti-IgE (+) or IgE alone (−) was measured by ELISA. A result performed with triplicate was shown as means ± SD and similar result was observed in another independent experiment in A, B, and C.



	mock	PU.1/BMMC	BMDC
Gab2	85.7	23.8	19.7
Akt3	28.0	6.3	13.5
Inpp5d	1078.3	693.6	81.2
Vav1	138.2	66.2	27.3
Rac2	4267.5	2120.9	590.2
LAT	618.0	277.2	8.5

Fig. 4. Gene expression profiles in BMMC, PU.1-overexpressing cells, and BMDC. Representation of mRNA expression levels in control BMMC (left), PU.1-overexpressing BMMC (middle), and BMDC (right). One row of colored bars represents one gene and the color bars capture the magnitude of the response for each gene according to the scale shown. A cluster of genes that were highly expressed in BMMC, a low level in BMDC and reduced by overexpression of PU.1 is shown.

some points such as production of TNF-α and PGD_2 in response to IgE-stimulation, the effect of PU.1 appears to be complex. However, considering that TNF-α and PGD_2 are abundantly produced from activated monocytes, increased transcription magnitude of these genes may be reasonable. We will further study regulation of PU.1 function in activated monocytes to clarify this observation.

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NK cells contribute to the skin graft rejection promoted by CD4$^+$ T cells activated through the indirect allorecognition pathway

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Keywords: NKG2D, transplantation

Abstract

Rejection of solid organ allografts is promoted by T cells. Recipient T cells can directly recognize intact allo-MHC molecules on donor cells and can also indirectly recognize processed donor-derived allo-peptides presented by recipient antigen-presenting cells in the context of self-MHC molecules. Although CD4$^+$ T cells primed through the indirect allorecognition pathway alone are sufficient to promote acute allograft rejection, it is unknown how they can mediate graft destruction without cognate recognition of donor cells. In this study, we analyzed the indirect effector mechanism of skin allograft rejection using a mouse model in which SCID recipients bearing MHC class II-deficient skin allografts were adoptively transferred with CD4$^+$ T cells. Histologically, entire graft necrosis was preceded by mononuclear cell infiltration in the graft epithelia with epithelial cell apoptosis, indicating cell-mediated cytotoxicity against donor cells as an effector mechanism. Beside CD4$^+$ T cells and macrophages, NK cells infiltrated in the rejecting grafts. Depletion of NK cells as well as blocking of the activating NK receptor NKG2D allowed prolonged survival of the grafts. Expression of NKG2D ligands was up-regulated in the rejecting grafts. These results suggest that NK cells activated through NKG2D contribute to the skin allograft rejection promoted by indirectly primed CD4$^+$ T cells.

Introduction

Rejection of solid organ allografts is promoted by recipient T cells recognizing donor allo-antigens. Allorecognition can occur by two distinct, but not mutually exclusive, pathways (1). In the direct pathway, recipient T cells recognize intact allo-MHC molecules on donor antigen-presenting cells (APCs). In the indirect pathway, recipient T cells recognize donor-derived allo-peptides that have been processed and presented by recipient APCs in the context of self-MHC molecules. The direct pathway of sensitization generates CD4$^+$ and CD8$^+$ effector CTLs, either of which alone can mediate acute allograft rejection through cognate recognition of graft parenchymal cells (2). In addition, CD4$^+$ T cells primed through the direct pathway provide help for CD8$^+$ CTL generation and activate cells of the innate immune system, particularly macrophages and eosinophils, which may also contribute to the effector phase of allograft rejection (2).

CD4$^+$ T cells primed through the indirect pathway were also shown to provide help for CD8$^+$ CTLs (3). There was compelling evidence that indirectly primed CD4$^+$ T cells alone are sufficient to promote skin allograft rejection (4–9). In this setting, however, graft destruction cannot be mediated by direct cytotoxicity of the effector CD4$^+$ T cells through cognate recognition of graft parenchymal cells, because the determinants recognized by indirectly primed CD4$^+$ T cells were not expressed on donor cells (4–9). Macrophage-dependent delayed-type hypersensitivity (DTH) and eosinophil-mediated tissue damage were suggested as the indirect effector mechanisms (2, 6–9). In addition, other cells of the innate immune system including NK cells and

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neutrophils may also participate in allograft rejection (10–15). However, the role for the innate immune system in the skin allograft rejection promoted by indirectly primed $CD4^+$ T cells has not been firmly established yet.

It was reported that indirectly primed (cross-primed) $CD8^+$ T cells can also promote skin allograft rejection by themselves (16, 17). These studies demonstrated that the cross-primed $CD8^+$ T cells mediate skin allograft rejection through cognate recognition of vascular endothelial cells of recipient-derived blood vessels that cross-present donor allo-antigens in the context of self-MHC molecules (16, 17). The cross-primed $CD8^+$ effector CTLs damage recipient-derived blood vessels feeding the grafts through direct cytotoxicity that eventually results in ischemic injury leading to graft necrosis (16, 17). It remains unknown, however, whether similar effector mechanism could be operative also in the skin allograft rejection promoted by indirectly primed $CD4^+$ T cell, because vascular endothelial cells can directly activate $CD8^+$ T cells (18) but are poor APCs for $CD4^+$ T cells (19).

In this study, we analyzed the effector mechanism of skin allograft rejection promoted by indirectly primed $CD4^+$ T cells. Here we show that skin allograft rejection promoted by indirectly primed $CD4^+$ T cells is primarily mediated by NK cells rather than by other cells of the innate immune system or by ischemic injury through vascular damage. Indirectly primed $CD4^+$ T cells may trigger activation of NK cells through the activating NK receptor NKG2D in the allografts by up-regulating NKG2D ligands on donor cells.

Materials and methods

Mice

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan (Yokohama, Japan). C.B-17/Icr-*scid*/*scid* (SCID) mice (H-2^d) were obtained from CLEA Japan (Tokyo, Japan). B6;129S-*H2*$^{dlAb1\text{-}Ea}$ (MHC class II-deficient) mice (20) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a C57BL/6 background. Female mice were used at 6–10 weeks of age. All procedures have been approved by the Animal Care Committee of Niigata University following the institutional guidelines.

Skin grafting, $CD4^+$ T-cell purification and adoptive transfer

SCID mice were transplanted with full-thickness trunk skin allografts from MHC class II-deficient or wild-type C57BL/6 mice as described (21). In some experiments, allogeneic (MHC class II-deficient) and syngeneic control (BALB/c) skin grafts were placed side by side on the same recipient mice. The skin grafts were allowed to heal for at least 14 days before $CD4^+$ T-cell reconstitution.

$CD4^+$ T cells were prepared from BALB/c spleen cells using Mouse T Cell CD4 Subset Column Kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. The purity of the $CD4^+$ T cells was >92% and contaminating $CD8^+$ T cells were <1%, as determined by flow cytometry.

