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# Notch-1 Regulates NF- $\kappa$ B Activity in Hemopoietic Progenitor Cells<sup>1</sup>

Pingyan Cheng,\* Andrew Zlobin,<sup>†</sup> Veronica Volgina,<sup>‡</sup> Sridevi Gottipati,<sup>¶</sup> Barbara Osborne,<sup>¶</sup> Erica J. Simel,<sup>†‡</sup> Lucio Miele,<sup>†§</sup> and Dmitry I. Gabrilovich<sup>2\*</sup>

We investigated the interaction between two elements critical for differentiation of hemopoietic cells, the Notch-1 receptor and the transcription factor NF- $\kappa$ B. These factors were studied in hemopoietic progenitor cells (HPC) using Notch-1 antisense transgenic (Notch-AS-Tg) mice. DNA binding of NF- $\kappa$ B as well as its ability to activate transcription was strongly decreased in HPC from Notch-AS-Tg mice. NF- $\kappa$ B-driven transcriptional activity was completely restored after transduction of the cells with retroviral constructs containing activated Notch-1 gene. HPC from Notch-AS-Tg mice have decreased levels of several members of the NF- $\kappa$ B family, p65, p50, RelB, and c-Rel and this is due to down-regulation of the gene expression. To investigate functional consequences of decreased NF- $\kappa$ B activity in transgenic mice, we studied LPS-induced proliferation of B cells and GM-CSF-dependent differentiation of dendritic cells from HPC. These two processes are known to be closely dependent on NF- $\kappa$ B. B cells from Notch-AS-Tg mice had almost 3-fold lower response to LPS than B cells isolated from control mice. Differentiation of dendritic cells was significantly affected in Notch-AS-Tg mice. However, it was restored by transduction of activated Notch-1 into HPC. Taken together, these data indicate that in HPC NF- $\kappa$ B activity is regulated by Notch-1 via transcriptional control of NF- $\kappa$ B. *The Journal of Immunology*, 2001, 167: 4458–4467.

Differentiation of hemopoietic progenitor cells (HPC)<sup>3</sup> is a complex process regulated by a network of different transcription factors and regulatory molecules. Elucidation of how various members of this network interact with each other is important for our understanding of the mechanisms of cell differentiation. Here, we investigate the interaction between Notch-1 and NF- $\kappa$ B in HPC. The Notch family is a group of unique molecules that function as both cell surface receptors and direct regulators of gene transcription. Notch-1 is a 300-kDa non-covalent heterodimer consisting of a 180-kDa extracellular domain and a 115-kDa transmembrane domain. In some experimental systems, Notch signaling limits the number of cells that undergo differentiation, whereas some progenitors remain uncommitted (1, 2). Notch-1 is activated after binding of appropriate ligands on adjacent cells to the extracellular domain of Notch-1 on the surface of HPC. This results in proteolytic cleavage, release, and nuclear translocation of the Notch intracellular domain. This domain interacts with a number of cytoplasmic and nuclear proteins, permitting signal transduction through several pathways that include activation of CBF-1/Rbp-J $\kappa$  transcription factor and *E(spl)/HES* genes, which work as negative regulators of lineage-specific gene

expression. Another Notch pathway, mediated by different effector molecules such as Deltex, may regulate another set of target genes. Evidence accumulated in recent years suggests an important role for *Notch-1* in lymphocyte differentiation and T cell development (2–5). The signaling pathways involving NF- $\kappa$ B play an especially important role in differentiation and function of hemopoietic cells, and recent evidence of interaction between Notch and NF- $\kappa$ B suggests that Notch may influence lymphoid development (6–9).

NF- $\kappa$ B binds to specific DNA sequences and is composed of subunits from the family of Rel proteins, which share a 300-aa Rel homology domain. The members of this family are the proto-oncogenes *c-Rel*, *p50*, *p52*, *p65* (*RelA*), and *RelB*. These different family members can associate in various homo- or heterodimers through a highly conserved N-terminal Rel homology domain. In the cytoplasm of quiescent cells, they are associated with inhibitory molecules of the I $\kappa$ B family. Cell activation by various stimuli including TNF- $\alpha$ , LPS, IL-1, and CD40 results in serine phosphorylation and degradation of I $\kappa$ B with subsequent nuclear translocation and specific DNA binding of NF- $\kappa$ B dimers (7). It has also been suggested that tyrosine phosphorylation of I $\kappa$ B $\alpha$  can activate NF- $\kappa$ B without degradation (10). NF- $\kappa$ B is required for differentiation of dendritic cells (DCs) and B cells, and its role in T cell development has been demonstrated as well (8, 11–15).

There is conflicting evidence of a possible interaction between Notch and NF- $\kappa$ B. Like I $\kappa$ B, Notch proteins have a conserved cdc10 repeat domain. Interaction of Notch with NF- $\kappa$ B may mimic I $\kappa$ B function and block NF- $\kappa$ B activation (9). Recent evidence indicates that this inhibitory interaction takes place in the nucleus and requires a region of Notch-1 that is N-terminal to the ankyrin repeats. This region partially overlaps with the putative CBF-1/RBP-J $\kappa$  binding site (16). In two other reports, up-regulation of NF- $\kappa$ B by Notch was described. Notch-1 *trans*-activates the promoter of p52, one of the members of the NF- $\kappa$ B family (17), and constitutive activation of NF- $\kappa$ B by Notch-3 was observed in Notch-3-transgenic mice (18). This activation was reportedly due to increased phosphorylation and degradation of I $\kappa$ B $\alpha$ .

