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Notch1 Perturbation of Hemopoiesis Involves Non-Cell-Autonomous Modifications

Shin Kawamata, Changchun Du, Kaijun Li, and Catherine Lavau

To study the effects of Notch on hemopoiesis we used a bone marrow transduction/transplantation model and compared the transduced and nontransduced populations in reconstituted mice. While cells expressing a constitutively active form of murine Notch1 (Notch1IC) completely lacked B cells, a profound suppression of the B lineage was also seen in the nontransduced compartment. Experiments performed with retroviral supernatants of varying titers showed that the perturbations of B cell development among the nontransduced population correlated with the percentage of Notch1IC-transduced cells inoculated into the mice. The myeloid lineage of the Notch1IC-transplanted mice was altered as well, and this also affected the nontransduced population that had features of excessive maturation. To explore the basis of these non-cell-autonomous modifications we prepared conditioned medium from ex vivo cultures of Notch1IC-transplanted mice bone marrow and showed that it inhibited B cell maturation and promoted myeloid differentiation in a dose-dependent manner. Finally, we found that the T cell leukemia/lymphomas that occur in Notch1IC-transplanted mice were accompanied by abnormal maturation of nontransduced T cells in the bone marrow. These findings indicate that modifications of neighboring cells through non-cell-autonomous modifications take part in multiple facets of the activity of Notch on hemopoiesis.


The Notch proteins are highly conserved transmembrane receptors that regulate tissue patterning and morphogenesis in both vertebrates and invertebrates (1–4). In vertebrates, Notch is thought to initiate signaling upon ligand stimulation through the release of the intracellular domain of Notch (NotchIC) into the nucleus. There, NotchIC interacts with the transcription factor CBF1, and the NotchIC/CBF1 complex activates transcription of Hes genes in a CBF1-dependent fashion (5–7). In addition to this pathway, a CBF1-dependent NF-κB2 pathway (8) and CBF1-independent pathways (9) have been reported. Thus, the activation of Notch could trigger multiple pathways that induce various cellular responses in Notch-activated cells. Notch signaling can also be modified or regulated by other proteins, such as Fringe, so that only a subset of cells responds to Notch signals. Ectopic expression of Fringe during Drosophila eye or wing development linked boundaries of Fringe expression and Notch activation (10–12). In addition to cell-autonomous modifications, non-cell-autonomous modifications mediated by secreted factors from Notch-activated cells have been documented in Drosophila morphogenesis (13).

Notch receptors and ligands are expressed in the mammalian hemopoietic system, and several reports have demonstrated the involvement of Notch activation in hemopoietic cell fate choices (reviewed in Ref. 14). Transgenic overexpression of NotchIC (a constitutively active form of Notch1) was associated with an increased ratio of CD8 over CD4 single-positive (SP) thymocytes (15). Another study using the same proximal Lck promoter to drive the Notch1IC transgene similarly showed that Notch1 activation led to the generation of an excess of mature CD8 SP thymocytes, but the development of SP CD4 thymocytes was also shown to be stimulated (16). At an earlier time in T cell development, Notch1IC was shown to promote the adoption of a αβTCR phenotype in the TCRαβ vs TCRγδ cell fate decision process (17). More conclusively, complementary studies using gain- and loss-of-function of Notch1 activity showed that Notch1 signaling favored the commitment of common lymphoid progenitors to T cell fate in the T vs B lymphoid cell fate choices. Pear et al. (18, 19) demonstrated the development of T cell leukemia/lymphomas concomitantly with a block in B cell lymphopoiesis in mice transplanted with bone marrow (BM) overexpressing Notch1IC. Conversely, Radtke et al. (20, 21) demonstrated that T cell development was blocked at an early stage and that B cells accumulated in the thymus of conditional Notch1 knockout mice, suggesting an instructive role of Notch1 in T cell lineage induction. This was confirmed in transgenic mice overexpressing a modifier of Notch1 signaling, lunatic fringe, under control of the Lck promoter. Transgenic thymocytes acted non-cell-autonomously to inhibit Notch1 activation, which inhibited T cell commitment and promoted B cell development in the thymus (22).

