Regulation of dendritic-cell differentiation by bone marrow stroma via different Notch ligands

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Notch is a major factor mediating interaction between hematopoietic progenitor cells (HPCs) and bone marrow stroma (BMS). However its contribution to dendritic cell (DC) differentiation is controversial. We found that main Notch ligands Delta-1 and Jagged-1 had the opposite effect on DC differentiation. Delta-1 promoted generation of fully differentiated DCs, whereas Jagged-1 stimulated accumulation of DC precursors but prevented their transition to terminally differentiated DCs. BMS expressed a substantially higher level of Jagged-1 than Delta-1. Just the opposite expression pattern was observed in spleen stroma (SS). The BMS effect on DC differentiation was similar to that of Jagged-1, whereas the effect of SS was similar to the effect of Delta-1. Down-regulation of Jagged-1 in BMS substantially increased DC differentiation. Experiments in vivo with adoptive transfer of DC precursors further supported the different roles of BMS and SS in DC development. Jagged-1 and Delta-1 equally activated CBF-1/RBPJκ transcription factor, which is a major Notch target. However, they produced a different pattern of activation of Notch target gene *Hes1*. Overexpression of *Hes1* resulted in increased DC differentiation from HPCs. Thus, this study not only revealed the different role of Notch ligands in DC differentiation but also may provide a new insight into regulation of DC differentiation by BMS. (Blood. 2007;109:507-515)

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Introduction

It is widely accepted that differentiation of dendritic cells (DCs) critically depends on bone marrow microenvironment, which consists of 2 major components: a complex network of cytokines and direct physical interaction between hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs) with bone marrow stroma (BMS). Currently, the number of cytokines involved in DC differentiation has been identified.[1-4] However, surprisingly little is known about the direct effect of bone marrow microenvironment on DC differentiation and especially the role of direct cell-cell contact between HPCs and BMS. One of the major factors that mediates interaction between BMS and HPCs is the Notch family of receptors/transcriptional regulators. Notch signals influence multiple processes that govern lineage specification among progenitor cells, programmed cell death, and cellular proliferation. At present, 2 Notch ligand families, Delta and Jagged, have been described.[5] HPCs express primarily Notch-1 and Notch-2 receptors. Each member is a large single heterodimeric receptor composed of noncovalently associated extracellular, transmembrane, and intracellular subunits (ICNs). Notch signaling is initiated by the binding of the extracellular domain of Notch to a Notch ligand. After ligand binding to Notch receptor, ICN is cleaved and translocates to the nucleus.[6] All mammalian Notches seem to use the same basic signaling pathway via CBF-1/RBP-Jκ transcription factors.[7,8] This results in the activation of transcription of target genes including bHLH transcription factors HES, STAT3,[9-11] as well as p21[Cip/Waf], cyclin D1, cyclin A, NF-κB, and others.[8,12-14]

An important role of Notch signaling in differentiation of lymphocytes is established.[5,15-17] The role of Notch signaling in DC differentiation is more controversial. In vivo experiments with a conditional knock out of the Notch-1 gene showed no effect on myeloid development[15,18] or differentiation of plasmacytoid DCs (pDCs).[19] However, constitutive expression of the activated intracellular domain of mouse Notch-1 in 32D myeloid progenitors inhibits granulocytic differentiation and permits expansion of undifferentiated cells.[20] Conditional induction of the constitutively active intracellular domain of Notch-1 promoted myeloid differentiation via RBP-J transactivation[21] and DC differentiation via up-regulation of NF-κB.[12] Incubation of murine bone marrow precursors with Notch ligand Delta-1 and growth factors inhibited myeloid differentiation and promoted an increase in the number of precursors capable of short-term lymphoid and myeloid repopulation.[22] At the same time, immobilized Delta-1 inhibited differentiation of monocytes into mature macrophages with GM-CSF and permitted differentiation into mature DCs.[23] Recent studies have demonstrated that Delta-1 affected differentiation of pDCs[24,25] and Langerhans cells.[26] In contrast, another Notch ligand, Jagged-1, inhibited the differentiation of DCs and promoted accumulation of DC precursors.[27]

We sought to understand the role of BMS in DC differentiation in vitro and in vivo and the role of different Notch ligands in this process. Here, we report surprising findings that different Notch ligands had the opposite effect on DC differentiation. We have demonstrated that although both BMS and splenic stroma (SS) promoted accumulation of DC precursors, BMS but not SS prevented terminal differentiation of DCs and that this effect was mediated by Jagged-1. These experiments may suggest a model for spatial control of DC differentiation and the role of different Notch ligands in this process.

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Materials and methods

This study was approved by University of South Florida Institutional Animal Care and Use Committee (IACUC).

Animals

Six- to 8-week-old female BALB/c and C57B/6 mice were purchased from Harlan (Indianapolis, IN); female Swiss mice (6-8 weeks old), from Charles River Laboratories (Wilmington, MA); and B6.SJL-PtrcaPep3b/BoyJ mice (CD45.1[+]) mice, from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in pathogen-free units of the Division of Comparative medicine at H. Lee Moffitt Cancer Center, University of South Florida.

Reagents

The following antibody-producing hybridomas were purchased from the American Type Culture Collection (Manassas, VA) and used as supernatants: anti-CD4 (L3T4, TIB-207), anti-CD8 (Lyt-2.2, TIB-210), and anti–MHC II (TIB-120). Anti-IA[b], anti-IA[d], anti-CD11b, anti-CD86 (B7-2), anti-CD45, anti-CD45.2, anti-CD11c, anti-CD34, anti–Sca-1, anti–c-Kit, and isotype control antibodies were obtained from PharMingen (San Diego, CA). Anti-F4/80 and antineutrophil (Neu) (clone 7/4) antibodies were purchased from Serotec (Raleigh, NC); antiphycoerythrin (anti-PE) microbeads, from Miltenyi Biotec (Auburn, CA); and anti–mouse Jagged-1 and anti–mouse Delta-1, from Santa Cruz Biotechnology (Santa Cruz, CA). Low-Tox rabbit complement and Lympholyte M were purchased from Cedarlane Laboratories (Hornby, ON). Recombinant murine GM-CSF and IL-4 were obtained from Research Diagnostics (Flanders, NJ); and LPS, from Sigma (St Louis, MO). Mouse Jagged 1 smartpool siRNA was purchased from Dharmacon (Chicago, IL). The monomeric form of the Notch ligand Delta-1 was made by fusing the extracellular domain of Delta-1 to a series of myc epitopes Delta-1[ext-myc28] and was a gift from Dr I. Bernstein.