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<sup>3</sup> Abbreviations used in this paper: HPC, hemopoietic progenitor cells; DCs, dendritic cells; Notch-AS-Tg, Notch-1-deficient antisense-transgenic.

We have previously demonstrated abnormal differentiation of myeloid cells in cancer (19, 20). These functional effects were mediated, in major part, by NF- $\kappa$ B (21). To investigate molecular mechanisms of tumor-associated abnormalities in myeloid cells further, we used Notch-1-deficient antisense-transgenic (Notch-AS-Tg) mice. These mice contain a Notch-1 antisense transgene and have reduced level of Notch-1. These mice also demonstrated significantly reduced NF- $\kappa$ B activity indicating that Notch-1 signaling may regulate NF- $\kappa$ B activity *in vivo*. We have investigated this fact further and report here the novel finding that in HPC, NF- $\kappa$ B activity is highly dependent on the presence of Notch-1. Furthermore, we show that Notch-1 regulates NF- $\kappa$ B activity via transcriptional regulation of several NF- $\kappa$ B subunits.

## Materials and Methods

### Mice

Notch-AS-Tg mice were generated using a Notch-1 antisense construct expressed under the control of the mouse mammary tumor virus long terminal repeat promoter. This construct has been described in detail earlier (22) and has been shown to down-regulate Notch-1 expression and function in murine thymocytes (23). The genetic background of the founders was (C57BL/6  $\times$  SJL) $F_1$ , and the mice were then backcrossed for four generations with C57BL/6 mice. Hemizygous transgenic mice were then bred to each other, selecting homozygous transgenic and negative mice until two syngeneic strains derived from the same original litter were obtained: one homozygous Notch-AS-Tg and one nontransgenic (control). Transgene integration and expression were confirmed by PCR and RT-PCR, respectively. Notch-1 protein levels were determined by Western blotting and flow cytometry using anti-Notch-1 Ab (Fig. 1). The specificity of Notch-1 down-regulation was tested by Western blotting for Notch-2, -3, and -4 (Fig. 1). Control and transgenic mice were housed in pathogen-free units of the Comparative Medicine Facility at Loyola University (Chicago, IL). All mice used were between 10 and 16 wk. BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN).

### Abs and reagents

The following Ab-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-TER-119, anti-CD45 receptor antagonist (B220), anti-Gr-1 (anti-Ly-6G), anti-IA<sup>b</sup>-FITC, anti-CD86 (B7-2)-PE, anti-CD11c-APC, biotinylated goat anti-rabbit Ab, streptavidin-FITC, and isotype control Abs were obtained from PharMingen (San Diego, CA). Anti-p65, p52, p50, c-Rel, Rel-B, I $\kappa$ B $\alpha$ , and Notch-1, -2, -3, and -4 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pI $\kappa$ B $\alpha$  Ab was obtained from Cell Signaling Technology (Beverly, MA). Low-Tox rabbit complement and Lympholyte M were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Recombinant murine GM-CSF, IL-4, and TNF- $\alpha$  were obtained from Research Diagnostics (Flanders, NJ). LPS from Sigma (St. Louis, MO), and poly(dI-dC) from Pharmacia (Piscataway, NJ). Anti-GADPH Ab was obtained from Chemicon International (Temecula, CA) and was used at a dilution of 1/10000. Cells were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics (Life Technologies, Gaithersburg, MD).

### Preparation of HPC

Bone marrow cells were harvested from the femurs and tibias of mice and enriched for HPCs by depletion of lineage-specific cells. Briefly, bone marrow cells were incubated with mixture of Abs (TIB-207, TIB-210, TIB-120, anti-TER-119, anti-Gr-1, anti-B220, and anti-mouse Ig) for 30 min on ice, washed, and treated with complement for 1 h at 37°C. Dead cells were then removed by centrifugation over a Lympholyte M gradient. The resulting fraction contained <20% of lineage-positive cells as was detected by flow cytometry.

### Electrophoretic mobility shift assay

EMSA was performed as previously described (21). Briefly, double-stranded oligonucleotides containing the specific binding site for NF- $\kappa$ B were made by annealing the appropriate single-stranded oligonucleotides at 65°C for 10 min. The probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (6000  $\mu$ Ci/mmol; Amersham Life Sciences, Arlington Heights, IL) using Klenow DNA polymerase. Two probes were used: wild type (5'-AGTTGAGGGGACTTTCCAGG-3'); and mutant (5'-AGTTGAGGCGACTTTCCAGG-3').

HPCs were cultured overnight with 20 ng/ml GM-CSF, washed, and incubated for 2 h in serum-free medium. Cells were then treated for 15 min with either PBS or 10 ng/ml TNF- $\alpha$ . Our preliminary experiments demonstrated that this time of exposure and TNF- $\alpha$  concentration provided a maximum response. HPCs were collected, and nuclear extracts were prepared as previously described (21). Ten micrograms of nuclear extract were incubated with labeled probe in binding buffer containing 20 mM HEPES, 5% glycerol, 0.2 mM EDTA, 1 mM DTT, 5 mM MgCl<sub>2</sub>, and 4  $\mu$ g poly(dI-dC) to prevent nonspecific DNA binding. Specific competition assays were performed with a 200-fold excess of unlabeled probes. For supershift experiments, 2–4  $\mu$ g of the corresponding Ab were added to nuclear extracts and left on ice for 30 min before incubation with the probe. The samples were separated on 4% polyacrylamide gels, and bands were visualized by overnight exposure to x-ray films (Fuji, Stamford, CT) at –70°C.