In contrast to the numerous reports describing the role for Notch in lymphopoiesis (reviewed in Refs. 1, 14, and 23), there has not been any study demonstrating the effect of Notch on myeloid development in vivo. In vitro studies based on the myeloid cell line 32D have been described, and these have generated contradictory results on the effects of Notch1 activation on myeloid differentiation (24, 25).

In this report we describe the non-cell-autonomous effects of Notch1 on hemopoiesis by transplanting irradiated mice with a mixture of cells overexpressing Notch1IC and normal bystander cells and explore the function of Notch in mammalian hemopoiesis.
Materials and Methods

Constructs, Northern blotting, and semiquantitative PCR

The murine Notch1IC cDNA encoding the totality of the intracellular domain (from codons 1474–2531; a gift from Dr. M. Cleary, Stanford University School of Medicine, Stanford, CA) was cloned into the retroviral vector MIE (MSCV-ires-eGFP) (26, 27).

The enhanced green fluorescence protein (eGFP $^+$ or eGFP $^-$ cells) were separated from BM of Notch1IC-transplanted mice by sorting the cells for high or no expression of eGFP, respectively, using a FACS Vantage BD Biosciences, San Jose, CA). The reanalysis of the sorted eGFP population from Notch1IC BM showed that 95% of the cells were negative for eGFP expression. Total RNA was isolated from respective sorted fraction with Stat-60 RNA extraction reagent (Tel-Test B, Friendswood, TX). A total of 15 μg of total RNA was fractionated by electrophoresis through 1.2% agarose/formaldehyde denaturing gel and transferred to a nylon membrane (Roche, Indianapolis, IN). The membrane was hybridized with 12p-labeled Notch1IC probe covering DNA sequence 5441–5961 and with a G3PDH probe (Clontech Laboratories, Palo Alto, CA).

cDNA was generated from total RNA with random hexamers and Superscript (Life Technologies, Gaithersburg, MD). PCR was performed using primers coding for murine Notch1 DNA sequences 5441–5460 and 5942–5961, and TaqPlus polymerase (Stratagene, La Jolla, CA).

Retroviral transduction of BM cells and reconstitution of mice

C57BL/Ka-Ly5.2, Thy1.1 mice (known as BA.1), C57BL/Ka-Ly5.1, Thy1.1 mice (known as BS/BA), and BALB/c were bred and maintained in the animal facility at SyStemix (Palo Alto, CA). Four-week-old BA.1 (Ly5.2) mice were injected i.v. with 150 mg/kg 5-fluorouracil (5-FU) in PBS 5 days before sacrifice. BM cells from 4-wk-old BA.1 mice were collected, followed by RBC lysis. Primitive hematopoietic precursors were enriched by depleting lineage-positive cells using a panel of Abs directed against CD3, CD5, CD8a, CD11b (Mac-1), Gr-1, and B220 (BD Pharmingen, San Diego, CA), followed by a step of negative selection using Dynabeads M-450 (Dynal Biotech, Oslo, Norway). The retroviral transduction of the resulting Lin$^-$/low cells was performed as described previously (28). The transduction efficiency was determined by flow cytometry measuring percentage of Lin$^-$/low cells among the Lin$^-$/low population on the day following the second round of spinoculation. Lethally irradiated (1050 rad, one total gamma irradiation) BS/BA. (Ly5.1) recipient mice were transplanted with 15,000–20,000 Lin$^-$/low-transduced BA.1 cells together with 100,000 syngenic whole BM (Ly5.1) cells for short-term irradiation protection.

Flow cytometric analysis and methylationcell assay

The cells harvested from BM, PB, spleen, and thymus or following culture in vitro were stained with Abs directed against Ly5.1, CD4, CD8a, CD3, CD11b (Mac-1), CD19, CD24, CD43, BP-1, IgM, or Gr-1 (BD Pharmingen). Stained cells were resuspended with propidium iodide (5 μg/ml) to exclude nonviable cells and analyzed on a FACS Calibur (BD Biosciences) or a FACS Vantage BD Biosciences. Light scatter gating was set to include all nucleated cells. The percentage of cells from MIE control or Notch1IC-transplanted mice were seeded in 1.1 ml of Methocult M3320 methylcellulose medium (StemCell Technologies, Vancouver, Canada) supplemented with murine IL-3, IL-6, GM-CSF (all at 10 ng/ml; R&D Systems, Minneapolis, MN), and stem cell factor (SCF; 100 ng/ml; supplied by Novartis Pharmaceuticals, East Hanover, NJ). After 2 wk of culture, colonies were scored, and analyzed by flow cytometry for eGFP expression and myeloid maturation markers.

