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Regulation of Dendritic Cell Differentiation in Bone Marrow during Emergency Myelopoiesis

Hao Liu,1 Jie Zhou,1,2 Pingyan Cheng, Indu Ramachandran,3 Yulia Nefedova,3 and Dmitry I. Gabrilovich3

Although accumulation of dendritic cell (DC) precursors occurs in bone marrow, the terminal differentiation of these cells takes place outside bone marrow. The signaling, regulating this process, remains poorly understood. We demonstrated that this process could be differentially regulated by Notch ligands: Jagged-1 (Jag1) and Delta-like ligand 1 (Dll1). In contrast to Dll1, Jag1, in vitro and during induced myelopoiesis in vivo, prevented DC differentiation by promoting the accumulation of their precursors. Although both ligands activated Notch in hematopoietic progenitor cells, they had an opposite effect on Wnt signaling. Dll1 activated Wnt pathways, whereas Jag1 inhibited it via downregulation of the expression of the Wnt receptors Fzr (Fzd). Jag1 suppressed fzd expression by retaining histone deacetylase 1 in the complex with the transcription factor CSL/CFB1 on the fzd promoter. Our results suggest that DC differentiation, during induced myelopoiesis, can be regulated by the protein of the Notch ligand expressed on adjacent stroma cells. The Journal of Immunology, 2013, 191: 1916–1926.

Dendritic cells (DCs) are professional APCs critically important for the induction of immune responses (1, 2). The regulation of DC differentiation is a complex, spatially controlled process. Although it is initiated in bone marrow (BM), most DCs became terminally differentiated cells in the peripheral lymphoid organs or tissues. There are only a few differentiated, functionally competent DCs in BM (3). This plays an important biological role by limiting the possible inflammatory reaction in BM. The molecular mechanisms regulating this process remained largely unclear.

DC differentiation in BM is controlled by a complex network of soluble factors and cell surface–bound molecules. Among the latter, the Notch family of transcriptional regulators plays a major role. Notch signaling is initiated by the binding of the Notch receptor to specific ligands that result in the proteolytic cleavage of the intracellular domain (ICN), followed by the ICN translocation to the nucleus, where it interacts with the transcriptional repressor CSL/CFB-1 (4, 5). One of the most puzzling questions is the role played by different Notch ligands in defining the biological effects of Notch. At present, two major Notch ligand families, Delta (Dll1, Dll3, and Dll4) and Jagged (Jag1 and Jag2), have been described in mammals (6). Although these ligands activate Notch signaling, in recent years, evidence has emerged of the contrasting effects of Delta and Jagged on different cells (7–13). However, the mechanisms these effects remain largely unclear.

It is known that the Notch pathway is involved in the differentiation and function of DCs. However, its role remains controversial. Most of the studies with “gain-of-function” experiments have demonstrated an upregulation of DC differentiation, whereas many studies with “loss of function,” using knockout (KO) mice, showed either a lack of the effect or an inhibition of DC differentiation (reviewed in Ref. 14). Dll1 was shown to potently promote DC differentiation (11, 15, 16), whereas Jag1 prevented the terminal DC differentiation in vitro by inducing an accumulation of immature myeloid cells (IMCs) and DC precursors (11). The mechanism of such an opposite effect remained unclear. The goal of this study was to understand the mechanisms that prevent DC differentiation in BM and possible role of Jag1 in this process.

Materials and Methods

Mice

All mouse experiments were approved by the University of South Florida Institutional Animal Care and Use Committee. Female C57BL/6 mice (aged 6–8 wk) were obtained from the National Cancer Institute. Mx1-Cre mice (mouse strain number 003556), β-catenin−/− mice (mouse strain number 004152 on C57BL/6 background), and CD45.1+ congenic mice (B6.SJL-Ptch1aPep3b/BoyJ) were purchased from The Jackson Laboratory. Jagged1−/− mice were provided by Dr. J. Lewis (London Research Institute, Cancer Research UK, London, U.K.) (10). The conditional β-cat or Jag1 KO mice were generated by crossing homzygous floxed β-cat or Jag1 mice with Mx1-Cre mice, and the offspring carrying a floxed β-cat or Jag1 allele and Mx1-Cre were backcrossed to the homozygous floxed β-cat or Jag1 mice. Homozygous floxed β-cat or Jag1 mice carrying Mx1-Cre transgene were selected and were referred to as β-cat−/−Cre+ or Jag1−/−Cre+ mice. Jag1−/−Cre− or β-cat−/−Cre− mice were used as controls. To induce the β-cat or Jag1 deletion, 250 μg polyinosinic-polyribocytidylic acid [poly(I:C)] were injected i.p. every other day for three or five times. Mice were used within 3 wk after their last poly(I:C) injection. For adoptive transfer experiments, Jag1−/− mice were further backcrossed for eight generations with C57BL/6 mice.
Reagents