### Expression of NF- $\kappa$ B-specific mRNA

Total RNA was extracted using the GlassMAX RNA microisolation spin cartridge system (Life Technologies). Traces of DNA were removed by treatment with DNase I. The cDNA was synthesized from 1  $\mu$ g total RNA by using random hexamers as primer and Superscript II reverse transcriptase (Life Technologies) according to manufacturer's protocol. Samples were subjected to initial denaturation at 94°C for 3 min and 24 cycles (for *p65* and *c-rel*) or 28 cycles (for *hprt*) of PCR (94°C for 30 s, 55°C for 30 s, 72°C for 45 s) with final extension for 7 min at 72°C. The number of cycles was selected after preliminary experiments to avoid saturation of the PCR products.

PCR primer pairs used in this study: *p65*: forward, 5'-GCTCAGCGGG CAGTATTCCT; reverse, 5'-CGGCAGATCTGAGCTCGGCAGTG. *c-Rel*: forward, 5'-CAGGGGAGCGCAGCACAGACA; reverse, 5'-AGTATTT GGGGCACGGTTATCA; *hprt*: forward, 5'-GATTCAACTTGCGCT CATCTTAGGC; reverse, 5'-GTTGGATACAGGCCAGACTTTGTTG.

The PCR products were visualized on 1% agarose gel. The sizes of PCR products were 445 bp for *c-rel*, 472 for *p65* and 164 for *hprt*. PCR products were transferred in an alkaline transfer buffer (0.4 N NaOH, 1 M NaCl) onto Hybond N<sup>+</sup> nylon transfer membranes (Amersham, Highland Park, IL), and probed with <sup>32</sup>P-labeled oligonucleotide probes: *c-Rel*, 5'-GAAGACT GCGACCTCAATG-3'; *p65*, 5'-TTAGCCAGCGCAGCACAGACA-3'; *HPRT*, 5'-GTTGTTGGATATGCCTTGAC-3'.

Gene expression was quantitated by using UN-Scan-IT software (Silk Scientific, Orem, UT). Expression of *c-rel* and *p65* in each sample was normalized for *hprt* and was expressed as an arbitrary unit ((*c-rel* *p65*)/*hprt*)  $\times$  100.