Coculture and conditioned medium experiments

Lethally irradiated BA.1 (Ly5.2) mice were transplanted with Notch1IC-transduced Lin$^-$/low BA.1 BM cells. BA.1 mice transplanted with Lin$^-$/low BA.1 BM cells transduced with empty vector (MIE) were used as a control. BM cells from BA.1 Notch1 IC mice (1 million cells/ml) were used as coculture. BM cells from BA.1 Notch1 IC mice (1 million cells/ml) were cocultured with normal Lin$^-$/low BS/BA (Ly5.1) BM cells (0.2 million cells/ml) in IMDM complete medium (Life Technologies, Gaithersburg, MD). 10% FCS (Gemini Bioproducts, Calabasas, CA), and 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO) in the presence of IL-7 (10 ng/ml), SCF (100 ng/ml), and Flk-L (50 ng/ml). The total cell number was determined every 2 days, and cells were split at a density of 1–1.5 million cells/ml. The phenotype of the cells in coculture was examined by flow cytometry. Supernatant from cultured BM cells of BA.1 Notch1 IC mice or BA.1 MIE mice (1 million cells/ml at the initiation of culture) was harvested and filtered every 3 days and added to the test Lin$^-$/low BS/BA BM cells cultured at a density of 0.2–1 million cells/ml in the presence of IL-7, SCF, and Flk-L. The supernatant of BM cells from transplanted mice was diluted in fresh medium as indicated to evaluate a dose-dependent effect.

Results

Perturbation of B cell development through non-cell-autonomous modifications

Early BM hematopoietic precursors from 5-FU-treated BA.1 (Ly5.2) donor mice were enriched by depletion of the lineage-positive cells. The resulting Lin$^-$/low donor population consisted of ~2% of the 5-FU-treated whole BM. These cells were transduced with a constitutively active form of Notch1, Notch1 intracellular domain (Notch1IC), using the MIE retroviral vector (26) that encodes an internal ribosomal entry site (IRES) and the eGFP. The transduced Lin$^-$/low cells were inoculated to lethally irradiated BS/BA (Ly5.1) recipient mice together with syngenic (Ly5.1) BM to ensure short-term radioprotection (Fig. 1A). The initial transduction efficiencies of Lin$^-$/low cells with MIE or Notch1IC at the time of transplantation were 65 or 25%, respectively. The BM, spleen, thymus, and peripheral blood from the transplanted mice were harvested for flow cytometric analyses at 3–4 wk post-transplantation. The phenotypes of the BM among the transduced and the nontransduced populations are shown in Fig. 1B; similar results were found in the spleen and peripheral blood cells (data not shown). As described in an earlier report using a similar transplantation model performed with BALB/c mice (19), B cell development in the transduced fraction of Notch1IC mice was completely blocked. Strikingly, we also found that virtually no B cells were present in the eGFP$^*$ population of Notch1IC mice (Fig. 1B). This absence of B cells among the nontransduced congenic (Ly5.2) and the cotransplanted syngenic (Ly5.1) donor cells (see Fig. 1A) suggested that Notch1IC could modify the lineage determination of cells through a non-cell-autonomous mechanism(s). To further explore this hypothesis we examined the expression of Notch1IC in the eGFP$^*$ population. BM cells from a Notch1IC mouse were sorted by FACS in eGFP$^+$ and eGFP$^-$ populations from which total RNA was extracted to assess the expression of Notch1IC message by Northern blotting. As shown in Fig. 1C, no Notch1IC message could be detected in the eGFP$^+$ population. Semiquantitative PCR assay performed on the same eGFP$^+$–sorted populations revealed that the estimated expression of Notch1IC message in the eGFP$^+$–sorted fraction was ≤6.25% of the amount present in the eGFP$^+$ population (data not shown). This percentage is in the same range as the fraction of eGFP$^+$ cells that contaminates the eGFP$^-$ population obtained by FACS. These results indicate that the alterations seen in the eGFP$^+$ cells cannot be accounted for by the intracellular expression of Notch1IC but are truly non-cell-autonomous.