Cell lines expressing Notch ligands

The expression plasmid of murine Delta-1 (pEF-BOS neo/Delta1-gene 10) was a gift from Dr Honjo (Kyoto University, Kyoto, Japan). The NIH 3T3-Delta-1–expressing cell line (3T3-Delta-1) was established by transfection of NIH 3T3 cells with pEF-BOS neo/Delta1-gene 10 and selection with 1 mg/mL G418. A control fibroblast cell line (3T3-EF) for 3T3-Delta-1 was made using pEF-BOS neo. NIH-3T3-Jagged-1–expressing cell line (3T3-Jagged) was a gift from Irwin D. Bernstein (Fred Hutchinson Cancer Center, Seattle, WA) and the generation of a control fibroblast cell line for 3T3-Jagged (3T3-MSCV) was described previously.[27]

Bone marrow and spleen stromal cell culture

Bone marrow cells were enriched for HPCs using depletion of lineage-specific cells as described previously.[27] Enriched population of HPCs contained less than 1% lymphocytes and less than 20% mature myeloid cells (Figure S1, available on the *Blood* website; see the Supplemental Figures link at the top of the online article). BMS and SS cells were obtained as described previously with some modification.[23,29] Briefly, 30 million bone marrow cells from Balb/c, C57BL/6, or B6.SJL-PtrcaPep3b/BoyJ mice were cultured in a T-25 culture flask using Iscoves medium (12.5% FCS, 12.5% horse serum, 1% MEM-nonessential amino acids, 1% MEM-vitamins, 10^6 M hydrocortisone, 10^1 M 2-ME, 100 mM Na-Pyruvate), at 34°C, 5% CO_2. Culture medium was changed every 4 days. Spleens from C57BL/6 or B6.SJL-PtrcaPep3b/BoyJ mice were cut into small pieces and cultured in a T-25 flask with RPMI medium (20% FBS, 1% MEM-nonessential amino acids, 1% MEM-vitamins, 10^6 M hydrocortisone, 10^1 M 2-ME, 100 mM Na-Pyruvate), at 37°C and 5% CO_2. Culture medium was changed every 4 days. Stromal cells were used in experiments after 2- to 3-week culture.

Generation of DCs, cell isolation, and the analysis of phenotype

Stroma cells were irradiated at 25 Gy and cultured overnight in 24-well plates (1.5×10^5 cells/well). Half million HPCs were placed into each well in 2 mL 10% fetal bovine serum (FBS) RPMI supplemented with 20 ng/mL GM-CSF (and in some experiments with 10 ng/mL IL-4) for 7 days. Every 3 days, cells were transferred onto new stroma and medium was replaced. In some experiments, 1 μg/mL LPS was added for 24 hours to achieve DC activation. CD45^{+} cells were isolated using microbeads and MiniMACS columns according to the manufacturer's protocol (Miltenyi Biotec). Purity for CD45^{+} was more than 93% in all samples. The phenotype of DCs and myeloid cells was analyzed on FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using a CellQuest program (Becton Dickinson, Mountain View, CA). In the experiments of DC coculture with 3T3 fibroblasts or bone marrow stroma, CD45^{+} or CD45.2^{+} cells were gated for the analysis.

Allogeneic mixed leukocyte reaction (MLR)

DCs were sorted using FACSVantage SE cell sorter (BD Biosciences) and were cultured for 4 days in triplicate in U-bottom 96-well plates with lymph node cells (10^5/well) obtained from allogeneic BALB/c or C57BL/6 mice depending on the nature of stimulator cells. [^{3}H]-thymidine (1 μCi [0.037 MBq]) was added to each well 18 hours before cell harvest. T-cell proliferation was measured by [^{3}H]-thymidine incorporation using a liquid scintillation counter (Packard Instrument, Meriden, CT).

Retroviral constructs

The retroviral bicistronic constructs containing EGFP were described previously: active form of Notch 1 (NIC1),[12] Notch-2 (NIC2; gift from Dr Klug, University of Alabama at Birmingham, Birmingham, AL),[30] and Deltex-1 (gift from Dr Pear, University of Pennsylvania Medical Center, Philadelphia, PA).[31] Human *HES1* was excised from pCMV-SPORT6-Hes1 (Open Biosystems, Huntsville, AL) and subcloned into pMSCV2.2. These constructs were cotransfected with pCL-Eco into the packaging cell line 293T cells, or transfected into Bosc 23. The retroviral supernatants were harvested 48 hours after transfection and used for infection of HPC-enriched bone marrow cells. On day 1 and day 2, HPCs were infected with retroviruses for 4 hours at 32°C in the presence of 4 μg/mL polybrene (Sigma). Viral supernatants were removed and culture medium supplemented with GM-CSF was added. Cells were cultured for 20 hours at 37°C, and then infection was repeated.

Luciferase reporter assay, electrophoretic mobility shift assay (EMSA), and Western blot assay

For the analysis of CBF-1 luciferase activity, we used Notch reporter construct containing CBF-1 responsive element. The retroviral reporter plasmid p6×CBF1-IL-2-Luc was constructed from pKA9 plasmid (IL-2-Luc) containing luciferase gene under control of minimal IL-2 promoter (a gift from T. J. Murphy, Emory University, Atlanta, GA) and described in detail previously.[32] To measure NF-κB transcriptional activity, plasmid 6×IFN-γtkLuc–containing luciferase reporter gene under NF-κB–dependent promoter from IFN-γ and pGL$_3$-basic plasmid–containing only luciferase gene as control were used as described previously.[12] EMSA was performed as previously described[27] using double-stranded oligonucleotides containing the specific binding site for CBF-1. Western blotting was performed as described previously.[12] For the detection of intercellular domain of Notch-1 and Notch-2, 25 μg nuclear protein per sample was used. Samples were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with specific antibody. Equal loading was determined by β-actin level for whole-cell lysates and anti–histone H3 for nuclear protein. The specific bands were visualized by an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, Arlington Heights, IL).

Assessment of gene expression by real-time PCR

RNA was extracted with Trizol (Invitrogen, Frederick, MD); cDNA was synthesized and used for the evaluation of gene expression as described previously.[32] Polymerase chain reaction (PCR) was performed with 2.5 μL cDNA, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and target gene assay mix containing sequence-specific primers and 6-carboxyfluorescein (6-FAM) dye–labeled TaqMan minor groove binder (MGB) probe (Applied Biosystems). Amplification with 18S endogenous control assay mix was used for controls. In order to determine the copy number of *Jagged-1* or *Delta-1,* partial sequences of these genes, which spanned the regions used for real-time PCR, were cloned into pCR2.1-TOPO and used as a standard for the calculation of copy number. PCR was carried out in triplicate for each sample. Data quantitation was performed using the relative standard curve method.[33] Expression levels of the genes were normalized by 18S mRNA.

To calculate the number of cDNA copies per cell, standard curve was established using the plasmids whose copy number was calculated as described previously, copies/ng = (base pairs of plasmid × 660 Da)/ (Avogadro constant × 10^{-9}).[34] The copy numbers of test RNA samples were obtained after real-time PCR amplification from regression of the corresponding standard curve, and then converted to copies per cell based on the RNA yield of per million cells.[35]

Adoptive transfer of Gr-1⁺ cells in vivo

Gr-1⁺ cells were isolated from bone marrow of C56BL/6 (CD45.2⁺) mice on MiniMACS columns using biotinylated anti–Gr-1 antibody and streptavidin magnetic beads. More than 92% of isolated cells were Gr-1⁺CD11b⁺. Immature myeloid cells (ImCs; 15 × 10⁶/mouse) were injected intravenously into congenic CD45.1⁺ mice. Spleen and bone marrow were collected 2 to 72 hours after the injection. Cells were labeled with antibodies against different surface markers, and the proportion of DCs, macrophages, and ImCs was evaluated within the population of CD45.2⁺ donor cells.