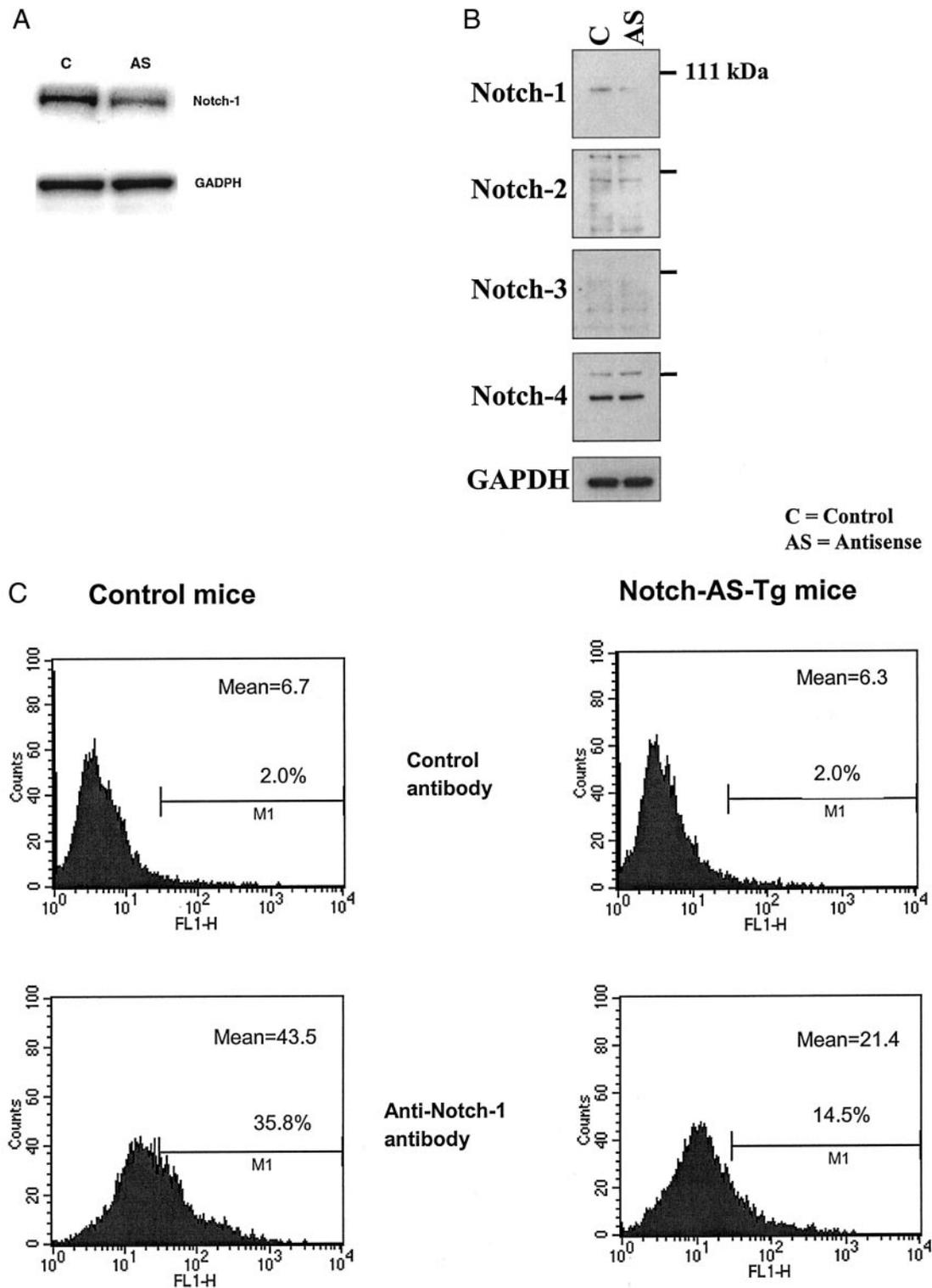
### Western blot assay

HPCs were lysed for 30 min on ice in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1% Triton X-100, 0.5% sodium deoxycholate, 100 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin). Cell debris was removed by centrifugation (14,000  $\times$  g, 15 min at 4°C). Samples (50  $\mu$ g total protein per lane) were subjected to electrophoresis on 8–16% gradient SDS-polyacrylamide gel followed by transfer to a polyvinylidene difluoride membrane. Membranes were blocked overnight with Western blocking reagent (Boehringer Mannheim, Indianapolis, IN) and then incubated with appropriate primary Ab for 2 h at room temperature, followed by incubation with anti-goat IgG HRP-conjugated Ab for 1 h at room temperature. The bands were visualized by ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL). To confirm equal loading of protein, the membranes were stripped with 2% SDS buffer and reprobed with Ab against  $\beta$ -actin.

Levels of proteins were quantitated by using UN-Scan-IT software (Silk Scientific, Orem, UT). Expression in each sample was normalized for  $\beta$ -actin and was expressed as an arbitrary unit.

### NF- $\kappa$ B transcription activity in HPCs

Three constructs were used to measure NF- $\kappa$ B transcriptional activity: plasmid 6 $\times$ IFN- $\gamma$ tLuc containing luciferase reporter gene under NF- $\kappa$ B-dependent promoter from IFN- $\gamma$  (21); pGL<sub>3</sub>-basic plasmid containing only luciferase gene was used to measure the background luciferase activity; pRL-TK plasmid containing *Renilla* luciferase gene was used for control of transfection efficiency. NF- $\kappa$ B transcription activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Briefly, HPCs were incubated with 20 ng/ml GM-CSF overnight, collected, and washed twice with serum-free RPMI 1640. HPCs (5  $\times$  10<sup>6</sup> in 1 ml serum-free RPMI 1640) were cotransfected with 20  $\mu$ g 6 $\times$ IFN- $\gamma$ tLuc or pGL<sub>3</sub> and 10  $\mu$ g pRL-TK by electroporation at 475 V and 330  $\mu$ F. After transfection, cells were incubated for 10 min at room temperature and then cultured for 5 h in complete culture medium supplemented with 20 ng/ml



**FIGURE 1.** Decreased expression of Notch-1 in Notch-AS-Tg mice. *A*, Whole cell lysates were prepared from splenocytes, and the presence of Notch proteins was determined by Western blotting as described in *Materials and Methods*. *B*, Thymic organ extracts from control and Notch-1 antisense mice (25  $\mu$ g protein per lane) were analyzed by Western blotting. Samples were run in five pairs (one control and one antisense each) on a single 7% polyacrylamide gel, and proteins were transferred to a polyvinylidene difluoride-PAGE membrane (Immobilon-P). The membrane was cut into strips containing a control lane and an antisense lane each, and strips were stained with Abs to the intracellular domain of Notch-1, -3 and -4, the extracellular domain of Notch-2 (Santa Cruz Biotechnology), and GAPDH (Chemicon). Intracellular Notch domains appeared as 1–3 bands in the 111- to 90-kDa range, whereas the extracellular domain of Notch-2 appeared as a ~180-kDa band accompanied by a number of lower bands that may represent degradation products. *C*, Bone marrow cells enriched for HPC were labeled for 30 min on ice with anti-Notch-1 polyclonal rabbit Ab that recognizes the extracellular portion of Notch-1. Cells were washed and stained with biotinylated goat anti-rabbit Ab followed by streptavidin-FITC. Notch-1 expression was analyzed on a FACSCalibur flow cytometer. Background staining was established after labeling of cells with nonspecific rabbit Ab (antiphosphorylated ERK; Cell Signaling Technologies), followed by biotinylated anti-rabbit Ab and streptavidin-FITC. Mean = mean of fluorescence (FL1-H); % = percent of Notch-1<sup>bright</sup>-positive cells. Three experiments with similar results were performed. M1 regions were set to exclude negative cells.

GM-CSF, followed by 20 h incubation with 5 ng/ml TNF- $\alpha$ . After that time, cells were collected, and luciferase activity was measured in duplicate in each experiment on a Lumat LB 9501 luminometer (Berthold, Germany). Values of relative light U were normalized to *Renilla* luciferase activity according to the manufacturer's protocol (Promega). The luciferase activity of samples containing the NF- $\kappa$ B-responsive element was compared with the luciferase activity in samples transfected with the control plasmid and reported as the fold increase.