The efficiency of Notch1IC transduction among donor cells influences the outcome of transplanted mice

To explore the mechanism underlying the absence of B cells in the eGFP$^+$ compartment of Notch1IC mice, we repeated the transduction/transplantation experiment in different genetic backgrounds and/or with retroviral supernatant of varying titers. When the initial transduction efficiency of Lin$^-$/low cells was 20%, we were able to reproduce our finding in BALB/c mice (Fig. 2). We found that the initial transduction efficiency had a drastic influence on the outcome of the experiment. While the block in B cell differentiation among the transduced (eGFP$^+$) cells was always virtually complete, the degree of inhibition of B cell development among the nontransduced compartment of the transplanted mice analyzed 3 wk post-transplantation clearly correlated with the percentage of donor cells expressing Notch1IC at the time of inoculation (Fig. 2). The proportion of eGFP$^+$ cells 3 wk post-transplantation displayed
a high variability from mouse to mouse. However, the high percentage of eGFP+/H11001 cells in the BM of some of the Notch1IC mice inoculated with BM progenitors infected at a lower efficiency probably reflects the growth advantage of the Notch1IC-transduced (eGFP+) cells. The limits defining the eGFP+ population are shown on the histograms with the percentages of cells included. The transduced and nontransduced populations are analyzed for expression of the B cell markers CD19 and surface IgM (left panel). The Notch1IC mouse shown was relatively healthy at the time of sacrifice and is representative of a group of 10 mice. The absolute numbers of B, T, and myeloid cells were determined by FACS based on the expression of CD19, CD3, and Gr-1 and/or Mac-1, respectively, in the eGFP+ and eGFP− fractions. The results are the mean ± SD of eight MIE and eight Notch1IC mice. The total numbers of BM cells harvested from four hind leg bones of MIE and Notch1IC mice were 24.3 ± 4.7 × 10^6 and 11.7 ± 2.8 × 10^6, respectively. C, Expression of Notch1IC message in the transplanted mice. BM from a Notch1IC mouse (mouse 5 in Table II) was collected 3 wk post-transplantation and sorted by FACS into eGFP+ and eGFP− populations. The limits defining the eGFP-positive and -negative BM fractions of the Notch1IC mouse are shown on the histogram (left). The initial Notch1IC transduction efficiency among Linneg/low donor cells at the time of transplantation was 25%. Expression of Notch1IC message in eGFP+ (Notch1IC eGFP+) or eGFP− (Notch1IC eGFP−) Notch1IC BM or in nonsorted MIE control BM (MIE control) was determined by Northern blotting (right). Fifteen micrograms of total RNA was hybridized with 32P-labeled Notch1IC probe or G3PDH. The band hybridizing with the Notch1IC probe corresponds to the expected size of the 5′ long terminal repeat-Notch1IC-IRES-eGFP-3′ long terminal repeat fragment.
To further explore the effects of Notch1 on myeloid development, we set up myeloid clonogenic assays in methylcellulose with BM cells from the Notch1IC-transplanted mice. After 2 wk of culture in the presence of IL-3, IL-6, GM-CSF, and SCF, the number of colonies was scored, and the cells were pooled, counted, and analyzed by flow cytometry for the expression of eGFP and myeloid markers. As shown in Table I and Fig. 3, Notch1IC BM cells had a very low seeding efficiency, and this activity was confined to the fraction of cells expressing low levels of eGFP. The lower eGFP intensity in these Notch1IC cells probably reflects the fact that the fraction of BM expressing higher levels of eGFP before culture was essentially made up of DP T cells and mature myeloid cells that do not survive the myeloid culture conditions used for the assay (data not shown). The lack of eGFP cells recovered from the myeloid clonogenic assay (Fig. 3) is consistent with the more mature phenotype of myeloid cells observed among the BM eGFP compartment, as these would be expected to have lost their colony-forming potential. In agreement with a previous study of FIGURE 2. The initial transduction efficiency of the Notch1IC construct influences the degree of B cell repression in the nontransduced (eGFP−) compartment. The initial percentage of Lin−/low cells expressing eGFP was determined by flow cytometric analysis on the day of transplantation. BM was harvested 3 wk post-transplantation to analyze eGFP expression and B cell maturation. The limits defining the eGFP+ population are shown on the histograms with the percentage of cells included. Expression of the B cell markers CD19 and surface IgM was determined on the gated eGFP+ and eGFP− populations. Mice transplanted with Lin−/low cells with 1.5 or 13% Notch1IC transduction efficiency were of C57BL/Ka background, while those transplanted with 20% Lin−/low cells transduced were BALB/c. The bar graphs represent the absolute number of B, T, and myeloid cells (determined as in Fig. 1B) present in the eGFP+/top) and eGFP− (bottom) fractions of BM from Notch1IC mice transplanted with a Lin−/low inoculum in which 1.5, 13, or 20% of the cells were transduced. The total numbers of BM cells harvested from the experimental groups were 15.7 ± 3.9 × 106, 16.8 ± 2.1 × 106, and 12.5 ± 2.5 × 106, respectively. Data are the mean ± SD from four or five mice for each transduction efficiency. Mice transplanted with Lin−/low cells with 2 or 18% Notch1IC transduction efficiency demonstrated essentially the same results.