Abs against mouse I-A/E, CD11b, CD86 (B7-2), CD45, CD45.1, CD11c, Gr1, B220, and isotype control Abs were obtained from BD Pharmingen (San Diego, CA). Siglec H, DEC-205, and DCIR-2 (3D1) Abs were from eBioscience (San Diego, CA). F/4/80 Ab was purchased from Serotec (Ratnagiri, NCR). A C-terminal combinator murine GM-CSF and IL-4 were obtained from Research Diagnostics (Flanders, NJ), and IL-3, FLT-3, M-CSF, recombinant human cytokines GM-CSF, TNF-α, and mouse Wnt3a were obtained from R&D Systems (Minneapolis, MN). Jagged-IgG was from Alexis Biochemical, SB216763, human IgG, and anti-human IgG were from Sigma-Aldrich (St. Louis, MO). β-Catenin, Jagged-1, and histone deacetylases (HDACs) 1, 2, 5, and 7 Abs were from Cell Signaling Technology (Beverly, MA). Abs against Frizzled (Fzd) 6, 7, and 10, β-actin, Delta-1, CBF1, and siRNA for Jagged-1, HDAC1, Delta-1, and CBF-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Primers for Real-time RT-PCR were synthesized by Integrated DNA Technologies (Coralville, IA). The monomeric form of the Notch ligand Delta-1 was made by fusing the extracellular domain of Delta-1 to a series of myc epitopesDelta-1myc2830 and was a gift from Dr. I. Bernstein.

Cell lines, cell culture, and flow cytometric analysis

BM and spleen (SPL) stroma cells were generated as described previously (11). The NIH/3T3 cell lines transfected with Delta-1, Jagged-1, or control plasmids were described previously (11). Mouse hematopoietic progenitor cells (HPCs) from mouse BM were enriched with a lineage cell depletion kit from Miltenyi Biotec (Auburn, CA). Enriched HPCs, from naive BM, were placed onto 3T3 cells or primary stromal cells the following day and cultured in RPMI 1640 medium supplemented with 10% FBS and 20 ng/ml GM-CSF for various times. HPC progenies were transfected to new plate and coated with corresponding cell lines every 2–3 d. CD45 cells were gated so that hematopoietic cells could be distinguished from contaminating stroma. For the analysis of gene and protein expression, hematopoietic cells were isolated with biotin-conjugated CD45 Ab and magnetic beads (Miltenyi Biotec) (17). 32D cells (murine hematopoietic cell line) were cultured in RPMI 1640 medium with 10% FBS and 20% conditioned medium containing IL-3 (WEHI-3B cell line). The incubation of 32D cells with different 3T3 cell lines was similar to HPCs. Cells was analyzed by flow cytometry on a FACScalibur flow cytometer (BD Biosciences, Mountain View, CA) or LSR II (BD Biosciences), and data were analyzed with the CellQuest program (BD Biosciences), or FlowJo program (Tree Star, Ashland, OR).

The immobilization of Notch ligands on plastic was performed, as described previously (18). In brief, 20 μg/ml Ab against human IgG was placed into each well of a 24-well plate and incubated for 30 min at 37˚C. Wells were blocked with complete culture medium for 30 min and then incubated for 3 h at 37˚C with 7.5 μg/ml DII, Jag1, or IgG in complete culture medium.

Plasmid construction and transfection

The 5’-regulated sequence (~1233 to +235 bp) of the mouse fzd10 gene (NM_175284) was cloned into pGL3 enhancer vector (Promega, Madison, WI), using the following pair of oligonucleotides: forward primer, 5'-CGACGGTTTACTTCCACAAAACCAGCCA-3' and reverse primer, 5'-CCGGTCGGTAGTCTCGAAACCAGCCAATC-3'.

Transient transfections were performed on 32D cells using the GenePORTER2 transfection agent (Genlantis, San Diego, CA) and following the manufacturer’s guidelines. For the reporter activity assay, Renilla plasmid was cotransfected with luciferase plasmids and relative light units of luciferase produced were measured. The NIH/3T3 cotransfected with new plasmid was used as the loading control.

Hydrodynamic gene transfer

Hydrodynamic gene transfer (HGT) was performed as described previously (19). Briefly, GM-CSF plasmid (20) was purified with an Endofree plasmid max kit (Qiagen). Plasmid (80 μg), resuspended in 1.6 ml saline, was injected i.v. within 5 s.