#### B cell preparation and LPS-induced proliferation

Single-cell suspensions were obtained from spleens using a cell strainer with 70- $\mu$ m pores. RBC were removed by osmotic lysis with ACK buffer, and B cells were purified by positive selection using anti-B220 Ab and magnetic cell sorting. Briefly, cells were labeled with biotinylated anti-CD45R (B220) Ab on ice for 30 min, washed, and incubated with streptavidin microbeads followed by separation on a MiniMACS column (Miltenyi Biotec, Auburn, CA). B cells were cultured in triplicates in U-bottom 96-well plates ( $10^5$  cells/well) with 5, 10, or 20  $\mu$ g/ml LPS for 48 h. [ $^3$ H]Thymidine, 1  $\mu$ Ci, was added to each well 18 h before cell harvest. Thymidine incorporation was measured on a liquid scintillation counter (Packard Instrument, Meriden, CT).

#### Generation of DC from bone marrow progenitors and analysis of their function

DC were generated from HPC using a combination of GM-CSF and IL-4 as described earlier (24). Briefly,  $2 \times 10^5$  HPC were cultured for 5 days in 2 ml complete medium supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4 in 24-well plates. One-half of the medium was replaced on day 3 with fresh medium supplemented with growth factors. On day 5, TNF- $\alpha$  (5 ng/ml) was added, and cells were cultured for additional 48 h. After that time, nonadherent and loosely adherent cells were collected and analyzed.

The phenotype of DCs was analyzed on FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) using a combination of Abs (anti-CD11c, anti-MHC class II (IA<sup>b</sup>), anti-CD86 (B7-2), and anti-CD11b). Isotype control Abs were used for gate setup.

Allogeneic MLR was used to measure DC function. Briefly, different numbers of DCs generated as described above were cultured for 4 days in triplicates in U-bottom 96-well plates with lymph node cells ( $10^5$ /well) obtained from allogeneic BALB/c mice. T cell proliferation was measured by [ $^3$ H]thymidine uptake as described above.

#### Retroviral transduction

The retroviral constructs encoding the intracellular part of the *Notch-1* gene (MSCV-ICT-GFP) or control vector (MSCV-IRES-GFP) were kindly provided by J. C. Aster (Brigham and Women's Hospital, MA) (25). These constructs were transiently cotransfected with pCL-Eco (26) into the packaging cell line 293T cells using Fugene (Roche Diagnostics, Somerville, NJ) following the manufacturer's instructions. The retroviral supernatants were harvested 48 h post-transfection and used for infection of HPC-enriched bone marrow cells. HPCs were infected with retroviruses for 4 h at 32°C in the presence of 4  $\mu$ g/ml Polybrene (Sigma). After that time, viral supernatants were removed and complete culture medium supplemented with GM-CSF was added. Cells were cultured for 20 h at 37°C, and then infection was repeated. Cells were used 18 h after second infection for transient transfection with NF- $\kappa$ B construct. In experiments with DC generation, cells were infected four times with 24-h intervals and analyzed 48 h after the last infection.

#### Statistical methods

Statistical analysis was performed using parametric methods and JMP statistical software (SAS Institute, Cary, NC).

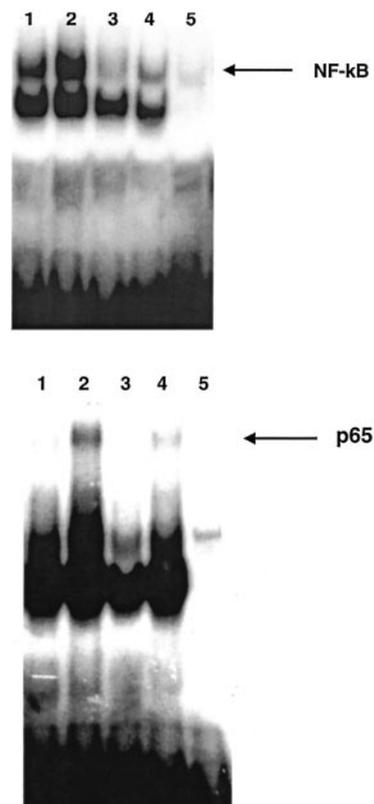
## Results

To confirm decreased Notch-1 production in Notch-AS-Tg mice, Notch-1 protein was evaluated in whole cell lysates from different organs by Western blot. In all experiments performed, a 1.5- to 2-fold-reduced amount of Notch-1 protein was detected in thymuses, spleens, and livers from Notch-AS-Tg mice. Fig. 1A illustrates the results of one representative experiment. Western blots for other Notch family members showed that the down-regulation of Notch-1 is specific (Fig. 1A). A detailed description of Notch-1 expression in different organs of these mice is provided elsewhere (B. A. Osborne, A. Zlobin, T. Palaga T., K. Fortner, L. Shelly, E.

Lizzio, E. Simel, and L. Miele, manuscript in preparation). To investigate whether the expression of Notch-1 on the surface of HPC was also affected in Notch-AS-Tg mice, HPC-enriched bone marrow cells were labeled with polyclonal rabbit anti-Notch-1 Ab able to recognize extracellular portion of Notch-1 (22) followed by staining with biotinylated anti-rabbit Ab and streptavidin-FITC. HPC from Notch-AS-Tg mice had a substantially lower level of Notch-1 expression than the cells from control mice (Fig. 1B). Because each experiment in this study would require a significant number of cells, use of highly purified stem cells would not be possible. Instead, we used a population of bone marrow cells enriched for HPC. To monitor the level of Lin<sup>+</sup> cell contamination, enriched HPC obtained from control and Notch-AS-Tg mice were labeled with FITC of PE-conjugated anti-CD3, CD19, CD11b, Gr-1, and TER-119 Abs and analyzed by flow cytometry. In both cell populations, the levels of CD3<sup>+</sup> and TER-119<sup>+</sup> cells were <1%, CD19<sup>+</sup> cells 1–2%, Gr-1<sup>+</sup> cells 2–4%, and CD11b<sup>+</sup> cells 8–10%. The level of Lin<sup>+</sup> cell contamination was monitored in each experiment; it was the same in control and Notch-AS-Tg mice (data not shown).