The SCID recipients bearing healthy skin grafts were reconstituted with 1×10^7 purified $CD4^+$ T cells. The skin grafts were observed daily and defined as rejected when complete loss of the intact epithelia occurred. At the time of rejection, spleen cells were harvested and analyzed by flow cytometry to confirm the $CD4^+$ T-cell reconstitution without detectable $CD8^+$ T cells (<1%).

In vivo NK-cell depletion and NKG2D-blocking

To deplete NK cells *in vivo*, recipient mice were injected intra-peritoneally (i.p.) with 200 μg of rabbit anti-asialo GM1 antibody (Wako Chemicals, Osaka, Japan) or control rabbit Ig every third day from 2 days before $CD4^+$ T-cell reconstitution until rejection. NK-cell depletion was confirmed by flow cytometric analysis of peripheral blood lymphocytes and spleen cells.

To block NKG2D *in vivo*, recipient mice were injected i.p. with 250 μg of neutralizing hamster anti-mouse NKG2D mAb (clone HMG2D) or control hamster Ig twice a week from 2 days after $CD4^+$ T-cell reconstitution until rejection. The HMG2D mAb was generated by immunizing an Armenian hamster with mouse NKG2D–Fc fusion protein (R&D Systems). This mAb blocks NKG2D-mediated killing of retinoic acid early inducible-1 (RAE-1)-transfected RMA cells by NK cells *in vitro* and reverses the rejection of RAE-1-transfected RMA cells in C57BL/6 mice *in vivo* (H. Yagita, unpublished data). NK cells were not depleted by *in vivo* treatment with the HMG2D mAb (Supplementary Figure 1, available at *International Immunology* Online).

Histology

The skin grafts were dissected 3, 10, 14, 16, 18 and 22 days after $CD4^+$ T-cell reconstitution or 14 and 100 days after transplantation on unreconstituted SCID mice. Formalin-fixed paraffin sections were stained with hematoxylin and eosin. Apoptosis was analyzed by TUNEL assay using *In Situ* Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany).

Graft-infiltrating cells

Graft-infiltrating cells were isolated as described previously (22). Briefly, the skin grafts were dissected 14 days after $CD4^+$ T-cell reconstitution and digested in Iscove's modified Dulbecco's medium containing 0.1 mg ml^{-1} DNase I, 3.3 mg ml^{-1} collagenase type I and 1 mg ml^{-1} hyaluronidase type IV-S (all from Sigma–Aldrich, St Louis, MO, USA) in a shaking water bath at 37°C for 90 min. The digested skin was sequentially filtered through 70- and 30-μm nylon meshes.

Flow cytometry

Cells were re-suspended in PBS supplemented with 2% FCS and 0.1% NaN_3. After pre-incubation with anti-CD16/CD32 mAb (clone 2.4G2; BD Biosciences, San Jose, CA, USA), cells were stained with saturating amounts of the following mAbs: biotin- or PE-conjugated anti-CD4 (H129.19, BD Biosciences), FITC-conjugated anti-CD8α (53-6.7, BD Biosciences), biotin- or PE-conjugated anti-CD49b (DX5, BD Biosciences), biotin-conjugated anti-F4/80 (CI:A3-1, Serotec, Oxford, UK), biotin-conjugated anti-NKG2D (C7, eBioscience, San Diego, CA, USA) and FITC- or PE-conjugated isotype

control mAbs. Biotin-conjugated mAbs were visualized with streptavidin–PE (Southern Biotechnology, Birmingham, AL, USA) or streptavidin–Quantum Red conjugate (Sigma–Aldrich). After gating on forward and side scatters and propidium iodide, viable cells were analyzed using the FACScan flow cytometer with the CellQuest Pro software (BD Biosciences).

RT–PCR

Total RNA was extracted from the cells and tissues and reverse transcribed using random hexamers (23). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene expression levels in each cDNA preparations were semi-quantified as described (23). The amount of cDNA was normalized according to the GAPDH mRNA levels, and 10-fold serial dilutions of cDNA were amplified using RAE-1-specific primers. The GAPDH and RAE-1 primers were described elsewhere (23, 24). PCR conditions for 30 cycles were 94°C for 45 s, 55°C for 45 s and 72°C for 1.5 min. PCR products were separated on 1.5% agarose gels and visualized with ethidium bromide. RAW264.7 (25) and Pam 212 (23) tumor cells were used as positive and negative controls for RAE-1 expression, respectively.

Statistical analysis

Differences in the numbers of graft-infiltrating-cell subpopulations were evaluated by *t*-test. Mann–Whitney's *U*-test was used to compare the graft survival time. Differences were considered significant if two-tailed *P*-values were <0.05. All statistical analyses were performed using the JMP software (SAS Institute, Cary, NC, USA).

Results

Skin allograft rejection promoted by indirectly primed CD4$^+$ T cells exhibits histological features indicating cell-mediated cytotoxicity against graft epithelial cells as an effector mechanism

To analyze the effector mechanism of skin allograft rejection promoted by indirectly primed CD4$^+$ T cells, we used an adoptive transfer model with SCID mice bearing MHC class II-deficient skin allografts (5), in which graft rejection is mediated exclusively by transferred CD4$^+$ T cells that can recognize allo-antigens only through the indirect pathway. In the original report (5), adoptive transfer of 5×10^6 CD4$^+$ T cells into SCID recipients bearing MHC class II-deficient skin allografts was shown to cause slow rejection or sometimes to be insufficient for rejection. We confirmed that this was, at least in part, because of the inefficient reconstitution of SCID recipients by CD4$^+$ T cells (data not shown). Therefore, we increased the number of transferred CD4$^+$ T cells in this study. When we transferred 1×10^7 CD4$^+$ T cells, all SCID recipients were successfully reconstituted by CD4$^+$ T cells and all grafts were uniformly rejected within 22 days, while all grafts survived indefinitely (>100 days) in unreconstituted SCID mice. Macroscopic rejection based on visual inspection usually started 16–18 days after CD4$^+$ T-cell reconstitution, at that time entire graft necrosis was observed histologically (data not shown). Histological analysis of the rejecting allografts at an earlier time point (14 days after CD4$^+$ T-cell reconstitution) revealed epidermal thickening, mononuclear cell infiltration in the graft epidermis and hair follicles and epithelial cell apoptosis (Fig. 1A). Eosinophils or neutrophils were not observed in the rejecting allografts (Fig. 1A). Syngeneic control skin grafts placed on the same recipient mice as well as surviving allografts on unreconstituted SCID mice exhibited essentially normal histology (Fig. 1B and C). Because entire graft necrosis was preceded by intra-epithelial infiltrates with epithelial cell apoptosis, it is unlikely that the allograft rejection was primarily mediated by DTH or ischemic injury through vascular damage. Rather, these histological findings indicate cell-mediated direct cytotoxicity against graft epithelial cells as an effector mechanism.