FIGURE 3. Notch1IC alters the phenotype and maturation potential of untransduced myeloid progenitors. The upper histograms represent eGFP expression in the BM of MIE or Notch1IC-transplanted mice at 3 wk. The gates delimit the eGFP+ and eGFP− populations studied for the expression of Gr-1 and Mac-1. The percentages of DP T cells and B cells in the BM of the Notch1IC mouse shown were 68 and 1% for the eGFP+ fraction and 7 and 1% for the eGFP− fraction, respectively. The Notch1IC mouse shown was relatively healthy at the time of sacrifice and is representative of a group of 10 mice. The lower histograms show eGFP expression in the cells recovered from the myeloid clonogenic assay. The expression of the Notch1IC mRNA among the eGFP+ population was confirmed by RT-PCR using specific primers for Notch1IC (data not shown). The results shown are representative of three independent experiments.

Table I. Myeloid clonogenic growth of BM from MIE or Notch1IC mice

<table>
<thead>
<tr>
<th>Transduced Genes</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MIE</td>
<td>Notch1IC</td>
<td></td>
</tr>
<tr>
<td>Percentage of eGFP+ cells at seeding (%)</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>No. of coloniesy</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>No. of cells (×107)y</td>
<td>13.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Percentage of eGFP+ cells following culture (%)</td>
<td>45</td>
<td>92</td>
</tr>
</tbody>
</table>

The results were obtained by seeding the BM shown in Fig. 3 and are representative of three independent experiments.

y Generated from 2 × 104 cells grown in methylcellulose with IL-3, IL-6, GM-CSF, and SCF.

z Total number of cells present at the end of culture.
FIGURE 4. Conditioned medium from ex vivo cultures of Notch1IC-transplanted BM inhibits B cell maturation and promotes myeloid maturation. A, Schematic of the experimental protocol used for the coculture and the conditioned medium experiments. B, Coculture experiment. Lin<sup>−</sup> cells from BS/BA (Ly5.1) mice were cocultured with BA.1 BM from MIE mice or Notch1 IC mice or with normal BA.1 BM. The cells were harvested after 5 days of coculture, and the Ly5.1-gated population was examined for the expression of CD19/CD24 and Gr-1/Mac-1. The Notch1IC BM used for the coculture experiment shown in the dot plot was harvested from a mouse transplanted with an inoculum in which 13% of the cells were transduced. The percentages of DP T cells in BM, B cells, and myeloid cells in the eGFP<sup>−</sup> BM fraction at the time of sacrifice were 16, 6, and 70%, respectively. The data shown are representative of four independent experiments. The bar graph represents the percentages of B cells (CD19<sup>+</sup>) and myeloid cells (Mac-1<sup>−</sup> and/or Gr-1<sup>−</sup>) among test (Ly5.1) cells cocultured with BM from BA.1 MIE, BA.1 Notch1IC, or BA.1 mice. Data are the mean ± SD from four or five mice for each group.
Notch1-expressing myeloid cell line 32D (11), the myeloid maturation of the eGFP+ cells recovered from the myeloid clonogenic assay was blocked (data not shown).