#### NF- $\kappa$ B activity in Notch-AS-Tg mice

The ability of NF- $\kappa$ B to bind DNA was examined using EMSA. The location of the specific bands was determined using a  $^{32}$ P-



**FIGURE 2.** Decreased DNA binding of NF- $\kappa$ B from Notch-AS-Tg mice. HPC-enriched bone marrow cells were obtained as described in *Materials and Methods*. HPCs isolated from control (lanes 1 and 2) or Notch-AS-Tg (3, 4) mice were incubated with 20 ng/ml GM-CSF overnight, incubated in serum-free medium for 2 h, and then either left in medium (lanes 1 and 3) or stimulated with 10 ng/ml TNF- $\alpha$  for 15 min (lanes 2 and 4). NF- $\kappa$ B binding was determined by EMSA as described in *Materials and Methods*. Lane 5, Inhibition of the specific binding with 200-fold excess of unlabeled probe. *Top*, Typical result of one EMSA. Three experiments with the same results were performed. *Bottom*, Supershift was performed using anti-p65 Ab as described in *Material and Methods*. Representative result from three independent experiments.

labeled mutant probe and confirmed in experiments with a 200-fold excess of unlabeled wild-type probe (data not shown). The basal level of NF- $\kappa$ B binding was substantially lower in Notch-1-AS-Tg mice than in control animals (Fig. 2, top, lanes 1 and 3). Treatment of HPCs from control mice with TNF- $\alpha$ , a potent NF- $\kappa$ B activator, resulted in increased NF- $\kappa$ B binding (Fig. 2, top, lane 2). In contrast, very weak up-regulation was detected in HPC from Notch-AS-Tg mice (Fig. 2, top, lane 4), and the level of binding was dramatically less than that in control mice (Fig. 2, top, lanes 2 and 4). These data were confirmed in supershift experiments. DNA binding of complexes containing one of the major members of NF- $\kappa$ B family, p65 (RelA), was substantially reduced in Notch-AS-Tg mice (Fig. 2, bottom). This reduction was observed both on a basal level and after stimulation with TNF- $\alpha$ .

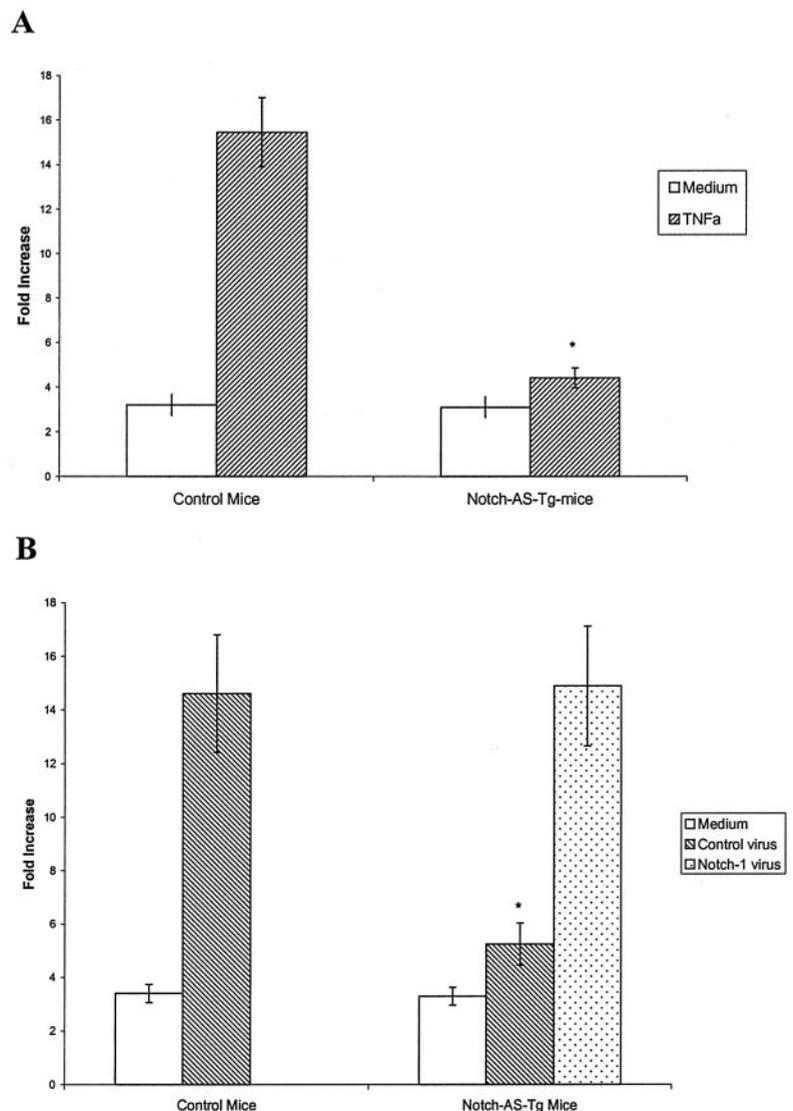
To investigate transcriptional activity of NF- $\kappa$ B in Notch-AS-Tg mice, we used a luciferase reporter gene construct under a NF- $\kappa$ B-dependent promoter (21). HPCs were transfected with the NF- $\kappa$ B reporter or a control construct. Incubation of HPC from control mice with GM-CSF and TNF- $\alpha$  resulted in significant up-regulation of NF- $\kappa$ B activity (15-fold increase over basal level) (Fig. 3A). NF- $\kappa$ B activity in Notch-AS-Tg HPCs treated with TNF- $\alpha$  was almost 4 times lower than that in control HPCs receiving the same treatment (Fig. 3A). We asked whether this activity could be restored if HPC were transduced with Notch-1