Down-regulation of Jagged-1 in bone marrow stromal cells

One million BMS cells were mixed with Jagged-1 siRNA (Dharmacon) at 100-nM concentration in 100 μL mouse macrophage nucleofactor solution, and transfection was performed using Nucleofector I (Amaxa, Gaithersburg, MD). The expression of Jagged-1 was verified by Western blot.

Results

Jagged-1 and Delta-1 had opposite effects on differentiation of myeloid but not plasmacytoid DCs

To evaluate the effect of different cell-bound Notch ligands on HPCs, NIH 3T3 fibroblasts expressing Jagged-1 or Delta-1 were used. Fibroblasts transfected with corresponding empty vectors served as controls in all experiments (Figure 1A). Jagged-1 and Delta-1 cell lines expressed comparable level of these genes:
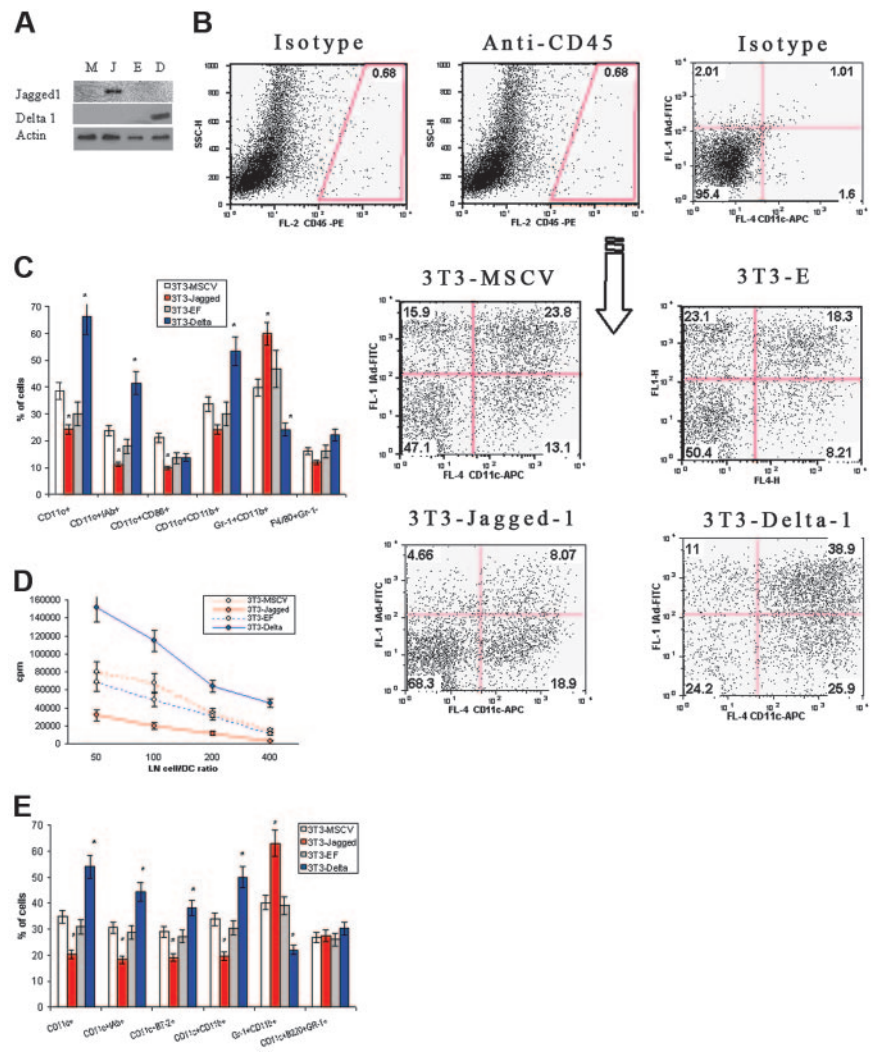

Figure 1. Jagged-1 and Delta-1 had opposite effects on DC generation. (A) Expression of Notch ligands on cell lines used in this study. Whole-cell lysates were prepared. Jagged-1 and Delta-1 proteins were detected by Western blotting using specific antibodies as described in "Materials and methods." M indicates 3T3-MSCV control cell line; J, 3T3-Jagged-1 fibroblasts; E, 3T3-EF control cell line; and D, 3T3-Delta-1 fibroblasts. (B) HPCs were cultured on irradiated 3T3 fibroblasts in 24-well plates with GM-CSF (20 ng/mL) for 7 days. A typical example of the analysis of cell phenotype is shown. (C) Phenotype of DCs. Cells were labeled with APC-conjugated anti-CD11c or anti–Gr-1 antibodies; PE-conjugated anti-CD45 antibody; FITC-conjugated anti-IA^d, anti–B7-2, and anti-CD11b; and PerCp-conjugated anti-B220 and anti-CD11b antibodies and analyzed on a FACSCalibur flow cytometer. Only CD45⁺ cells were evaluated. Values are the average ± SE from 7 experiments. (D) Allogeneic MLR. CD45⁺ cells from experiments described in panel C were isolated using PE-conjugated anti-CD45 antibody, anti-PE beads, and magnetic-activated cell sorting (MACS) columns. The isolated cells were irradiated at 25 Gy and cultured with lymph node cells isolated from control allogeneic C57BL/6 mice at different ratios. DCs generated from BALB/c using GM-CSF and IL-4 were used as a control. Cell proliferation was measured in triplicate with [³H]-thymidine uptake. Values are the mean ± SE. (E) HPCs were cultured on different fibroblasts in the presence of 100 ng/mL FL for 10 days and analyzed as described in Figure 2B. Mean ± SE of 3 experiments is shown.

3T3-Jagged-1 had 9.5 copies of *Jagged-1* cDNA per cell and 3T3-Delta-1 had 6.6 copies of *Delta-1* cDNA per cell. HPCs were cultured on the monolayer of fibroblasts and several experimental conditions were tested. First, cells were cultured in the presence of GM-CSF for 6 days. The total number of cells generated from HPCs was not changed (data not shown). However, striking differences were found in the proportion of generated DCs. Delta-1 significantly increased the proportion of DCs, whereas Jagged-1 substantially decreased it (Figure 1B-C). Jagged-1 substantially increased the population of Gr-1+CD11b+ ImCs. In contrast, Delta-1 significantly reduced the proportion of these cells (Figure 1C). The opposite effect of 2 Notch ligands on DC differentiation was confirmed in allogeneic MLR, the hallmark of DC activity. Cells generated on Delta-1 fibroblasts stimulated allogeneic T cells much more strongly than the cells generated on control fibroblasts, whereas just the opposite effect was observed in cells generated on Jagged-1 fibroblasts (Figure 1D).

The effect of Delta-1 on lymphocyte differentiation may depend on the density of the ligand.[36] Therefore, it was possible that lower Delta-1 density could mimic the effect of Jagged-1. To test this possibility, plates were coated with Delta[ext-myc] protein. Enriched HPCs were cultured for 6 days with GM-CSF. Delta[ext-myc] at 5-μg/mL concentrations induced DC differentiation similar to the effect of 3T3-Delta-1 cells. At a lower concentration of Delta[ext-myc], this effect has disappeared and the proportion of DCs returned to the control level. No decrease in the presence of DCs or the accumulation of ImCs was observed (data not shown).