**Conditioned medium from ex vivo cultures of Notch1IC-transplanted BM contained activities that inhibited B cell maturation**

The altered phenotype and growth potential of the eGFP+ compartment of the Notch1IC mice suggested that the cells with constitutively activated Notch1 could influence the cell fate of the nontransduced population. To investigate whether the inhibition of B cell maturation in the nontransduced population was mediated by direct cell-to-cell interaction or by secreted molecules, we set up ex vivo culture experiments that are schematically depicted in Fig. 4A. We cocultured normal Linneg/low cells from BS/BA (Ly5.1) mice with BM cells from BA.1 (Ly5.2) mice that had been transplanted 4 wk before with BA.1 (Ly5.2) progenitors transduced by Notch1IC with an efficiency of 8–13%. At the time of sacrifice these Notch1IC mice were healthy, and the percentage of DP T cells in their BM ranged from 18 to 42%, while the nontransduced fraction displayed a moderate to marked deficiency of B cells. As controls, we cocultured the test Linneg/low Ly5.1 cells with BM from MIE-transplanted BA.1 mice or with BM of normal BA.1 mice. We analyzed the expression of CD19 and CD24 in the Ly5.1 population at 5 days of culture, because the proliferative potential of immature Linneg/low cells is much higher than that of whole BM cells, and at later time points the progeny of the Linneg/low Ly5.1 cells greatly outgrew the Ly5.2 population. As shown in Fig. 4B, the presence of Notch1IC BM considerably inhibited the maturation of the test population along the B cell lineage. Additionally, a higher proportion of these cells differentiated toward the myeloid lineage, as evidenced by the increase in the percentage of cells expressing the Gr-1 and Mac-1 Ags (Fig. 4B). The percentages of B cells and myeloid cells among the test cells (Ly5.1) on day 5 of coculture are shown in Fig. 4B. We looked for the proliferation of neoplastic T cells in these cultures on day 4 by flow cytometric analyses using anti-CD3, anti-CD4, and anti-CD8 Abs. No T cells were found in either Ly5.1 or Ly5.2 fractions (data not shown), suggesting that even the Notch1IC-transduced T cells could not be maintained in the culture conditions used here.

To determine whether direct cell-to-cell contact was dispensable, we cultured the test Linneg/low cells with conditioned medium from Notch1IC BM and analyzed the expression of CD19 and surface IgM after 10 days of culture. As shown in Fig. 4, C and D, we observed that the Notch1IC BM supernatant inhibited B cell maturation in a dose-dependent manner, with a nearly total block when a 2-fold dilution of the medium was used. Again, we found that suppression of the B cell compartment was coupled with an expansion of the myeloid compartment.

**Effect of Notch1IC on T cell development**

In the BM of Notch1IC mice, DP T cells dominated among the eGFP+ fraction. As shown in Fig. 5, we also observed an interesting BM profile of CD4 and CD8 expression among the eGFP+ fraction. These nontransduced cells expressed unusually high levels of CD4 and CD8 compared with the double-negative population that normally resides in the BM (Fig. 5, see region R2 on FACS dot plot). The same CD4/CD8 profile was observed among the Ly5.1 host cells that were not exposed to the Notch1IC-encoding retrovirus, thus confirming that non-cell-autonomous phenomena underlie this unusual T cell maturation (Fig. 5 and Table II). It is noteworthy that the T cells present in the BM of the Notch1IC mice did not have the phenotype of normal mature lymphocytes, making it unlikely that their presence is due to the redistribution of circulating lymphocytes to the BM as can be seen in stressed animals.