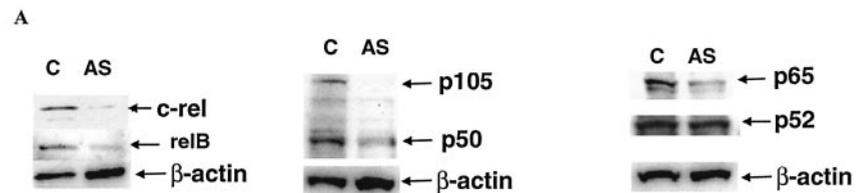
cDNA. HPCs from control and Notch-AS-Tg mice were infected either with control retrovirus or with retrovirus encoding a constitutively active form of Notch-1. After 48 h, later cells were transfected with reporter gene construct, and NF- $\kappa$ B transcriptional activity was measured using the Dual-Luciferase Reporter Assay System. As shown in Fig. 3B, expression of Notch-1 completely restored NF- $\kappa$ B activity in HPC from Notch-AS-Tg mice. Thus, these data indicate that presence of Notch-1 is critically important for NF- $\kappa$ B activity.

#### Synthesis of NF- $\kappa$ B subunits is regulated by Notch-1

Decreased NF- $\kappa$ B activity in Notch-AS-Tg mice could be a result of decreased synthesis of NF- $\kappa$ B subunits or their sequestration in cytoplasm preventing nuclear translocation of NF- $\kappa$ B. To clarify the mechanism of the decreased NF- $\kappa$ B activity in Notch-AS-Tg mice, we examined the level of NF- $\kappa$ B proteins in HPC-enriched bone marrow cells using Western blotting analysis. HPC from Notch-AS-Tg mice had a substantially lower level of c-Rel (2-fold), p50 (2.1-fold), RelB (1.5-fold), and p65 (2.2-fold) than those cells from control mice. In control mice, anti-p50 Ab also recognized p105 precursor that can generate the p50 subunit of NF- $\kappa$ B by proteolytic processing. This band was not seen in cells from Notch-AS-Tg mice (Fig. 4A). Activation of NF- $\kappa$ B is normally controlled by the family of inhibitory molecules I $\kappa$ B. I $\kappa$ B $\alpha$  is the

**FIGURE 3.** Decreased NF- $\kappa$ B transcriptional activity in Notch-AS-Tg mice. **A**, HPCs were isolated from bone marrow of control and Notch-AS-Tg mice and incubated overnight with GM-CSF. After that, cells were washed and transfected with either control (pGL<sub>2</sub>) or NF- $\kappa$ B-specific luciferase reporter gene plasmids (6 $\times$ IFN- $\gamma$ tLuc) together with pRL-TK (*Renilla* luciferase) plasmid as described in *Materials and Methods*. Cells were then cultured for 36 h either with GM-CSF alone (Medium) or with GM-CSF and TNF- $\alpha$  (TNF- $\alpha$ ). After that time, cell lysates were prepared, and luciferase activity was measured using dual-luciferase reporter assay system as described in *Materials and Methods*. Values of relative light U were normalized to *Renilla* luciferase activity. The luciferase activity of samples containing NF- $\kappa$ B-responsive element was compared with luciferase activity in samples transfected with the control plasmid and are reported as the fold increase. Each experiment was performed in duplicates. Two experiments showed identical results. \*, Statistically significant differences from control ( $p < 0.05$ ). **B**, HPC were isolated from control and Notch-AS-Tg mice and after overnight incubation with GM-CSF were infected with either control retrovirus (control) or retrovirus containing an active form of Notch-1 (Notch-1 virus). Cells were incubated with GM-CSF, and infection was repeated 24 h later. Eight hours after second infection, cells were transfected with NF- $\kappa$ B reporter gene construct and incubated with GM-CSF and TNF- $\alpha$  as described above. Luciferase activity was measured 36 h later. Medium = cells incubated with GM-CSF alone without viral infection. Result of one experiment is shown. Two experiments with similar results were performed.

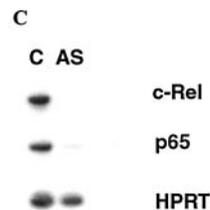
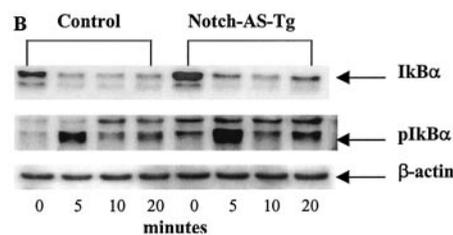




**FIGURE 4.** Decreased expression of NF- $\kappa$ B in Notch-AS-Tg mice. *A*, Total cellular lysates were prepared from HPCs isolated from control (C) and Notch-AS-Tg (AS) mice. Protein levels were detected by Western blotting assay as described in *Materials and Methods*. At least three experiments with similar results for each protein have been performed. *B*, HPCs were isolated from control and Notch-AS-Tg mice and stimulated with 15 ng/ml TNF- $\alpha$  for the indicated period of time. Whole cell lysates were prepared, and the levels of I $\kappa$ B $\alpha$ , phosphorylated I $\kappa$ B $\alpha$  (pI $\kappa$ B $\alpha$ ), and  $\beta$ -actin were detected by Western blotting using specific Abs as described in *Materials and Methods*. *C*, Total RNA was isolated from HPCs. RT-PCR and Southern blot were performed as described in *Materials and Methods*.