Incubation of HPCs with GM-CSF results in differentiation of myeloid DCs. To evaluate possible effects of Notch ligands on pDCs, HPCs were cultured with FLT-3 ligand (FL). Effect of Notch ligands on myeloid cells was the same as in the presence of GM-CSF. However, neither Jagged-1 nor Delta-1 changed the proportion of CD11c+Gr-1+B220+ pDCs (Figure 1E).

To investigate whether DCs generated in the presence of different Notch ligands had a similar function, enriched HPCs were cultured for 5 days with GM-CSF on top of the monolayer of fibroblasts expressing different Jagged-1 or Delta-1. CD11c+ DCs were isolated and then activated by incubation with LPS for 24 hours. No differences in the expression of MHC class II and costimulatory molecule (CD86, CD40) or the ability of these cells to stimulate allogeneic T cells were found (data not shown). To investigate whether cell-bound Notch ligands could induce DC activation, DCs were generated from HPCs using 5-day culture with GM-CSF and IL-4. CD11c+ DCs were isolated and plated on top of 4 different fibroblast cell lines. After 24- to 72-hour incubation, cells were collected and evaluated. Neither Jagged-1 nor Delta-1 induced DC activation (data not shown).

Effect of bone marrow and spleen stroma on DC differentiation

Next, we studied the effect of BMS and SS on DC differentiation using a congenic system to distinguish between stroma cells and HPCs. BMS or SS was generated from CD45.1+ mice. Enriched CD45.2+ HPCs were placed on the monolayer of CD45.1+ BMS or SS and incubated for 7 days with GM-CSF. CD45.2+ cells were analyzed. The proportion of DCs differentiated from HPCs on BMS was significantly lower than the cells cultured without stroma. This was associated with a significant increase in the proportion of ImCs (Figure 2A). These results resembled those obtained with cell-bound Jagged-1. Similar data were obtained when enriched HPCs were cultured with GM-CSF and IL-4 (data not shown). In parallel, we evaluated the effect of BMS on DC differentiation without addition of cytokines. No viable cells were
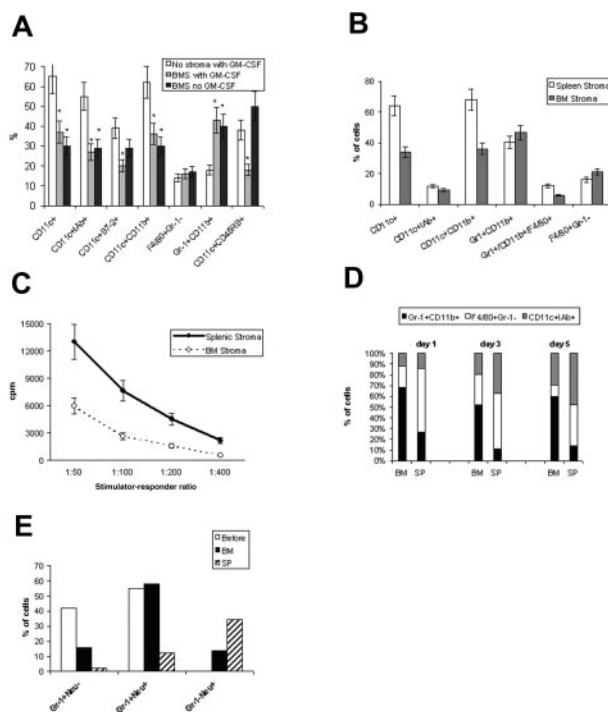

Figure 2. Effect of BMS and SS on DC differentiation. (A) Differentiation of DCs on the cultured BMS. BMS was prepared as described in "Materials and methods." HPCs were placed on top of the BMS monolayer and cultured for 7 days with or without GM-CSF. Cells were collected and analyzed by flow cytometry as described in Figure 1. To eliminate the effect of possible contamination by fibroblasts, only CD45.2+ hematopoietic cells were analyzed. Values are the mean ± SE from 3 experiments. (B) Differentiation of HPCs on BMS or SS with GM-CSF. Cells were labeled with APC-conjugated anti-CD11c or anti–Gr-1 antibodies; FITC-conjugated anti-CD45.2 antibody; PE-conjugated anti-IA[b], anti-B7-2, CD11b, and F4/80; and PerCp-conjugated anti-B220 and anti-CD11b antibodies and analyzed on a FACSCalibur flow cytometer. Only CD45.2+ cells were evaluated for cell phenotype. Values are the mean ± SE from 3 experiments. (C) Allogeneic MLR. Cells from the cultures described in panel B were isolated using PE-conjugated anti-CD45.2 antibody, anti-PE beads, and MACS columns. The isolated cells were irradiated at 25 Gy and cultured with lymph node cells isolated from control allogeneic C57BL/6 mice at different ratios. Cell proliferation was measured in triplicate by [3H]-thymidine uptake. Values are the mean ± SE. (D) Gr-1+ cells were isolated from bone marrow of control C57BL/6 mice using magnetic bead separation technique. More than 92% of these cells were Gr-1+CD11b+. Fifteen million of the cells were injected intravenously into congenic CD45.1+ mice. Spleen and bone marrow were collected 24, 48, and 72 hours after the injection. Each time point included 3 mice. Cells were labeled with antibodies against different surface markers, and the proportions of CD11c+IA[b]+ DCs, F4/80+Gr-1− macrophages, and Gr-1+CD11b+ immature myeloid cells were evaluated within the population of CD45.2+ donor cells. (E) Splenocytes (SP) and bone marrow cells (BM) were collected 24 hours after transfer of Gr-1+CD11b+ ImCs as described in panel D. Cells were labeled with APC-conjugated anti–Gr-1, PE-conjugated anti-Neu, PerCp-conjugated anti-CD11b, and FITC-conjugated CD45.2 antibodies and analyzed on a FACSCalibur flow cytometer. The proportions of cells were calculated within the population of donor's CD45.2+ cells. Before indicates cells analyzed prior to the transfer. Results of 2 performed experiments are shown.

present after a 7-day culture of HPCs in medium alone. The total number of hematopoietic cells recovered after a 7-day culture on BMS without cytokines was very small (5.2×10^4 per well) compared with 1.22×10^6 cells/well ($P < .001$) recovered from cultures on BMS with GM-CSF. No differences in the proportion of DC subsets were seen between the cells cultured with and without GM-CSF with the exception of a population of CD11c+CD45RB+ regulatory DCs, which was significantly higher among cells generated on BMS without cytokines (Figure 2A). It was consistent with a recent report that cultured SS could support differentiation of regulatory DCs from HSCs without the presence of cytokines.[29]

We compared side by side the effect of BMS and SS on DC differentiation. No difference in the total number of cells was found

(data not shown). The proportion of $CD11c^+$ DCs generated from HPCs on SS was similar to that in cells cultured without stroma and significantly ($P < .01$) higher than on BMS (Figure 2B). Cells generated on SS had significantly higher activity in stimulation of allogeneic T cells than cells generated on BMS (Figure 2C).