**Discussion**

This is the first study describing the ability of Notch activation to alter the fate of neighboring cells through non-cell-autonomous mechanisms in mammalian hemopoiesis. These findings were surprising because they had not been described by other investigators.
using a very similar experimental approach (19). One explanation for this discrepancy could result from the fact that the intensity of the cell-autonomous effect is dependent on the proportion of infused cells transduced with the Notch1IC virus. Alternatively, it is possible that qualitative differences between the Notch1IC transgenes used in the two studies could account for the differences in biological activities observed. The Notch1IC cDNA we worked with is identical in length to the one used by Pui et al. However, ours is a murine gene instead of a human sequence, and the percentage of identical amino acids between the two proteins encoded is 74%. Thus, it is possible that the murine cDNA is more potent.

Because the non-cell-autonomous effects are seen in transplanted mice that eventually succumb to fatal neoplasia, we cannot formally rule out that the malignant clones do not secrete or induce the secretion of substances in the terminally ill mice that underlie the effects we see in the nontransduced cells independently of Notch activation. However, several facts render this hypothesis unlikely. Firstly, the activity capable of suppressing B cell maturation in vitro was measured in the conditioned medium of BM harvested from Notch1IC mice before they displayed signs of disease. Similarly, the mice transplanted with an inoculum in which 13% of the cells were transduced by Notch1IC displayed a considerable suppression of B cells among the nontransduced population as early as 3 wk post-transplantation when they were healthy (see Fig. 2). Second, the Ly5.2 Notch1IC mice used for the coculture and the conditioned medium experiments were still healthy when terminated at 4 wk post-transplantation, yet we found a clear suppression of B cells among the Ly5.1 test cells (see Fig. 4). Third, when the mice that had been transplanted with a population of progenitors of which 1.5% were transduced did eventually develop tumors, nontransduced B cells were still present in their BM, although this fraction was greatly suppressed by the malignant DP T cell population (data not shown). We cannot attribute the suppression of the B cell compartment to a mass effect of the proliferating T cells in the BM either, because the total number of BM cells at 3 wk post-transplantation was actually lower than that in the MIE control mice (Fig. 1B). Furthermore, the BM T cells could not survive in the culture with IL-7, Flk ligand, and SCF, yet we found a clear suppression of B cells in the coculture and conditioned medium experiments (see Fig. 4). Our results demonstrate, rather, that the level of non-cell-autonomous B cell suppression at 3 wk post-transplantation correlates with the initial Notch1IC transduction efficiency at the time of transplantation and not with the clinical stage of the disease or the degree of proliferation of DP T cells in the BM. This suggests that the Notch1IC-expressing cells could influence the cell fate of the replicating progenitors in a dose-dependent manner by acting shortly after transplantation.

The non-cell-autonomous effects of Notch signaling described in this report are reminiscent of developmental phenomena described in the fly. Seckted morphogens such as Wingless have been shown to result from Notch activation and activate signaling of neighboring cells in a paracrine fashion (13). Similarly, a recent report by Koch and colleagues (22) showed that the overexpression of Lunatic Fringe, a modulator of Notch, in thymocytes could inhibit Notch1 activation in a non-cell-autonomous fashion, and thus increase B cells and decrease T cells in the thymus. During the development of fly wings and eye, Fringe also restricts Notch activation in a non-cell-autonomous fashion to cells at the dorsal-ventral boundary to ensure proper morphogenesis. Although the mechanisms by which Notch1 or Fringe exerts non-cell-autonomous effects in hemopoiesis need to be elucidated, these findings point to the conserved mechanisms of Notch activation between Drosophila morphogenesis and mammalian hemopoiesis.