NK cells infiltrate in the rejecting skin allografts

To identify the cytotoxic effector cells other than CD4$^+$ T cells that mediated epithelial cell damage, we analyzed graft-infiltrating cells isolated from the rejecting skin allografts 14 days after CD4$^+$ T-cell reconstitution. Flow cytometric analysis of the graft-infiltrating cells revealed that beside CD4$^+$ T cells and macrophages, considerable numbers of NK cells infiltrated in the rejecting allografts (Fig. 2, upper panels). No CD8$^+$ T cells were detected in the graft-infiltrating cells (data not shown).

In vivo depletion of NK cells delays the skin allograft rejection promoted by indirectly primed CD4$^+$ T cells

Recent studies demonstrated contribution of NK cells to rejection of vascularized cardiac and liver allografts (12–15). To determine whether NK cells also participate in the skin allograft rejection promoted by indirectly primed CD4$^+$ T cells, NK cells were depleted by *in vivo* treatment with anti-asialo GM1 antibody. Survival of the skin allografts from wild-type mice, which can be recognized by CD4$^+$ T cells through both direct and indirect pathways, was not affected by NK-cell depletion (Fig. 3A). In contrast, survival of MHC class II-deficient skin allografts, which can be recognized by CD4$^+$ T cells only through the indirect pathway, was significantly prolonged by NK-cell depletion (Fig. 3B).

Although anti-asialo GM1 antibody reacts not only with NK cells but also with a subset of alloreactive CTLs (26), flow cytometric analysis of the graft-infiltrating cells revealed that NK cells were selectively depleted by the *in vivo* treatment with anti-asialo GM1 antibody (Fig. 2, middle panels). Despite nearly complete depletion of NK cells in the peripheral blood and spleen of anti-asialo GM1 antibody-treated mice (data not shown), a small number of NK cells could still be detected in the skin allografts (Fig. 2, middle panel). The incomplete depletion of NK cells may explain the prolonged survival, but not permanent acceptance, of the grafts by the *in vivo* treatment with anti-asialo GM1 antibody (Fig. 3B). Supporting this, the rejecting allografts in anti-asialo GM1 antibody-treated mice exhibited histological features similar to that of untreated mice (Supplementary Figure 2, available at *International Immunology* Online). Alternatively, other cells of the innate immune system, in particular, macrophages, which infiltrated

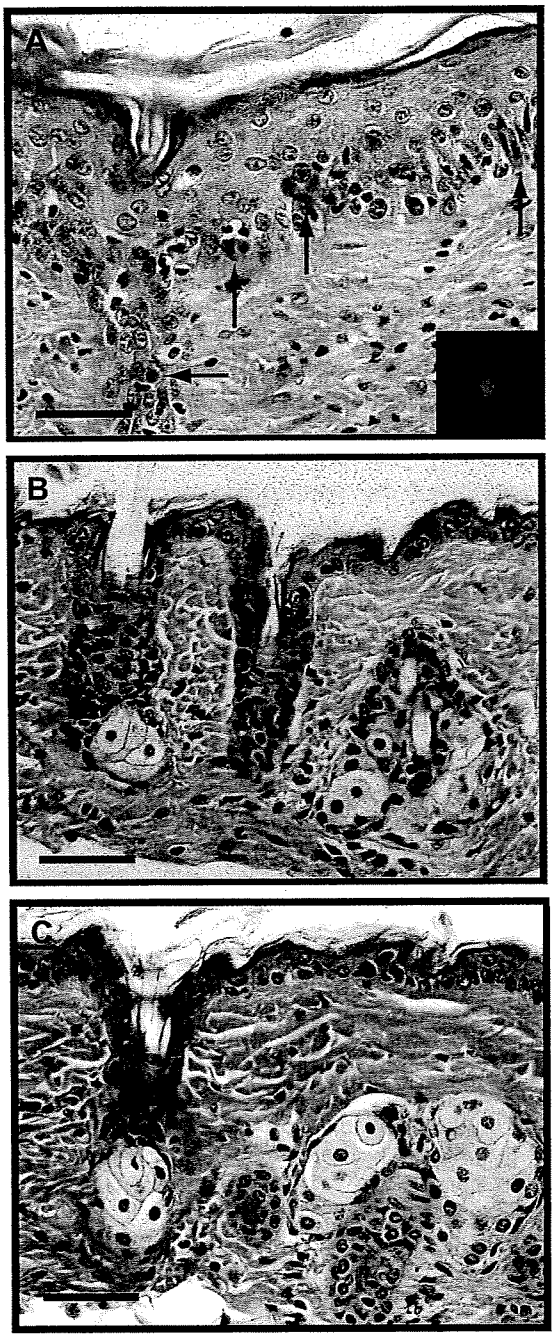

Fig. 1. Histology of the rejecting skin allografts. SCID mice were transplanted with MHC class II-deficient skin allograft (A and C) and syngeneic skin graft (B). The skin grafts were dissected 14 days after $CD4^+$ T-cell reconstitution (A and B) or 100 days after transplantation on unreconstituted SCID mice (C) for histological analysis. (A) Rejecting skin allograft showed epidermal thickening and mononuclear cell infiltration into the graft epidermis and hair follicles. Arrows indicate epithelial cell apoptosis that was confirmed by TUNEL assay (inset). (B and C) Syngeneic control skin graft on the same recipient mouse as well as surviving allograft on unreconstituted SCID mouse showed essentially normal histology without intra-epithelial infiltrates or apoptotic epithelial cells. The photographs are representative of the histological findings for three mice. Bars, 50 μm.

in the rejecting allografts (Fig. 2), might also contribute to the graft rejection as suggested by the prior studies (6–9). Nevertheless, these results indicate that NK cells play an essential role in the skin allograft rejection promoted by indirectly primed $CD4^+$ T cells.