These in vitro experiments demonstrated that BMS and SS had similar effects on generation of precursors of myeloid cells. However, their effect on DC differentiation was very different. BMS, in contrast to SS, prevented transition of ImCs to terminally differentiated DCs. We asked whether similar effects could be seen in vivo. $CD45.2^+$ $Gr-1^+CD11b^+$ ImCs were isolated from BM of C57BL/6 mice with a purity more than 92% (Figure S2), and 1.5×10^7 cells were transferred intravenously into $CD45.1^+$ congenic mice. BM cells and splenocytes were analyzed 2 hours, 24 hours, 72 hours, and 120 hours after intravenous injection. After 2 hours, slightly more $CD45.2^+$ donor cells migrated to BM than to spleens. However, those differences were not statistically significant (Table 1). Twenty-four hours after the transfer, ImCs represented the majority of donors' cells in BM but only 20% of donors' cells in spleens (Figure 2D). The total number of ImCs in BM was 5-fold higher than in spleen (Table 1). Most of the donors' cells in spleen differentiated into $F4/80^+Gr-1^-$ macrophages or $CD11c^+IA^{b+}$ DCs within 3 days, whereas in BM most of the cells retained the phenotype of ImCs (Table 1; Figure 2D). More than 50% of $CD45.2^+$ ImCs expressed neutrophil marker Neu. To investigate the fate of neutrophils after the transfer, we evaluated 3 populations of the cells: immature $Gr-1^+Neu^-$, intermediate $Gr-1^+Neu^+$, and mature $Gr-1^-Neu^+$ cells. In 24 hours after the transfer, the proportions of both population of immature cells ($Gr-1^+Neu^-$ and $Gr-1^+Neu^+$) among donors' cells in spleens was substantially reduced. In BM, the decrease was much smaller ($Gr-1^+Neu^-$) or even absent ($Gr-1^+Neu^+$) (Figure 2E). In contrast, the proportion of mature $Gr-1^-Neu^+$ cells increased substantially in spleen. In BM, this increase was significantly smaller (Figure 2E). These data supported the hypothesis that the BM environment, however, promoted the accumulation of precursors of DCs, while preventing their terminal differentiation. In this regard, what is the possible role of Notch ligands on BMS?

The role of Notch ligands in the effects of stroma on DC differentiation

We examined the expression of the 2 ligands in cultured BMS. Both proteins were present in BMS derived from BALB/c and C57BL/6 mice. However, the level of Delta-1 was significantly lower than Jagged-1 (Figure 3A). These results were confirmed by gene expression analysis. The expression of *Jagged-1* in BMS was much higher than *Delta-1* as assessed by cDNA copies per cell

basis in real-time reverse-transcription (RT)–PCR. In BALB/c BMS, 1.43 copies of *Jagged-1* cDNA per cell were detected, compared with only 0.002 copies of *Delta-1*. In C57BL/6 mice, 2.29 copies per cell of *Jagged-1* cDNA were detected compared with only 0.009 copies of *Delta-1* (Figure 3B). Both Jagged-1 and Delta-1 were present in SS. However, substantially more Delta-1 was found in SS than Jagged-1 (Figure 3C). As a result, the ratio of Jagged-1 to Delta-1 protein levels in BMS was more than 2.5, whereas in SS it was less than 0.5. The level of Jagged-1 protein in BMS was similar to that in 3T3-Jagged-1 cells, whereas 3T3-Delta-1 cells had a higher level of Delta-1 expression than both BMS and SS (Figure 3C). A similar expression pattern of Notch ligands was observed when whole cell populations from bone marrow and spleens were studied. Expression of Notch ligands in lymph node cells was similar to that in spleen (Figure 3D).

To investigate further the potential contribution of different Notch ligands in DC differentiation, HPCs were cultured with GM-CSF on a monolayer formed from a mixture of Jagged-1 and Delta-1 fibroblasts. As a control, we used a mixture of Delta-1 and control fibroblasts. The proportion of $CD11c^+$ DCs among $CD45^+$ hematopoietic cells generated after 6-day culture was calculated. Jagged-1 suppressed DC differentiation stimulated by Delta-1 fibroblasts only if present on more than 50% of the cells (Figure 3E). Of interest, those proportions were similar to those observed in BMS but not in SS (Figure 3C-D).

To clarify the role of Jagged-1 in BMS effect on DC differentiation, Jagged-1 was knocked down by siRNA (Figure 3F). BMS cells cultured for 2 weeks were transfected with control or Jagged-1 siRNA. HPCs were cocultured on the monolayer of transfected BMS in the presence of GM-CSF. Cells were transferred to a new monolayer of freshly prepared stroma cells every 2 days and analyzed on day 7. Down-regulation of Jagged-1 in BMS resulted in moderate but significant increase in the proportion of differentiated DCs (Figure 3G). The results were more dramatic when cell function was evaluated in allogeneic MLR. Down-regulation of Jagged-1 in BMS significantly increased the presence of cells able to stimulate allogeneic T cells, the function specifically attributed to DCs (Figure 3H). Taken together, these data indicate that Jagged-1 may play an important role in BMS-mediated suppression of DC differentiation.

Effect of Jagged-1 and Delta-1 on downstream targets of Notch

HPCs express primarily 2 members of the Notch family: Notch-1 and Notch-2. Using bicistronic retroviral constructs containing GFP and active forms of Notch 1 or Notch 2, we evaluated their effect on DC differentiation. The active form of Notch-1 and Notch-2 significantly and equally increased the proportion of

Table 1. Differentiation of myeloid cells in vivo

	CD45.2 cells, × 10⁶, by time after cell transfer							
	2 h		**24 h**		**72 h**		**120 h**	
Cells	**Bone marrow**	**Spleen**	**Bone marrow**	**Spleen**	**Bone marrow**	**Spleen**	**Bone marrow**	**Spleen**
All cells	1.27 ± 0.13	0.97 ± 0.10	2.5 ± 0.29	1.82 ± 0.18	1.53 ± 0.16	0.85 ± 0.09*	3.12 ± 0.32	1.06 ± 0.11*
$Gr-1^+CD11b^+$	0.55 ± 0.04	0.43 ± 0.04	0.93 ± 0.09	0.14 ± 0.04*	0.77 ± 0.06	0.02 ± 0.01*	2.52 ± 0.26	0.3 ± 0.31*
$CD11c^+1Ab^+$	0.10 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.37 ± 0.03	0.28 ± 0.02	0.35 ± 0.04	0.37 ± 0.04	0.67 ± 0.06*
$F4/80^+Gr-1^-$	0.32 ± 0.03	0.36 ± 0.03	0.75 ± 0.08	1.15 ± 0.12	0.42 ± 0.04	0.48 ± 0.05	0.43 ± 0.04	0.54 ± 0.06

$Gr-1^+$ cells were isolated from bone marrow of control C57BL/6 mice using magnetic beads. More than 92% of these cells were $Gr-1^+CD11b^+$. Fifteen million of the cells were injected intravenously into congenic $CD45.1^+$ mice. Spleen and bone marrow were collected 2, 24, 48, and 72 hours after the injection. Each time point included 3 mice. Cells were labeled with antibodies against different surface markers and the total number of $CD45^+$ donors' cells as well as $CD45^+CD11c^+1A^{b+}$ DCs, $CD45^+F4/80^+Gr-1^-$ macrophages, and $CD45^+Gr-1^+CD11b^+$ immature myeloid cells were calculated. Mean ± SE of 3 performed experiments is shown.