Quantitative real-time PCR, immunoprecipitation, and immunoblotting

PCR was performed by using SYBR Green RT-PCR reagent kit (Applied Biosystems, Foster City, CA) and target gene-specific primers. Amplification of endogenous cyclophilin or hprt was used as an internal control. Primers sequence was reported previously (18). The analysis of gene expression by immunoblotting was performed as described previously (11). For immunoprecipitation, 500 μg precleared cell lysate was incubated with specific Ab for overnight and the Ag–Ab complex was precipitated by using protein A/G–agarose beads (Santa Cruz Biotechnology, Dallas, TX) and then subjected to electrophoreses in 8% NaDodSO4-polyacrylamide gels. The specific bands were visualized by an ECL detection kit (Amer sham Life Sciences, Arlington Heights, IL).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed with the reagents and protocol from Millipore (Billerica, MA). In brief, we fixed 2 × 106 32D cells by adding formaldehyde into culture media to a final concentration of 1%. The cells were then lysed and sonicated to shear the chromatin DNA. Cell lysate was precleared with protein G–agarose before immunoprecipitation overnight at 4˚C with 2 μg specific Ab or normal mouse IgG. The precipitated Ab–chromatin complex was collected by incubation with protein G–agarose for 1 h at 4˚C and subsequently washed and eluted in elution buffer. DNA, in the precipitated samples, was recovered by reverse cross-linking at 65˚C for 4 h. Ten percent of lysate, before Ab precipitation, was used as the input. Real-time PCR was carried out with Fzd10-specific primers. Amplification of cyclophilin from the input was used as the loading control.

Primers used in ChIP of Fzd10

The following primers were used (boldface suggests the CBF-1 binding site): CHIP primer set 1, 5'-ACCTATGACTGCTGACCTAC-3' and 5'- ACCCGAGTCTCAGATGCTCC-3'; CHIP primer 2, 5'-GTTGAGAAGCT- CTTTGTCTTCCAT-3' and 5'-GGCTGTGTTTCTTCAAC-3'; CHIP primer 3, 5'-CCCTAAATCCTGGCCTC-3' and 5'-GGGATTGTCGGTTT- TATGACC-3'; CHIP primer 4, 5'-GCAAGTGATAGTAAAGCTG-3' and 5'-AACACGAGAAGGCAAGGAGG-3', Gaphid, 5'-TACAGGGTTTAC- CATGACC-3' and 5'-TGGAGACGAGGAGAGGAGA-3'; and putative CBF-1 binding sites: site 1, 5'-GCTCTATTCACGAGCCAA-3'; site 2, 5'-GGTAGTTTTCCAGACACT-3'; site 3, 5'-GGCTGGTTTTCCACGAC-3'; site 4, 5'-TGGGCTTCACAGTCCC-3'; and site 5, 5'- TGGAGACGAGGAGAGGAGA-3'.

Allogeneic MLR

The C57BL/6 BM HPCs were cultured on immobilized Jag-1, on plate, in the presence of SB216763 plus GM-CSF or GM-CSF only for 5 d. The HPC progenies were cocultured with T cells (105/well) from allogeneic BALB/c mice for 4 d. in triplicate, in U-bottom 96-well plates at different ratios. [3H]Thymidine (1 μCi) was added to each well 18 h prior to cell harvesting. T cell proliferation was measured by [3H]thymidine incorporation in a liquid scintillation counter (Packard Instrument, Meriden, CT).

Statistical methods

The data were analyzed with a two-tailed Student t test using GraphPad Software. P value < 0.05 was considered to be statistically significant.

Results

Jag1 negatively regulates DC differentiation in BM

To evaluate the role of Jag1 in DC differentiation, we used mice with conditional KO of this protein. Jag1 KO (10) were crossed with Mx1-Cre mice, and the Jag1 deletion was induced by repeated injections of poly(I:C). As a control, we used Jag1−/−cre−/− littersmates treated with poly(I:C). To symbolize abbreviation, in this paper, Jag1−/−cre−/− with indelible deletion of Jag1 are referred as Jag1 KO and control mice as wild-type (WT). We generated stroma from BM (BMS) and SPL (SPS) with inducible deletion of Jag1 (Supplemental Fig. 1) and evaluated their effect on DC differentiation from enriched HPCs in the presence of GM-CSF. SPS supported DC differentiation substantially better than BMS (control in Fig. 1A versus Fig. 1B). The absence of Jag1 in SPS did not affect DC differentiation (Fig. 1A). In contrast, lack of Jag1 on BMS significantly (p < 0.05) promoted DC differentiation (Fig. 1B). Absence of Jag1 on BMS had no effect on differentiation of DCs generated in the presence of FLT3L (Fig. 1C).
Next, we evaluated the DC differentiation in Jag1-deficient mice in vivo. In steady state, no differences in the total number or the proportions of different DC populations in BM, SPL, or lymph nodes (LNs) were found (Supplemental Fig. 2A). Because our in vitro experiments showed that Jag1−/− BMS had an effect on DC differentiation only in the presence of GM-CSF, which is associated with emergency hemtopoiesis, we evaluated the role of Jag1 in a model of inflammation-induced myelopoiesis caused by...
injection of CFA. In both, WT and Jag1 KO mice, CFA induced rapid and equal increase of IMCs in peripheral blood (PB) (Fig. 1D). In contrast, Jag1 KO mice had significantly higher numbers of CD11c+ cells than WT mice (Fig. 1E). CD11c+ cells (21) are composed of DCs (CD11c+MHC II+) (23), DC progenitors (mainly CD11c+MHC II+) (22), and few activated leukocytes (reviewed in Ref. 23). Analysis of the population of CD11c+MHC II+ DCs in PB confirmed significant increase in the presence of these cells in Jag1 KO mice (Fig. 1F). These results might reflect differences in the mobilization of DCs. To test this possibility, we evaluated DCs in BM 8 d after CFA injection (at the peak of DC presence in PB). The Jag1 KO mice had a significantly higher proportion and absolute number of plasmacytoid DCs and CD11c+MHC II+ DCs, but not MΦs, than their control littermates (Fig. 1G). An increased presence of DCs in BM of Jag1 KO mice was confirmed in allogeneic MLR, a hallmark of DC activity (Fig. 1H). Jag1 KO mice also had a significantly higher presence of DCs in LNs (Fig. 1I).