In vivo blocking of NKG2D delays the skin allograft rejection promoted by indirectly primed $CD4^+$ T cells

Activation of NK cells is regulated by the balance between positive and negative signals through various activating and inhibitory receptors (27). Recent studies demonstrated critical roles for the activating NK receptor NKG2D (28) in NK-cell-mediated rejection of bone marrow grafts (29) as well as in vascularized cardiac allograft rejection in the absence of CD28-co-stimulation (30) that is mediated by NK cells and $CD4^+$ and $CD8^+$ T cells (12, 13, 31). To determine the role for NKG2D in NK-cell activation in the skin allograft rejection promoted by indirectly primed $CD4^+$ T cells, NKG2D was blocked by *in vivo* treatment with neutralizing anti-NKG2D mAb. As shown in Fig. 4, NKG2D-blocking significantly prolonged survival of MHC class II-deficient skin allografts in SCID recipients reconstituted with $CD4^+$ T cells. *In vivo* treatment with neutralizing anti-NKG2D mAb did not affect the infiltration of NK cells in the allografts (Fig. 2, lower panels). Therefore, NKG2D plays a critical role in NK-cell activation also in the skin allograft rejection promoted by indirectly primed $CD4^+$ T cells. As similar to NK-cell depletion, NKG2D-blocking could not induce permanent graft acceptance (Fig. 4), probably because of incomplete blocking by the *in vivo* antibody treatment, involvement of activating NK receptors other than NKG2D or contribution of effector cells other than NK cells.

Expression of NKG2D ligands is up-regulated in the rejecting skin allografts

In mice, members of the RAE-1 protein family, minor histocompatibility antigen H60 and murine UL-16-binding protein-like transcript-1 act as ligands for NKG2D (28, 32). Most normal healthy tissues including the skin do not express NKG2D ligands, but their expression is up-regulated in a wide variety of conditions (24, 28, 32). Expression of NKG2D ligands was demonstrated in human renal and pancreatic allografts (33) as well as in mouse skin and cardiac allografts (30, 34). To determine whether NKG2D ligands are also induced in the rejecting skin allografts in our model, we analyzed RAE-1 mRNA expression, because MHC class II-deficient donor mice on a C57BL/6 background can express RAE-1δ and RAE-1ε but not H60, and expression of murine UL-16-binding protein-like transcript-1 is regulated at a level other than transcription (28, 32). RT–PCR using the primers capable of amplifying all five RAE-1 family members revealed that before $CD4^+$ T-cell reconstitution, RAE-1 was not expressed in the allografts or syngeneic grafts that had been allowed to heal for 14 days on unreconstituted SCID mice (Fig. 5). Up-regulation of RAE-1 expression was observed only in the rejecting allografts 10–14 days after $CD4^+$ T-cell reconstitution, but not in the syngeneic grafts placed on the same recipient mice (Fig. 5).

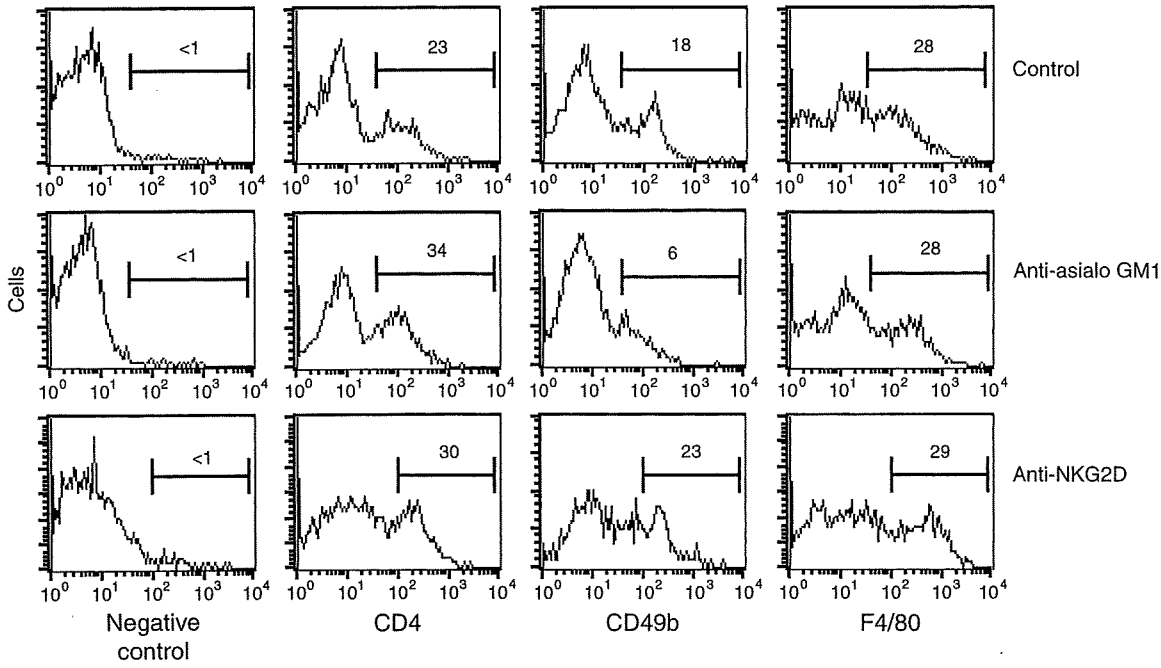

Fig. 2. Graft-infiltrating cells. Graft-infiltrating cells were isolated from MHC class II-deficient allografts on SCID recipients 14 days after $CD4^+$ T-cell reconstitution. Cells were stained with streptavidin–PE alone (negative control) or biotin-conjugated anti-CD4, anti-CD49b and anti-F4/80 mAbs. In control mice without antibody treatment, graft-infiltrating cells consisted of $CD4^+$ T cells, $CD49b^+$ NK cells and $F4/80^+$ macrophages (upper panels). *In vivo* treatment with anti-asialo GM1 antibody selectively depleted NK cells in the graft-infiltrating cells (middle panels). *In vivo* treatment with neutralizing anti-NKG2D mAb did not affect the infiltration of NK cells in the allografts (lower panels). Representative profiles from three independent experiments are shown. Mean ± SD (%) of each sub-populations (control versus anti-asialo GM1 antibody-treated versus anti-NKG2D mAb-treated mice, $n = 3$) were 24 ± 6 versus 31 ± 8 versus 24 ± 9 for $CD4^+$ T cells, 20 ± 3 versus 5 ± 1 versus 21 ± 4 for NK cells and 26 ± 4 versus 25 ± 5 versus 26 ± 5 for macrophages. Statistically significant difference in sub-population of the graft-infiltrating cells was evident only for NK cells in control versus anti-asialo GM1 antibody-treated mice ($P = 0.008$).

Discussion

In this study, we have shown that NK cells contribute to the skin allograft rejection promoted by indirectly primed $CD4^+$ T cells and that NKG2D plays an essential role in the NK-cell activation in the rejecting allografts.