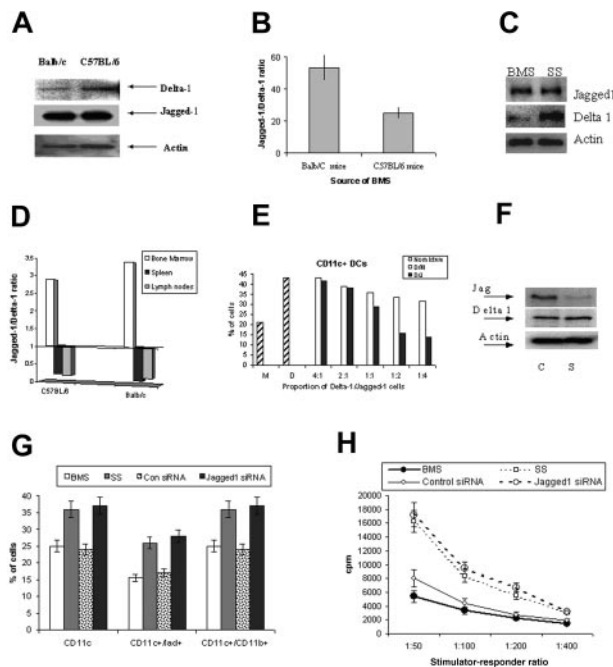*Statistically significant differences between bone marrow and spleen ($P < .05$).

Figure 3. Role of Notch ligands in the effect of BMS and SS on DC differentiation. (A) Expression of Notch ligands in cultured BMS. Whole-cell lysates of BMS (2-week culture) were prepared and used to determine the expression of Jagged-1 and Delta-1 by Western blot. (B) RNA samples were extracted from BMS; cDNA was synthesized and used to determine expression of *Jagged-1* and *Delta-1*. The number of gene copies per cell was determined using *Jagged-1* and *Delta-1* plasmids as described in "Materials and methods." The Jagged-1–Delta-1 ratio was presented based on copy number per cell. (C) Expression of Jagged-1 and Delta-1 in 3T3 cell lines expressing Jagged-1 or Delta-1, BMS, and SS. Whole-cell lysates of 2-week cultured BMS or SS were prepared and used to determine the expression of Jagged-1 and Delta-1 by Western blotting as described in "Materials and methods" using indicated antibodies. BMS indicates bone marrow stroma; and SS, spleen stroma. Quantification was performed using densitometry. Levels of proteins were quantitated by using UN-Scan-IT software (Silk Scientific, Orem, UT). Expression in each sample was normalized for β-actin and was expressed as an arbitrary unit. It was calculated as follows: (intensity of Jagged-1 or Delta-1 bands)/(intensity of β-actin bands) × 100. (D) Expression of *Jagged-1* and *Delta-1* in bone marrow, spleens, and lymph nodes. RNA was extracted from tissues, and gene expression was evaluated as described in Figure 4B. (E) 3T3-Jagged-1 and 3T3-Delta-1 cells were mixed together at indicated ratios and then used to form a monolayer in 24-well plates. HPCs were isolated from bone marrow and cultured on a monolayer of fibroblasts in the presence of GM-CSF as described in the legend to Figure 1. Cells were collected on day 6 and analyzed by flow cytometry. Only CD45+ hematopoietic cells were analyzed, and the proportion of CD11c+ DCs was calculated. M indicates cells cultured on control 3T3-MSCV fibroblasts; D, cells cultured on Delta-1 fibroblasts; D/M, mixture of 3T3-Delta-1 and control 3T3-MSCV fibroblasts; and D/J, mixture of 3T3-Delta-1 and 3T3-Jagged-1 cells. (F) BMS cells were transfected with Jagged-1 or control siRNA. Twenty-four hours later, whole-cell lysates were prepared and the level of Jagged-1 in the cells was evaluated in Western blotting. The membranes were also probed with anti–Delta-1 antibody to assess potential off-target effect of siRNA. (G) HPCs were cultured on a monolayer of BMS transfected with Jagged-1 or control siRNA as well as on the monolayer of SS for 7 days in the presence of 20 ng/mL GM-CSF. Cells were transferred every 2 days on freshly prepared BMS. On day 7, cells were collected, labeled with different antibodies, and analyzed by flow cytometry. Only hematopoietic CD45.2+ cells were evaluated. Average ± SE of 3 experiments is shown. (H) CD45.2+ cells from the experiments described in Figure 4F were isolated using magnetic beads and cultured with allogeneic lymph node cells at different ratios. Cell proliferation was measured in triplicate using [3H]-thymidine uptake. Data represent the average ± SE of 3 experiments.

CD11c+ DCs as well as the populations of CD11c+IA^d+ or CD11c+CD86+ activated DCs ($P < .05$) (Figure 4A). We compared the ability of Delta-1 and Jagged-1 to activate Notch signaling via transcription factor CBF-1/RBP-Jκ. HPCs were cultured for 16 hours on the monolayer of different 3T3 cell lines, CD45+ hematopoietic cells were isolated, and nuclear protein was extracted and used for evaluation of CBF-1 binding activity in

EMSA. Both Jagged-1 and Delta-1 strongly and equally increased CBF-1 binding to the specific DNA probe (Figure 4B). Similar results were obtained in CBF-1–luciferase reporter assay using HPCs transduced with CBF-1–luciferase retrovirus (Figure 4C). Transcription factor NF-κB plays a critical role in DC differentiation. Previously, we have demonstrated that Notch-1 may regulate expression levels of different NF-κB members.[12] We tested the hypothesis that Jagged-1 and Delta-1 induced different levels of NF-κB activation. Both Jagged-1 and Delta-1 equally activated NF-κB in HPCs (Figure 4D), and the expression of NF-κB members was not changed until at least after a 72-hour culture (Figure 4E).