The total number of cells in WT and Jag1 KO mice was the same (Supplemental Fig. 2B), and as a result, the total number of DCs showed the same changes as the proportion of the cells.

Similar effect of Jag1 was observed in the different model of induced myelopoiesis caused by HGT of GM-CSF plasmid in vivo. An equal increase in IMCs in the PB of both WT and Jag1KO mice was seen (Fig. 2A). In Jag1 KO mice, GM-CSF effect on DC presence in PB (Fig. 2B), BM (Fig. 2C), and LNs (Fig. 2D) was similar to the effect of CFA. There were no difference in the absolute cell number between WT and Jag1 KO BM.

To clarify the role of Jag1 in DC differentiation, we used an adoptive transfer of BM cells from congenic CD45.1+ WT mice to lethally irradiated Jag1 KO or WT CD45.2+ mice. Jag1 deletion was induced by the poly(I:C) injections. Three weeks later, mice were irradiated, and congenic BM cells were transferred. Six weeks after the transfer, recipient mice were injected with CFA and evaluated 8 d later. Jag1 KO and WT mice were equally

![Graphs and images](http://www.jimmunol.org/)

**FIGURE 2.** Jag1 negatively regulates DC differentiation in BM after GM-CSF stimulation. (A and B) The kinetic of IMCs (A) and CD11c+ I-A/E+ DCs (B) in PB of Jag-1 KO and WT mice after treatment with GM-CSF HGT. Each group included three to four mice. *p < 0.05. (C) The proportion and absolute number of indicated populations of cells in BM of mice obtained on day 4 after HGT. Mean ± SEM from five mice per group are shown. *p < 0.05. (D) Absolute number of indicated cell populations in LNs of the same mice as in (C). (E) Lethally irradiated CD45.2+ Jag1 KO or WT mice were reconstituted with 10⁶ CD45.1+ donors' HPCs from WT mice. After 6 wk, mice were treated with CFA as described in the legend to Fig.1G, and the presence in BM of indicated donor’s cells was evaluated 8 d after CFA injection. Data are presented as mean ± SEM (n = 5 mice/group). *p < 0.05.
reconstituted with donor’s BM (Supplemental Fig. 3). However, Jag1 KO mice had a significantly higher presence of donor’s DCs in BM (Fig. 2E, Supplemental Fig. 3) than WT mice. Thus, taken together these results indicated that Jag1 in BM inhibited DC differentiation.

**DII1 and Jag1 have opposite effect on wingless (Wnt) signaling in HPCs**

What could be the mechanism of Jag1 negatively regulating the DC differentiation? Activation of the canonical Wnt pathway in HPC positively regulates DC differentiation; and the DII1 effect on DC differentiation could be mediated via activation of Wnt signaling (17). Canonical Wnt pathway is activated by Wnt proteins binding to Fzd receptors and coreceptors, which leads to stabilization and translocation of β-catenin to the nuclei to form coactivators with T cell factor (TCF)/lymphoid enhancer factor (LEF) to regulate the target genes (24–26). We asked whether Wnt signaling is involved in Jag1-mediated effects on DC differentiation. Enriched BM HPCs were incubated for 18 h on a monolayer of NIH3T3 fibroblasts overexpressing DII1, Jag1, or the corresponding control vectors (11). Jag1 cells did not significantly increase expression of any Fzd receptors but inhibited the expression of number of them (Fig. 3A). This was in contrast to the effect of DII1, which dramatically upregulated members of Fzd family (17). The downregulation of several Fzd receptors was confirmed by Western blotting (Fig. 3B). The inhibition of Wnt signaling was determined by downregulation of β-catenin (Fig. 3C), Wnt target genes (Fig. 3D), and TCF/LEF reporter activity (Fig. 3E). To avoid a possible confounding effect of fibroblasts, we used an experimental system where Notch signaling was activated by Notch ligands directly immobilized on plastic in the presence of reagents in the system where Notch signaling was activated by Notch ligands. Dll1 significantly upregulated the reporter (Fig. 6A) and restored the expression of β-catenin (Fig. 6B). Jag1 also decreased the activity of the TCF/LEF reporter induced by DII1 (Fig. 6A). In contrast, DII1 induced upregulation of Wnt signaling (Fig. 3G, 3H).