Although the crucial role for NK cells in rejection of bone marrow grafts is well established, involvement of NK cells in rejection of solid organ allografts has been shown only in limited studies (10–15). Among these studies, the most compelling evidence for NK-cell contribution in acute allograft rejection was provided by the vascularized cardiac allograft model in CD28-deficient mice (12, 13). The cardiac allograft rejection in CD28-deficient mice depends on NK cells (12, 13) as well as both $CD4^+$ and $CD8^+$ T cells (31), but the precise role for NK cells in this model is unclear. It was proposed that NK cells may participate in the sensitization phase of rejection by providing help for CTL generation that in normal mice would be provided by CD28 (10–13). Using the same model, Kim *et al.* (30) recently demonstrated that *in vivo* blocking of NKG2D prolongs survival of cardiac allografts without affecting the infiltration of NK cells or $CD8^+$ T cells in the allografts. Based on these findings, they concluded that $NKG2D^+$ cells act as cytotoxic effector cells directly mediating graft destruction in the effector phase of rejection (30). However, it remains unknown whether the prolonged survival of cardiac allografts was due to NKG2D-blocking on NK cells or $CD8^+$ T cells (30), because NKG2D is expressed not only on NK cells but also on activated $CD8^+$ T cells (28, 32). Our results suggest that in our model, NK cells mediate graft destruction through cognate recognition of and cytotoxicity against graft epithelial cells in the effector phase of rejection. Although we could not directly show NKG2D expression on the graft-infiltrating NK cells because of the sensitivity of NKG2D to enzymatic digestion during the isolation steps of graft-infiltrating cells (23), in our model, in which $CD8^+$ T cells are absent, NK cells would be the only cell population in the rejecting allografts that can express NKG2D on the cell surface (32).

Kim *et al.* (30) reported that expression of NKG2D ligands is up-regulated in a biphasic fashion in skin and cardiac grafts. The early transient up-regulation occurs both in syngeneic grafts and allografts (30). Therefore, it would be triggered by nonspecific inflammation and/or ischemia–reperfusion injury after transplantation (30). In contrast, the late up-regulation that occurs only in the rejecting allografts is mediated by T-cell-dependent adaptive immune response (30). Because we used an adoptive transfer model, in which skin grafts had been well-healed before $CD4^+$ T-cell reconstitution, the early transient up-regulation of NKG2D ligands in syngeneic grafts and allografts had returned to basal levels at the time of $CD4^+$ T-cell reconstitution (30). Up-regulation of NKG2D ligands in the rejecting skin allografts, but not in syngeneic grafts, at the late phase of rejection agrees with the observation by Kim *et al.* (30). In our model, the late

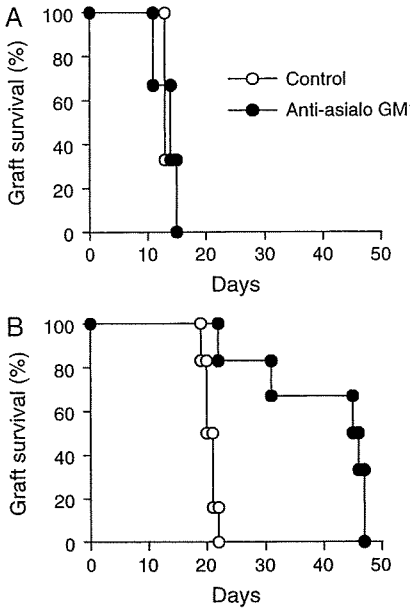

Fig. 3. NK-cell depletion by *in vivo* treatment with anti-asialo GM1 antibody prolongs survival of MHC class II-deficient, but not wild-type, skin allografts in $CD4^+$ T-cell-reconstituted SCID recipients. SCID mice were transplanted with skin allografts from wild-type (A) or MHC class II-deficient (B) mice and treated with control rabbit Ig (open circles) or rabbit anti-asialo GM1 antibody (filled circles) after $CD4^+$ T-cell reconstitution. (A) Survival of wild-type skin allografts was not affected by NK-cell depletion. Median survival time (MST) of six mice per group was 14 (range 11–15) days for control and 13 (13–15) days for anti-asialo GM1 antibody-treated mice (P = 1.000). (B) Survival of MHC class II-deficient skin allografts was significantly prolonged by NK-cell depletion. MST of six mice per group was 21 (19–22) days for control and 46 (22–47) days for anti-asialo GM1 antibody-treated mice (P = 0.002).

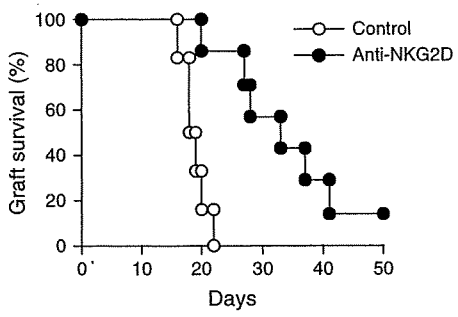

Fig. 4. NKG2D-blocking by *in vivo* treatment with neutralizing anti-NKG2D mAb prolongs survival of MHC class II-deficient skin allografts in $CD4^+$ T-cell-reconstituted SCID recipients. SCID mice were transplanted with MHC class II-deficient skin allografts and treated with control hamster Ig (open circles) or hamster anti-NKG2D mAb (filled circles) after $CD4^+$ T-cell reconstitution. NKG2D-blocking significantly prolonged skin allograft survival. Median survival time was 19 (range 16–22) days for control (n = 6) and 33 (20–75) days for anti-NKG2D mAb-treated mice (n = 7) (P = 0.002).

up-regulation would be mediated by indirectly primed $CD4^+$ T cells infiltrating the allografts, because it corresponded with the kinetics of $CD4^+$ T-cell infiltration in the allografts. Notably, the early transient up-regulation of NKG2D ligands after transplantation alone cannot lead to NK-cell-mediated

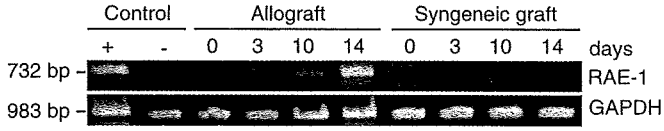

Fig. 5. Up-regulation of RAE-1 expression in the rejecting skin allografts. SCID mice were transplanted with MHC class II-deficient skin allograft and syngeneic skin graft. The skin grafts were dissected 14 days after transplantation (=0 day before $CD4^+$ T-cell reconstitution) and 3, 10 and 14 days after $CD4^+$ T-cell reconstitution. RT-PCR for RAE-1 mRNA was performed using cDNA diluted 1:10 for semi-quantification and compared with GAPDH housekeeping gene expression levels. Representative data from two independent experiments are shown.

graft rejection in unreconstituted SCID recipients. Therefore, sustained expression of NKG2D ligands in the allografts that is mediated by T-cell-dependent adaptive immune response may be required for NK-cell activation leading to graft destruction. In our model, indirectly primed $CD4^+$ T cells may induce inflammation by DTH and/or ischemic injury through vascular damage that may up-regulate and maintain the expression of NKG2D ligands in the allografts. It is also possible that inflammation induced by indirectly primed $CD4^+$ T cells may be required for recruitment of NK cells into the allografts in addition to NK-cell activation through up-regulation of NKG2D ligands on donor cells.