Hes1 is one of the main target genes regulated by Notch. We evaluated *Hes1* expression after the incubation of HPCs on fibroblasts with different Notch ligands. Jagged-1 and Delta-1 induced *Hes1* expression that oscillated following different patterns. Jagged-1 induced repeated peaks of expression with 2-hour intervals, which then slowly decreased after 12 hours in culture (Figure 5A). Delta-1 induced much stronger *Hes1* expression. It followed the pattern of 3 hours of oscillation and remained strong
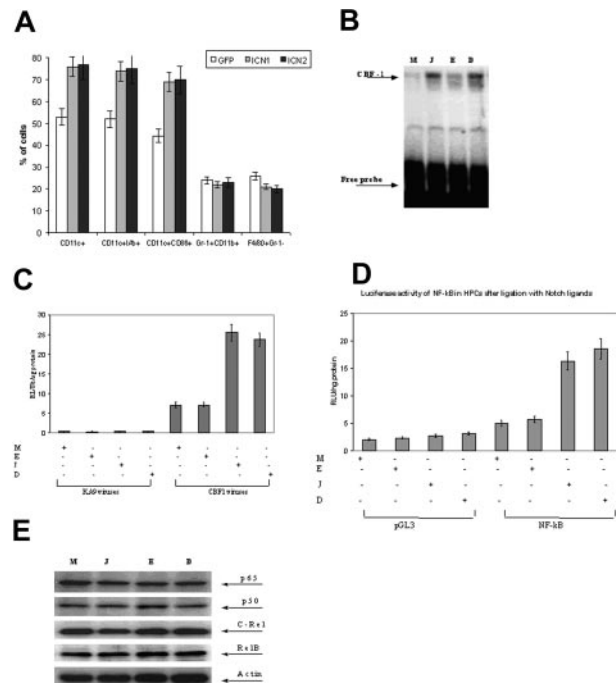

Figure 4. Effect of Notch ligands on downstream targets of Notch signaling. (A) Effect of ICN1 and ICN2 on differentiation of DCs. HPCs were transduced with either ICN1 or ICN2 using retroviruses as described in "Materials and methods" and differentiated to DCs with GM-CSF for 7 days. After that time, cells were collected and the phenotype of GFP-positive cells was evaluated by flow cytometry. The results of 4 experiments are shown. (B) CBF-1 binding activity. HPCs were cultured on a monolayer of different 3T3 fibroblast cell lines for 8 hours. Nuclear protein was extracted and used for evaluation of CBF-1 binding to 32P-labeled specific DNA probe in EMSA. (C) CBF-1 reporter luciferase activity induced by Notch ligands. HPCs from Balb/c mice were infected with control KA9 virus or CBF-1 reporter virus twice on day 0 and day 1 with a 16-hour interval. After second infection, cells were cultured for 48 hours. To activate Notch signaling, infected HPCs were cultured for 8 hours on 3T3 fibroblasts expressing different ligands. Luciferase activity was normalized on protein concentration. Mean ± SE of 3 performed experiments is shown. (D) HPCs were electroporated with pGL3 or NF-κB reporter plasmids and 40 hours later were placed on top of a monolayer of different fibroblast cell lines. M indicates 3T3-MSCV; J, 3T3-Jagged-1; E, 3T3-EF; and D, 3T3-Delta-1. After overnight incubation, cells were collected, CD45+ cells were isolated using magnetic beads, whole-cell lysates were prepared, and luciferase activity was measured as described in "Materials and methods." The results were normalized for protein concentrations. (E) HPCs were cultured for 72 hours on different cell lines, whole-cell lysates were performed, and protein level was evaluated by Western blotting using indicated specific antibodies. Data represent the average ± SE of 3 experiments.
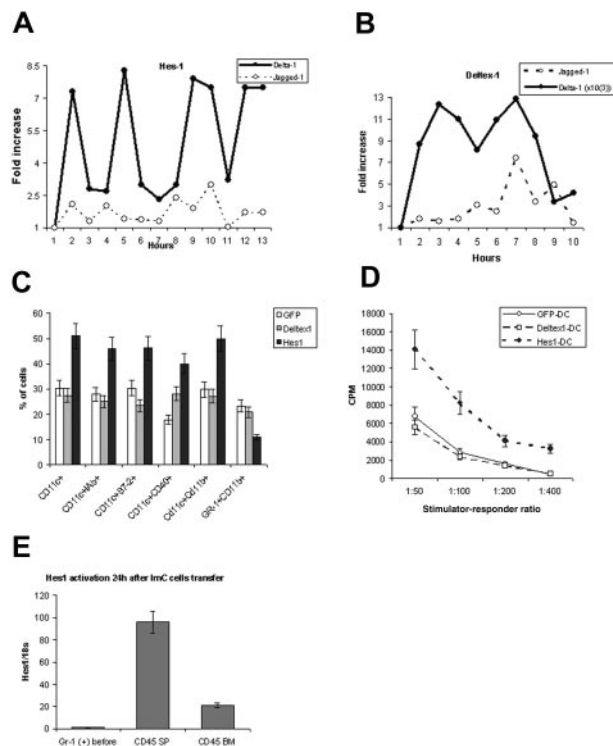
Figure 5. Effect of Notch ligands on Hes-1 and Deltex-1. (A) Expression of *Hes1* was evaluated in HPCs cultured on Jagged-1 or Delta-1 fibroblasts at indicated time points using real-time RT-PCR. The data are presented as fold increase over the level of *Hes1* expression in HPCs cultured on control fibroblasts. Two experiments with the same results were performed. (B) Expression of *Deltex-1* was measured in HPCs at indicated time points using real-time RT-PCR. The data are presented as fold increase over the level of *Deltex-1* expression in HPCs cultured on control fibroblasts. Two experiments with the same results were performed. (C) HPCs were transduced with *Hes1* or *Deltex-1* and cultured with GM-CSF for 7 days. The phenotype of transduced GFP-positive cells was evaluated using multicolor flow cytometry. The results of 2 experiments are shown. (D) GFP-positive cells from experiments described in panel C were sorted and used as stimulator of allogeneic lymph node cells. Cell proliferation was evaluated in triplicate using [³H]-thymidine uptake. Two experiments with similar results were performed. (E) Gr-1⁺ ImCs isolated from BM of CD45.2⁺ mice were injected intravenously into CD45.1⁺ recipients as described in Figure 2D. Donors' CD45.2⁺ cells were isolated from spleens and BM of recipients 24 hours after the transfer. RNA was extracted and expression of *Hes1* was measured in triplicate in real-time PCR as described in "Materials and methods." *Hes1/18S* ratio is shown. Data represent the average ± SE of 3 experiments.

after 12 hours of culture (Figure 5A). Deltex-1 is another important factor of Notch signaling pathway with possible antagonistic activity against Notch-1. In our experiments, both Delta-1 and Jagged-1 induced *Deltex-1* transcription. However, the effect of Delta-1 was much faster and much stronger (more than 1000-fold) than that of Jagged-1 (Figure 5B).

Since cell-bound Delta-1 induced substantially higher levels of *Hes1* and *Deltex-1* expression than Jagged-1, we investigated the possibility that *Hes1* or *Deltex-1* could be directly involved in DC differentiation using bicistronic retroviral constructs expressing GFP and either *Hes1* or *Deltex-1*. HPCs infected with different retroviruses were cultured for 7 days with GM-CSF and the phenotype of GFP-positive cells was analyzed. There was no difference between cells infected with control virus and *Deltex-1* virus (Figure 5C). Although the total cell number remained unchanged, the proportion of DCs differentiated from HPCs transduced with *Hes1* was significantly higher (Figure 5C). This was observed for the total population of CD11c⁺ cells as well as for the populations of mature DCs (CD11c⁺IA^b⁺, CD11c⁺CD86⁺, and CD11c⁺CD40⁺). Not surprisingly, the proportion of ImCs was significantly reduced (*P* < .05). The effect of Hes-1 on DC

differentiation was confirmed in allogeneic MLR. GFP-positive cells were sorted and then used as stimulators of allogeneic T cells. HPCs transduced with *Hes1* produced cells with much greater ability to stimulate allogeneic T cells than HPCs transduced with control or *Deltex-1* vectors (Figure 5D).