Our findings raise the question of the identity of the factor(s) secreted by Notch1IC-transduced cells that mediates the multiple hemopoietic alterations described in this report. In attempt to identify such a factor(s) we performed ELISA to look for the presence of certain cytokines in the conditioned medium of the BM of Notch1IC mice. The positive effect seen on the expansion of myeloid progenitors (Fig. 4C) prompted us to measure the concentration of GM-CSF in this supernatant. This cytokine was detected in the BM supernatant of three of the 11 Notch1IC mice tested by ELISA and in none of the 11 MIE controls studied (data not shown). Although additional testing would perhaps help clarify the issue, this result suggests that GM-CSF is unlikely to be a key mediator of the non-cell-autonomous effect of Notch. The similar B cell impairment seen in the Notch1IC-transduced and nontransduced fractions suggests that Notch activation might interfere with Notch modulators such as Lunatic Fringe that act non-cell-autonomously to alter the T/B lineage decision (22).

Notch1IC overexpression has been shown to induce thymic-independent accumulation of DP T cells in the BM at the expense of B cell precursors, suggesting that Notch1 signaling could instruct T cell development in a common lymphoid precursor (19). We show here that among the nontransduced BM compartment of the Notch1IC-chimeric mice, both a defect in B cell maturation and abnormal DN T cells are observed. This indicates that non-cell-autonomous mechanisms might also play a role in the ability of Notch1 to direct T lineage commitment from multipotent progenitor cells. Secreted factors from Notch1IC-transduced cells could contribute to the ectopic development of T cells by modifying the BM microenvironment and/or the developmental potentials of

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**Table II.** T cell subpopulations among host (Ly5.1) BM cells of Notch1IC transplanted mice

<table>
<thead>
<tr>
<th>Mouse (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BM Cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% of eGFP&lt;sup&gt;c&lt;/sup&gt; Cells</th>
<th>% of Ly5.1&lt;sup&gt;c&lt;/sup&gt; Cells</th>
<th>R1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R3&lt;sup&gt;c&lt;/sup&gt;</th>
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<th>R5&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Notch1IC no. 1 (25)</td>
<td>13.1 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>46</td>
<td>25</td>
<td>49</td>
<td>35</td>
<td>5</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Notch1IC no. 5 (25)</td>
<td>10.8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>33</td>
<td>13</td>
<td>51</td>
<td>39</td>
<td>2</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Notch1IC no. 6 (25)</td>
<td>12.3 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>40</td>
<td>17</td>
<td>60</td>
<td>27</td>
<td>2</td>
<td>1</td>
<td>10</td>
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<td>Notch1IC no. 12 (13)</td>
<td>14.9 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>43</td>
<td>38</td>
<td>67</td>
<td>20</td>
<td>6</td>
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<tr>
<td>Notch1IC no. 19 (13)</td>
<td>16.5 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>36</td>
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<td>3</td>
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</tr>
<tr>
<td>Notch1IC no. 21 (1.5)</td>
<td>17.3 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>30</td>
<td>15</td>
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<td>8</td>
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<td>Notch1IC no. 29 (1.5)</td>
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<td>24</td>
<td>29</td>
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<td>MIE no. 7 (65)</td>
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<td>1</td>
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<sup>a</sup> The initial transduction efficiency of the cells inoculated to the mice is indicated in parentheses.

<sup>b</sup> Total number of BM cells harvested from four hind leg bones 3 wk post-transplantation.

<sup>c</sup> Percentage of Ly5.1 cells in the different regions. The gates defining R1, R2, R3, R4, and R5 are shown in Fig. 5.
precursors. These non-cell-autonomous modifications could cooperate with the cell-autonomous consequences of Notch1 overexpression, such as the antiapoptotic effect of Notch1 on the transduced-T cell population (29, 30), to generate T cell leukemia/lymphomas in Notch1IC-transplanted mice.

The alteration of hematopoiesis in the nontransduced cells obtained in our experimental model as a result of Notch1 activation is certainly supraphysiological. It is more likely that local concentrations of Notch1-activated cells in the BM microenvironment could affect the fate of surrounding cells in a paracrine fashion. It is also possible that secreted factors act in an autocrine manner and further modify the hematopoietic potentials of Notch1-activated cells. Altogether, our in vivo and in vitro data suggest that tight regulation of notch signaling is indispensable to ensure proper distribution and development of hematopoietic cells and that this probably involves both intracellular and non-cell-autonomous mechanisms.

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References