In summary, this study demonstrates that indirectly primed $CD4^+$ T cells, which cannot directly interact with donor cells, can induce sustained expression of NKG2D ligands in the skin allografts. This may trigger activation of NK-cell cytotoxicity against graft epithelial cells through NKG2D that eventually results in graft destruction. This conclusion implies that NK-cell depletion or NKG2D-blocking may be an additional strategy for the therapeutic intervention of allograft rejection. However, contribution of NK cells to tolerance induction rather than graft rejection has also been demonstrated in the prior studies (10, 11). Another implication of this study is that $CD4^+$ T cells may collaborate with NK cells to eliminate infected or tumor cells with which $CD4^+$ T cells cannot directly interact due to down-regulation or lack of MHC class II molecules, because the indirect pathway of allorecognition is analogous to the physiological pathway of foreign antigen recognition.

Supplementary data

Supplementary figures are available at *International Immunology* Online.

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Abbreviations

APC	antigen-presenting cell
DTH	delayed-type hypersensitivity

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
i.p.	intra-peritoneally
RAE-1	retinoic acid early inducible-1

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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 $MHC\text{-}II^+OX40L^{high}$ 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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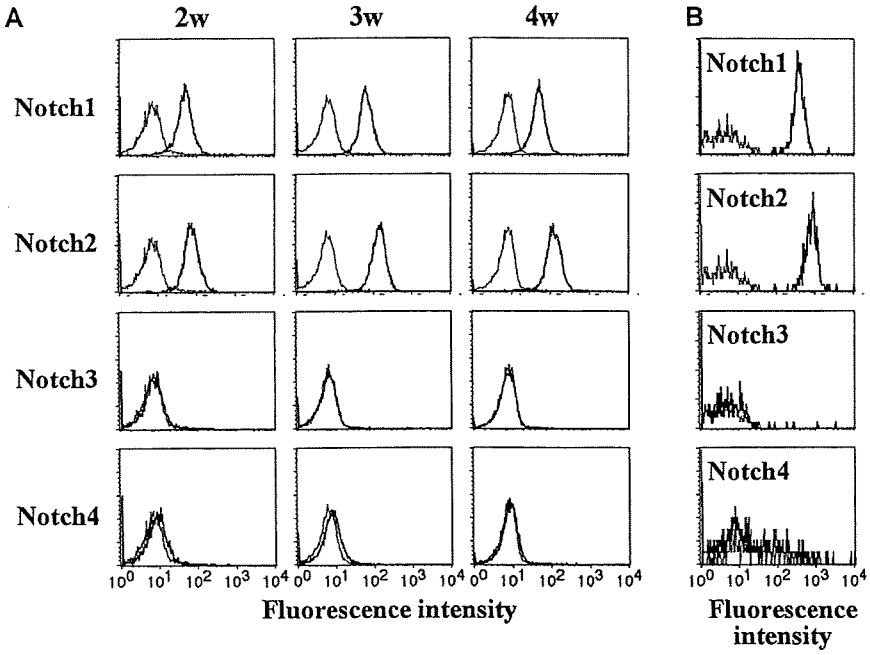

FIG 1. Cell surface expression of Notch receptors on mast cells. The expressions of Notch 1, 2, 3, or 4 *(bold lines)* on c-Kit$^+$FcεRIα$^+$ BMMCs cultured for 2, 3, or 4 weeks (**A**) or fresh c-Kit$^+$FcεRIα$^+$ PMCs (**B**) were analyzed by flow cytometry. *Thin lines* represent isotype-matched control.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

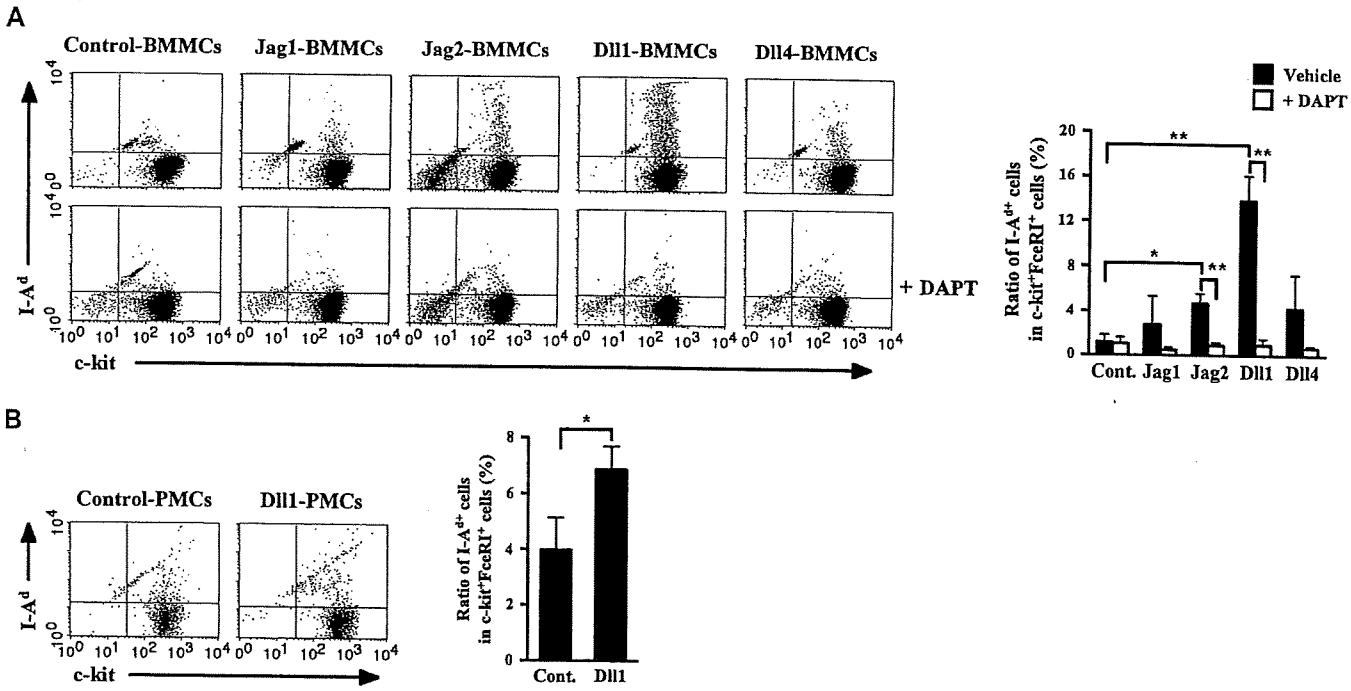

FIG 2. Notch signaling induces MHC-II expression on mast cell surface. BMMCs (A) or PMCs (B) were cocultured with the indicated CHO-Notch ligand—expressing cell lines or CHO control cells in the presence or absence of DAPT. *Cont.*, Control. *Dot blots* show the expressions of MHC-II (I-A) and c-Kit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