We asked whether Jag1 can neutralize activating effect of DII1 on Wnt signaling. DII1-IgG and Jag1-IgG were mixed at a 1:1 ratio and immobilized on plastic. As a control, DII1-IgG were mixed at a 1:1 ratio with control IgG. Jag1 did not inhibit DII1-inducible upregulation of hes5 expression, indicating that mix of these two ligands did not affect Notch signaling (Fig. 4A). In contrast, the presence of Jag1 dramatically reduced the expression of fzd6 and fzd10, as well as wisp1 and wisp2 induced by DII1 (Fig. 4A). Jag1 also decreased the activity of the TCF/LEF reporter induced by DII1 (Fig. 4B). Thus, Jag1 was able to neutralize upregulation of Wnt pathway caused by DII1.

Incubation of HPC on BMS generated from Jag1 KO mice resulted in increased expression of Wnt-targeted genes (Fig. 4C). HPCs isolated from BM of CFA-treated Jag1 KO mice (described in Fig. 1) had a significantly higher expression of Wnt target genes and fzd10 as well as β-catenin and Fzd10 proteins than the HPCs from control mice (Fig. 4D). These results were confirmed in experiments in vitro using BMS with Jag1 downregulated with specific siRNA (Fig. 4E). Thus, Jag1 in contrast to DII1 substantially reduced Wnt signaling in HPC via downregulation of Fzd receptors.

**Jag1 negatively regulate DC differentiation via downregulation of Wnt signaling**

Next, we investigated the mechanism of Jag1 mediated regulation of DC differentiation. Consistent with previous observations (11, 15, 16, 27), Dll1 promoted DC differentiation from HPCs (Fig. 5A). Addition of Jag1 to DII1 completely abrogated this effect (Fig. 5A). We evaluated the effect of activation of Wnt pathway downstream of Fzd receptors on the DC differentiation. Wnt pathway was activated by overexpression of a constitutively active β-catenin mutant (28) (Fig. 5B) or by inhibition of the GSK-3β kinase, which is critically important for activation of Wnt signaling, using specific inhibitor SB216763 (29) (Fig. 5C). In both cases, activation of Wnt pathway abrogated the inhibitory effect of Jag1 on DC differentiation (Fig. 5B, 5C) and restored the proportion of DCs to the control level. It also restored the ability of cells differentiated in the presence of Jag1 to stimulate alloimmune T cells, the function attributed to DCs (5D).

Next, we investigated the consequences of downregulation of Wnt signaling for DC differentiation, using mice with a conditional KO of the β-catenin gene. β-catenin null mice were crossed with Mx1-Cre mice, and β-catenin deletion was achieved by repeated injections of poly(I:C) (Fig. 5E). These mice were further referred as β-catenin KO. BM cells from β-catenin KO mice and Cre+ WT littermates were adoptively transferred into lethally irradiated congenic CD45.1 mice. Four weeks after the transfer, the mice were treated with GM-CSF HGT or with a control vector, as described in Fig. 2C, and analyzed on day 4. No differences in DC differentiation between recipients of WT and β-catenin KO BM cells were seen in mice treated with the control vector. GM-CSF HGT caused significant increase in the presence of WT donors’ DCs in BM, which was consistent with the recent paper (30). In contrast, no increase was observed in recipients of β-catenin KO BM (Fig. 5F). Mφ differentiation was not impaired in the β-catenin KO BM (Fig. 5F).

To evaluate DC differentiation from their precursors, BM Gr-1+CD11b+ cDCs were isolated from WT and β-catenin KO mice (CD45.2+) and transferred to sublethally irradiated congenic CD45.1 recipients. Donors’ cells were evaluated 4 d later. The proportion and absolute number of splenic cDCs (MHCIIC+CD11c+) but not Mφs generated from β-catenin KO BM were significantly lower than that from WT BM (Fig. 5G). The differentiation of splenic cDC subset was also significantly impaired, which was consistent with previous observations that the development of splenic CD8α- cDCs was dramatically blocked in CBF-1 KO mice (31). The culture of enriched HPCs from β-catenin KO mice with GM-CSF and IL-4 produced smaller proportion of CD11c+CD11b+F4/80+ DCs than that of WT HPC (Fig. 5H). Treatment of mice with selective Wnt inhibitor ICG-001 (32) reduced the proportion of DCs in BM of Jag1 KO mice treated with CFA but not in control mice (Supplemental Fig. 4). Thus, these data support the hypothesis that Jag1 regulates DC differentiation in emergency hematopoiesis via inhibition of Wnt signaling.