We evaluated the effect of BM and spleen on *Hes1* expression in donors' cells after adoptive transfer. Twenty-four hours after transfer of ImCs, CD45.2⁺ donors' cells were collected from spleen and BM, RNA was extracted, and the level of *Hes1* mRNA was measured in real-time PCR. *Hes1* expression was found to be substantially higher in donors' cells recovered from spleens than from BM (Figure 5E).

Discussion

Notch signaling is an important element mediating the effect of BMS on HPCs. It is closely involved in DC differentiation.[12,21,27] However, the nature of this effect remains controversial. A number of studies have demonstrated that Notch ligand Delta-1 promotes differentiation of myeloid DCs,[23,37] pDCs,[25] and Langerhans cells.[26] In contrast, another Notch ligand Jagged-1 was shown to inhibit differentiation of DCs and promote accumulation of DC precursors. These precursors differentiated to DCs as soon as Jagged-1–mediated signaling was terminated.[27] It was not clear whether the observed differences were just the result of different experimental conditions or the complex nature of Notch signaling in HPCs. The biologic significance of these phenomena was also not clear since all of these experiments were performed with cell lines expressing artificially high levels of Notch ligands. To address these questions, we have compared side by side the effect of 2 main Notch ligands Jagged-1 and Delta-1 on DC differentiation from HPCs. These 2 ligands were expressed on the same type of cells (3T3 fibroblasts) at similar levels. However, their effect on DC differentiation was totally opposite. Delta-1 increased accumulation of differentiated DCs, whereas Jagged-1 decreased their presence. This was associated with accumulation of Gr-1⁺CD11b⁺ ImCs previously shown to be precursors of DCs, macrophages, and granulocytes.[38] Jagged-1–induced accumulation of ImCs was consistent with the previously published observation that Jagged-1 promoted increase in the formation of primitive precursor cell populations.[39]

Different effects of Notch ligands on lymphocytes have been reported previously. Delta-1 allowed the emergence of cells with characteristics of T/natural killer (NK) precursors, while Jagged-1 did not interfere with lymphoid development.[40] The majority of BM-derived stem cells did not respond to Jagged-1 signal, whereas the Delta-1–expressing stroma cells promote the proliferation and maturation of T-cell progenitors.[41] Expression of Delta on antigen-presenting cells induced Th1-type T cells, while expression of Jagged induced Th2-type T cells.[42] These data raised the question, how can distinct ligands of the same receptor family induce such diverse biologic outcomes of the Notch signaling pathway? Notch ligands share a conserved extracellular domain containing multiple EGF repeats and a Delta-Serrate-Lag2 (DSL) motif that is required for receptor binding. It is known that Jagged proteins have a distinct cysteine-rich region proximal to the transmembrane domain absent in Delta ligands.[16] This structural difference can represent one explanation for Delta and Jagged distinct biologic functions. Apparently, the whole ligand sequence is necessary to generate a specific signal by a given ligand.[43]

What could be the biologic significance of the observed effects of Jagged-1 and Delta-1 on DC differentiation? First, we asked,

what was the level of expression of Notch ligands in bone marrow and spleen stroma? We found that in contrast to SS, BMS had a much higher level of Jagged-1 than Delta-1. Of importance, the effect of BMS and SS on DC differentiation was also different. BMS promoted accumulation of ImCs but prevented their transition to differentiated DCs. This resembled the effects of Jagged-1 ligands. SS did not prevent DC differentiation. Down-regulation of Jagged-1 expression on BMS significantly improved DC differentiation. This was consistent with data obtained in vitro using the mixture of Delta-1 and Jagged-1 fibroblasts. This indicates that observed effects of different Notch ligands on DC differentiation could be biologically relevant and may suggest a model of Notch involvement in compartmentalization of DC differentiation. The bone marrow environment promoted accumulation of DC precursors but prevented terminal DC differentiation. This could be mediated by predominant expression of Jagged-1 on BMS. When DC precursors leave bone marrow they rapidly undergo final steps of differentiation. This could be due to the termination of Jagged-1 signaling as well as predominant expression of Delta-1 in peripheral lymphoid organs. The fact that normal bone marrow contains a significant proportion of ImCs (30%-40%), whereas spleen contains only 3% to 5% and lymph nodes contain less than 1%, partially supports this hypothesis. However, one can argue that those differences could be due to the high rate of cell production and turnover in bone marrow. Our experiments with adoptive transfer of ImCs demonstrated that most of the donors' cells in spleen differentiated into mature DCs or macrophages within 3 days after adoptive transfer, whereas the majority of donors' cells in bone marrow retained an immature phenotype. The analysis of an absolute number of donors' cells in spleens and BM as well as the data on phenotype of these cells strongly suggest that the observed differences between spleen and BM were not due to a preferential loss of cells in spleens but rather to a different pattern of cell differentiation in these sites. It is plausible that ImCs could migrate between bone marrow and spleen and thus affect the cell number in these organs during the experiment. However, this possibility does not contradict the overall hypothesis.

What could be the mechanism of different effects of Delta-1 and Jagged-1 on DC differentiation? Previous studies have shown the possibility that the different members of Notch family may have unique function.[44,45] However, our data indicate that both Notch-1 and Notch-2 promoted DC differentiation. Jagged-1 and Delta-1 equally activated CSL/CBF-1 and NF-κB transcription factors—important targets for Notch signaling. Notch signaling could be regulated by the quantity of Notch-ICN present in the nucleus via Deltex. Deltex can act as a positive or negative regulator of Notch.[46] However, in our study, Deltex-1 did not affect DC differentiation. Hes1 transcription factor is a major target for Notch signaling. Jagged-1 induced an oscillatory pattern of Hes1 expression consistent with a previous report.[47] The pattern of Delta-1–induced activation was different. The intervals between peaks were longer and the intensity of the signal was higher. The splenic environment provides much stronger up-regulation of *Hes1* than BM, which is consistent with a predominant effect of Delta-1 in spleens. Could these differences in Hes1 expression contribute to the different effects of Notch ligands on DC differentiation? Our data suggest that it may be the case. Overexpression of Hes1 in HPCs resulted in a substantial increase in DC differentiation similar to the effect of Delta-1 and Notch-1/2. It appears that low expression of Hes-1 prevents transition of ImCs to DCs. However, these data cannot fully explain the observed biologic effects since down-regulation of Jagged-1 in BMS was sufficient to significantly enhance DC differentiation, and addition of Jagged-1–containing fibroblasts to Delta-1–expressing cells blocked Delta-1–stimulated DC differentiation. It is likely that Jagged-1 directly or indirectly affects a different set of transcription factors and target genes important for DC differentiation. It is possible that in addition to a direct cell-cell contact Jagged-1–expressing cells could affect DC differentiation via soluble factors. More studies are needed to clarify this question.

Authorship

Contribution: P.C. collected data, analyzed data, and wrote the paper; Y.N. and C.A.C. collected data; D.I.G. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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