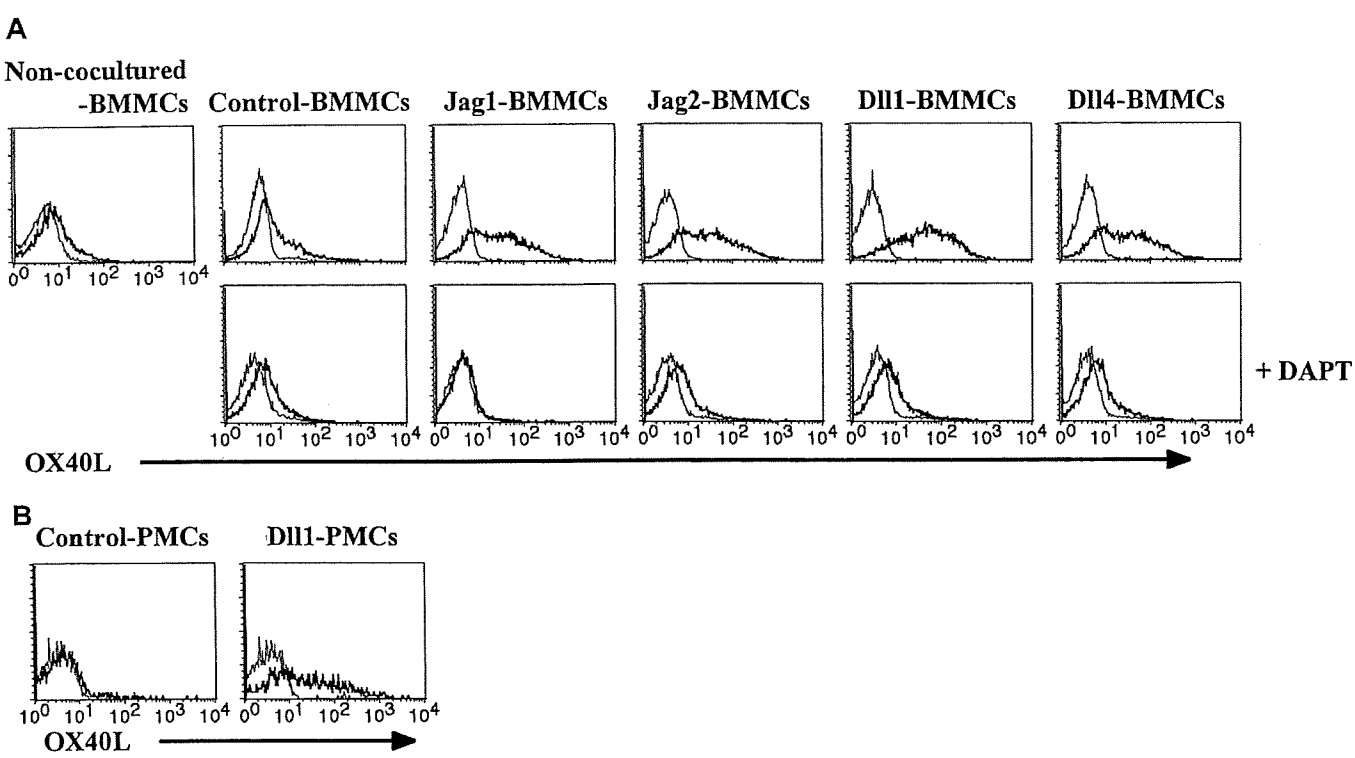

FIG 3. Notch signaling upregulates OX40L expression level on mast cell surface. BMMCs (A) or PMCs (B) were cocultured with the indicated CHO–Notch ligand–expressing cell lines or CHO control cells in the presence or absence of DAPT. The expression of OX40L *(bold lines)* on c-Kit$^+$FcεRIα$^+$ cells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