**Molecular mechanism of opposing effects of DII1 and Jag1 on Wnt signaling**

HDAC is a major component of the constitutive CSL/CBF-1 repressor complex. We explored the possible role of HDAC in the regulation of fzd expression by Jag1. HDAC inhibitor trichostatin A (TSA) completely abrogated the Jag1-inducible inhibition of fzd6 and fzd10 expression (Fig. 6A) and restored the expression of the Wnt target genes (Fig. 6B). We made a luciferase reporter construct by cloning the fzd10 promoter region into a pGL3 enhancer vector. 32D myeloid progenitor cells were transfected with this vector and placed on fibroblasts, overexpressing different Notch ligands. DII1 significantly upregulated the reporter (Fig. 6C), whereas Jag1 inhibited it (Fig. 6D). The TSA slightly upregulated the fzd10 reporter in the cells cultured on DII1 but completely restored its activity in 32D cells cultured on Jag1 (Fig. 6C, 6D).
To clarify these findings, ChIP assays were performed in 32D cells using Ab specific for acetylated histone H3 and primers specific for fzd10 promoter. Dll1 upregulated the association of acetylated H3 with the promoter, but it was significantly inhibited by Jag1 (Fig. 6E). The opposite effect was observed when chromatin was precipitated with HDAC1 Ab (Fig. 6F). Thus, in contrast to Dll1, Jag1 did not cause the displacement of HDAC1 from the fzd10 promoter, which could result on silencing of the gene expression.

Because both Jag1 and Dll1 induced CBF-1 activation, we asked whether these ligands differently regulated the physical interaction between HDAC1 and CBF-1. HPCs were transfected with HDAC1 siRNA (Fig. 6H, inset). The downregulation of HDAC1 completely abrogated the Jag1-inducible HDAC1 association with the fzd10 promoter (Fig. 6H), indicating that HDAC1 interacts with the fzd10 promoter in complex with CSL. HDAC1

FIGURE 3. Notch ligands differentially regulate Wnt signaling in HPCs. (A) Enriched HPCs were incubated on a monolayer of NIH3T3 fibroblasts overexpressing Jag1, or control MSCV-vectors in medium containing 20 ng/ml GM-CSF. CD45+ hematopoietic cells were isolated using magnetic beads after 18 h culture and expression of fzd gene family were evaluated using quantitative RT-PCR. Relative gene expression was calculated by normalizing to internal control (cyclophilin). Mean ± SEM of three experiments performed in triplicates are shown. (B and C) The amounts of selected Fzd proteins (B) and β-catenin protein (C) evaluated by Western blot after 3 d culture. Typical examples of three performed experiments are shown. (D) mRNA level of Wnt target gene were by measured by quantitative RT-PCR after 18 h culture. Mean ± SEM of three experiments performed in triplicates are shown. Please note differences in the scale. (E) HPCs were transfected with TCF/LEF reporter plasmid and control Renilla plasmid and then cultured on fibroblasts for 48 h. Reporter activity was measured using dual-luciferase reporter assay. Three experiments were performed in duplicates. (F–I) The effect of immobilized Notch ligands on Notch signaling in HPCs. (F) Enriched HPCs were transfected with luciferase CBF-1 reporter plasmid and control Renilla plasmid. Cells were cultured for 24 h on plates coated with 7.5 μg/ml control IgG, Dll1, or Jag1. Luciferase activity was measured in triplicates in dual-luciferase assay. Results of two experiments are shown. (G) Enriched HPCs were cultured on a 24-well plate coated with 7.5 μg/ml Δ-IgG, Jag-IgG, or control IgG protein in the presence of 100 ng/ml recombinant Wnt3A. Expression of fzd and Wnt-targeted genes evaluated using quantitative RT-PCR after 24 h culture. Fold changes in relative gene expression over the control IgG levels was calculated. The negative values indicate downregulation of genes expression. Mean ± SEM of three experiments performed in triplicates are shown. (H) The amount of selected Fzd proteins was evaluated in Western blotting using indicated Abs. (I) HPCs were transfected with TCF/LEF reporter plasmid and control Renilla plasmid before cultured on ligands coated plate. Reporter activity was measured 48 h later. Mean ± SEM of three experiments performed in duplicates are shown. *p < 0.05.
was knocked down in 32D cells by using siRNA (Fig. 6I, inset). This did not affect the Dll1-inducible upregulation of the expression of *fzd10* and Wnt target genes. However, the Jag1-inducible downregulation of these genes was abrogated (Fig. 6I).