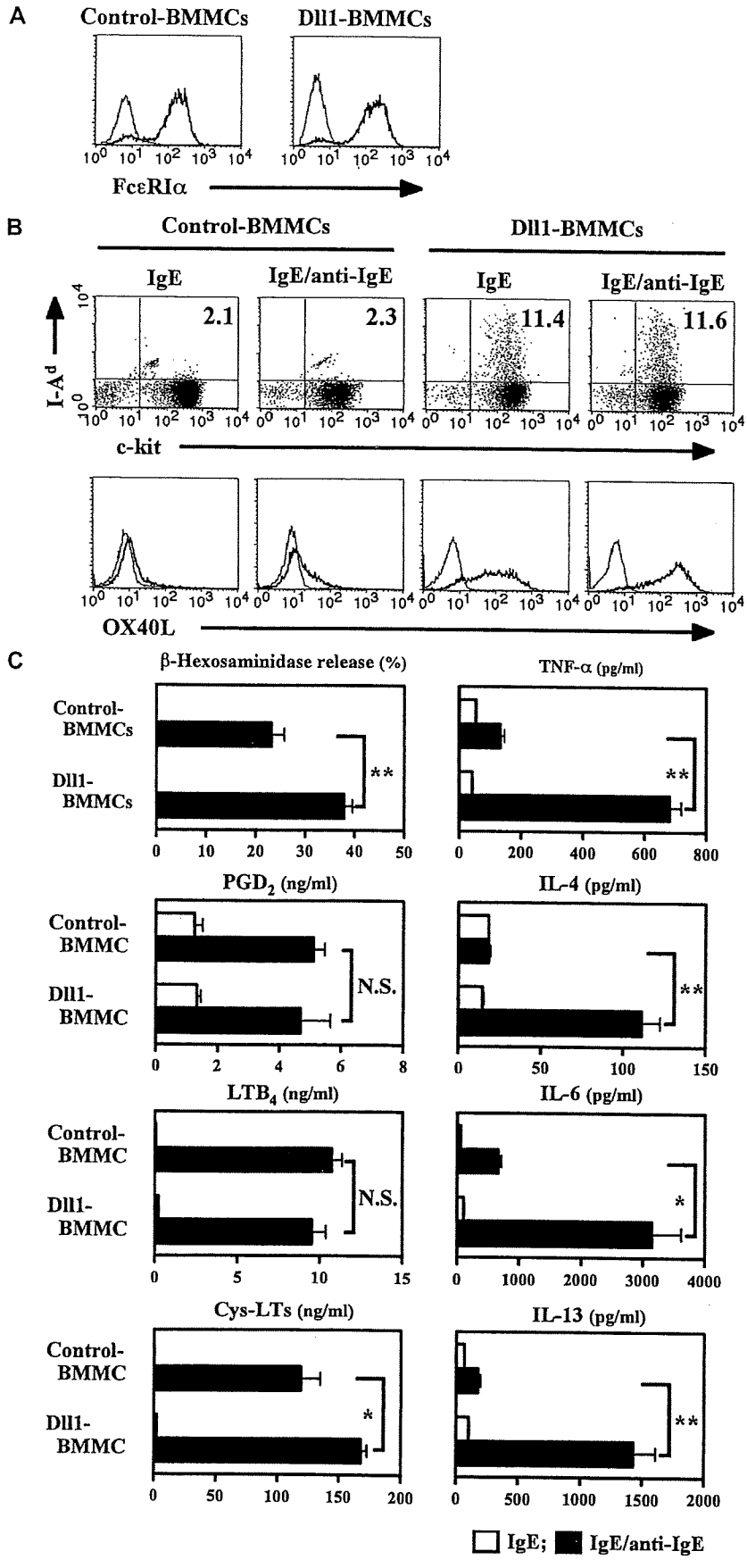

FIG 4. *(See next page for figure legend).*

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.

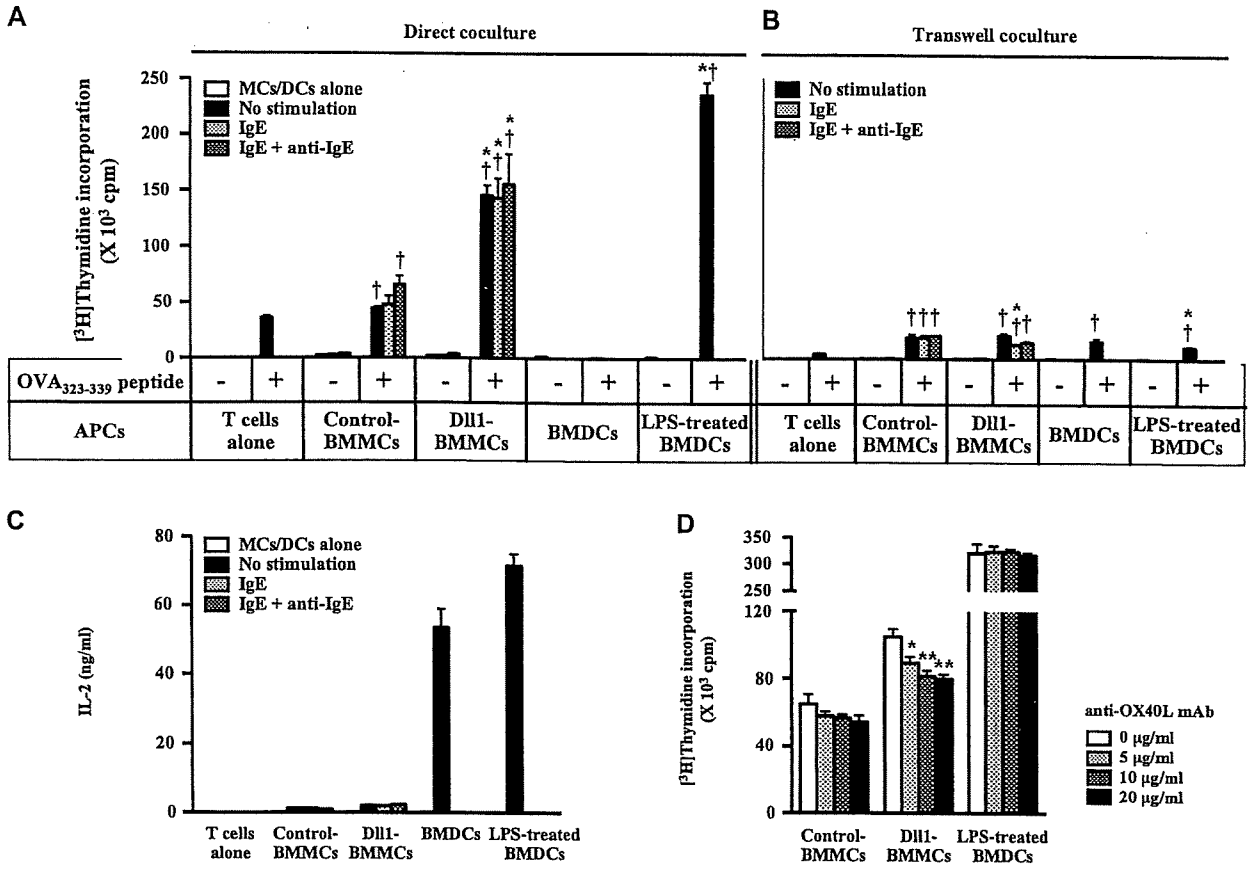

FIG 5. $CD4^+$ T-cell activation mediated by Dll1-BMMCs. [^{3}H]Thymidine incorporation into the $CD4^+$ T cells from DO11.10 mice cocultured together with BMMCs or BMDCs (**A**) or separated from them by a Transwell membrane (**B**) in the presence or absence of ovalbumin *(OVA)* peptide. Data are indicated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

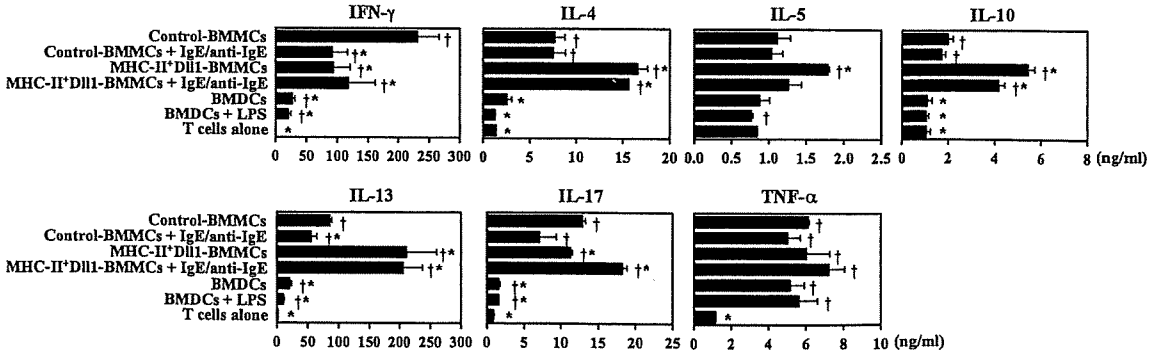

FIG 6. Dll1-BMMCs enhance IL-4, IL-10, and IL-13 production and suppress IFN-γ production by $CD4^+$ T cells. Purified $CD4^+$ T cells from DO11.10 mice were direct cocultured with BMMCs or BMDCs in the presence of ovalbumin peptide. After 4 days of coculture, the cocultured cells were restimulated with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