**Discussion**

This study suggests the novel mechanism of regulation of DC differentiation in BM, which depends on the expression of the Notch ligand, Jag1. Under steady-state conditions Jag1 was dispensable for DC differentiation. However, it was important in the conditions of forced myelopoiesis. Among several models of emergency myelopoiesis, we used two: inflammation-related caused by the injection of CFA; and induced by the administration of GM-CSF. Although both treatments caused an equal increase of IMCs in WT and Jag1 KO mice, the presence of DCs in blood and BM was significantly higher in Jag1 KO than in WT mice. These data were consistent with previous observations that Notch signaling had no effect on long-term, repopulating HSC self-renewal when assessed during homeostasis (33) but had a strong effect on the differentiation and enhanced generation of myeloid progenitors during stress hematopoiesis (34). The fact that the effect of Jag1 deletion on DCs was confined largely to BM could be explained by the fact that Jag1 expression is predominant in BMS but not in SPS (11). Jag1 was also previously shown to be the main Notch ligand that is expressed in the HSC niche in BM (35). It appears that Jag1 may play an important role in the inhibition of DC differentiation from the precursors inside BM. When DC precursors leave BM, they undergo a terminal differentiation in tissues where the expression of Jag1 is substantially reduced.

The main focus of this study was on the mechanism of the opposite effect of Jag1 and Dll1 on DC differentiation. Both are ligands for the same receptors and both are able to activate Notch signaling. One of the possible explanations is the different strength of the Notch signaling, mediated by Jag1 and Dll1. It was shown that Jag1 had the weakest ability to activate Notch1, whereas, Dll4 was the strongest Notch1 activator, followed by Dll1 and Jag2 (12). The different effect of Jag1 and Dll1 was also associated with the Lunatic-Fringe–mediated glycosylation of Notch1. Glycosylation...
FIGURE 5. Jag1 negatively regulates DC differentiation via downregulation of Wnt signaling. (A) Enriched HPCs were cultured on 24-well plates coated with a 1:1 mixture of Dll1 and Jag1 or IgG proteins in the presence of Wnt3a (100 ng/ml) for 5 d in medium with 20 ng/ml GM-CSF. Mean ± SEM of three experiments are shown. *p < 0.05, statistically significant differences between IgG+Dll1 and Jag1+Dll1 groups. (B) Enriched HPCs were transfected with β-catenin-GFP or GFP vector and then cultured on monolayer of fibroblasts for 5 d. The percentage of the indicated population of cells was calculated within gated CD45+GFP+ cells. Mean ± SEM of three experiments are shown. *p < 0.05 between Jag1 and Jag1+β-catenin groups. (C) Enriched HPCs were cultured on plates coated with IgG or Jag1-IgG for 4 d. SB216763 (10 nM) was added on day 0. The percentage of the indicated population of cells was evaluated on day 5. Cumulative results of two performed experiments. *p < 0.05, statistically significant differences between Jag1 and Jag1+SB216763 groups. (D) Allogeneic MLR. Cells from experiments described in (C) were cultured at different ratios with T cells isolated from allogeneic BALB/c mice for 4 d. Cell proliferation was measured in triplicate by [3H]thymidine uptake. Values are the mean ± SEM. (E) β-Catenin expression in splenocytes from β-cat^{fl/fl} Mx1-Cre^−/− KO or control β-cat^{fl/fl} Mx1-Cre^− (control) mice. (F) Lethally irradiated CD45.1^+ recipients were reconstituted with 1.5 × 10^6 cells BM from CD45.2^− β-cat KO or WT mice. Four weeks after the adoptive transfer, mice were treated with GM-CSF HGT or control vector and evaluated 4 d later. Data show the number of indicated populations of donors cells presented as mean ± SEM (n = 5 mice/group). *p < 0.05, statistically significant differences from control. (H) Differentiation of DCs in the presence of GM-CSF and IL-4 in serum free medium for 6 d from 1.5 × 10^5 enriched BM HPCs from β-cat KO or WT mice. Representative FACS profiles from four individual experiments and cumulative results of the total number of F4/80^+ CD11c^+CD11b^+ DCs are shown. **p < 0.01 between groups.
of Notch1 potentiated Notch signaling throughDlll ligands and Jag2, in contrast to Jag1 (12, 36). Upon glycosylation of Notch, Dll4-Notch signaling was enhanced, whereas Jag1 had a weak signaling capacity (7). Individual Notch receptors have different transcriptional activity (37). Jagged family members, in the presence of Fringe, can activate Notch2 (38-40). It was recently shown that Notch2 enhanced the rate of formation of HSCs while delaying myeloid differentiation in BM, during nonhomeostatic conditions, including after chemotherapy or during marrow regeneration after stem cell transplantation. However, both Jag1 and Dll1 activated Notch2 in the HSC-enriched population that inhibited myeloid differentiation and enhanced the generation of HSCs.
myeloid progenitors during stress hematopoiesis (34). In our study, lack of Jag1, during emergency hematopoiesis, did not affect the total number of myeloid cells. No decrease in the presence of IMCs or MOs was seen. This suggested that deletion of Jag1 did not result in a modified expansion of progenitor cells but rather influenced the specific differentiation of DCs in the compartment with prevalent Jag1 expression (BM). Jag1 was able to actively neutralize the effect of Dll1 on DC differentiation. These data argue that the inhibitory effect of Jag1 on DC differentiation was unlikely, because of its inability to reach the required Notch signal strength threshold to induce DC differentiation.

Our data suggest a novel mechanism of a negative regulation of DC differentiation by Jag1 that involves Wnt signaling. Previous studies have implicated the Wnt pathway in DC differentiation and activation (17) (28, 41). Dll1 promoted DC differentiation via up-regulation of the expression of the Fzd family of Wnt receptors and activation of Wnt signaling (17). This effect was different from the recently reported posttranslational inhibition of β-catenin in stem and colon cancer cells by membrane-bound Notch (42). To our surprise, Jag1 directly inhibited Wnt signaling in HPCs via the transcriptional downregulation of the expression of several Wnt receptors Fzd. Jag1 was able to neutralize the Dll1-inducible up-regulation of Fzd, despite the fact that Dll1 has a much stronger effect on Notch signaling.

These data suggest that Wnt signaling could affect the differentiation of DCs. However, recipients of β-caten KO BM cells, in steady state, had no defects in DC differentiation. This was consistent with a previous paper (43). However, when recipients of β-caten KO BM were treated with GM-CSF by HGT, they failed to upregulate DC production in BM, which suggested that β-caten may play an important role in DC differentiation during emergency hematopoiesis. In the absence of Notch activation, CBF-1 exists in nuclei as a repressor complex, in association with the transcriptional corepressors SMRT, NCoR, CIR, SHARP, KyoT2, and Skip. Some of these corepressors (SMRT, CIR, and SHARP) are shown to recruit HDACs to Notch target genes (44, 45). After activation, cleaved Notch ligands and Notch receptors are shown to recruit these corepressors (SMRT, CIR, and SHARP) to form HDAC complexes on Notch target genes. HDAC1 was able to neutralize the Dll1-inducible expression of Fzd, despite the fact that Dll1 has a much stronger effect on Notch signaling. These data suggest that Wnt signaling could affect the differentiation of Dll1, which directly inhibited Wnt signaling in HPCs via the transcriptional downregulation of the expression of several Wnt receptors Fzd. Jag1 was able to neutralize the Dll1-inducible up-regulation of Fzd, despite the fact that Dll1 has a much stronger effect on Notch signaling.
Figure S1. The efficiency of Jagged-1 deletion in Jag1^{fl/fl}Mx1-Cre^{+/−} mice. A.B. BM stromal cells were generated from poly (I:C) treated Jag1^{fl/fl}Mx1-Cre^{+/−} mice (KO) or control Jag1^{fl/fl}Mx1-Cre^{−/−} littermates (WT). Jag1 expression was examined by quantitative PCR in triplicates (A) or by Western blot (B). C. Spleen stroma cells were generated from poly (I:C) treated Jag1^{fl/fl}Mx1-Cre^{+/−} mice (KO) or control Jag1^{fl/fl}Mx1-Cre^{−/−} littermates (WT).
Figure S2. DCs differentiation in Jag1^{fl/fl}Mx1-Cre^{+/-} mice. A. The frequency of DC populations in BM, SPL, LNs from poly (I:C) treated Jag1 KO or WT mice. Mean ± SEM of three experiments are shown. B. The total number of cells in different organs 8 days after the treatment with CFA. Individual results are shown.
Figure S3. Typical gating of cells analyzed by flow cytometry. Lethally irradiated Jag-1 KO or WT mice were reconstituted with CD45.1$^+$ HPC for 6 weeks and injected with CFA ($n=3$ mice). Shown are the BM chimeras (left panel) and CD45.1$^+$ donors cells (right panel).
Figure S4. Effect of Wnt inhibitor on DC differentiation. A. C57BL/6 mice (n=4) were treated with ICG-001 (100 mg/kg i.p, twice a day) for 14 days. The total number of cells and the presence of DCs in BM were evaluated. Data presented as Mean ± SEM. B-C. Mice (n=4) were treated with ICG-001 (100 mg/kg i.p, twice a day) for 10 days. On day 2, mice were injected i.p. with 200 μl CFA. Mice were sacrificed on day 8 after CFA administration and the presence of DCs in BM was evaluated. Data presented as Mean ± SEM. * p<0.05 between the groups. C. The expression of wisp1 and wisp2 in BM cells * p<0.05 between groups.