Intensity of bands normalized for  $\beta$ -actin.

	Control	Notch-AS-Tg
c-Rel	33.7	12.7
Rel B	33.3	17.5
p 105	36.1	9.0
p 50	69.8	33.0
p 52	111.9	100.6
p 65	68.1	31.0



best known member of this family. TNF- $\alpha$  induces phosphorylation and degradation of I $\kappa$ B $\alpha$ , which results in nuclear translocation of NF- $\kappa$ B. We asked whether the observed decrease in NF- $\kappa$ B activity in Notch-AS-Tg mice could be due to the defects in I $\kappa$ B $\alpha$ . HPC isolated from control and Notch-AS-Tg mice were stimulated with TNF- $\alpha$  and I $\kappa$ B $\alpha$  degradation and phosphorylation was analyzed using Western blotting. The background levels of I $\kappa$ B $\alpha$  were equal in both groups of mice. TNF- $\alpha$  induced I $\kappa$ B $\alpha$  phosphorylation and degradation in HPC isolated from control and Notch-AS-Tg mice equally well (Fig. 4*B*). Thus, the decrease in NF- $\kappa$ B activation observed in Notch-AS-Tg mice was not mediated by the defects in I $\kappa$ B $\alpha$  degradation.

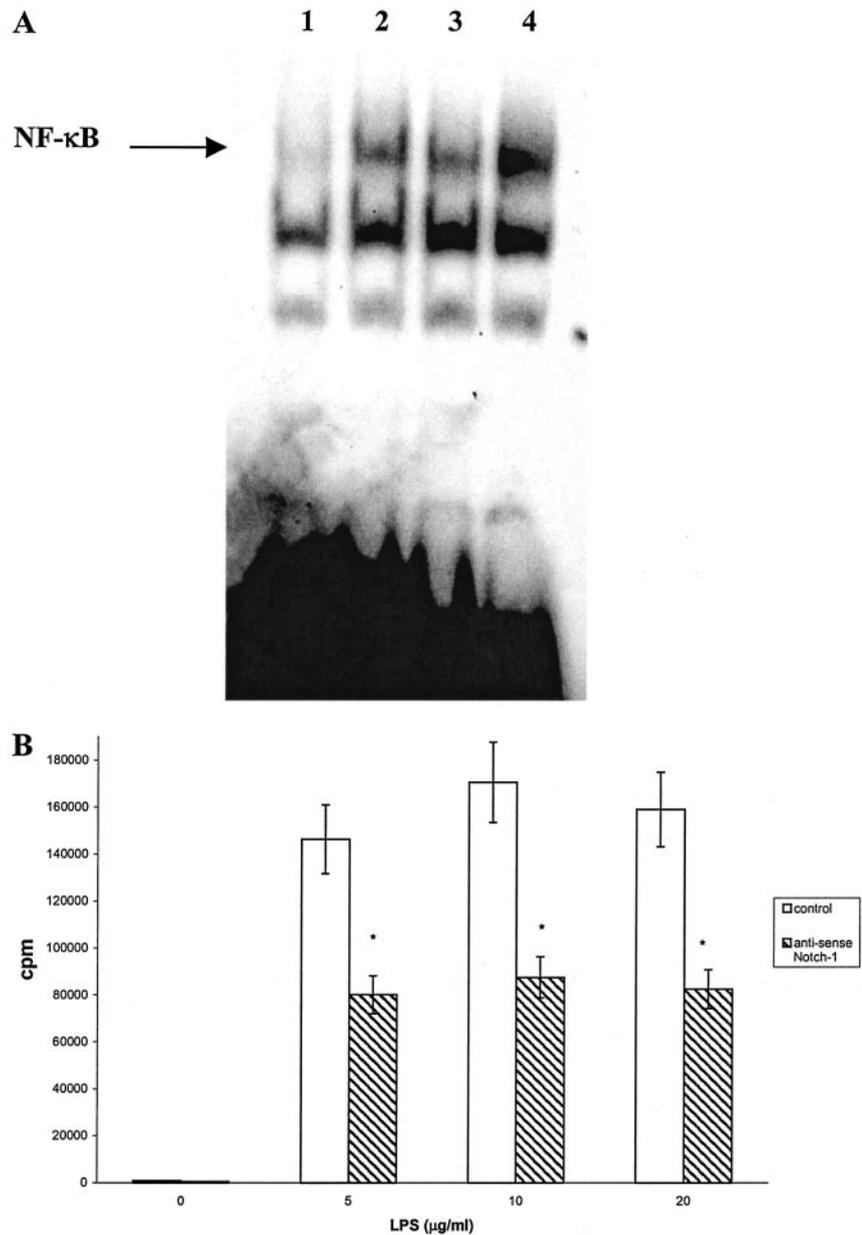
To determine whether decreased levels of NF- $\kappa$ B proteins were due to a transcriptional down-regulation, we evaluated mRNA specific for *p65* and *c-Rel* subunits of NF- $\kappa$ B using RT-PCR and Southern blotting. As shown in Fig. 4*C*, the expression of both *p65* and *c-Rel* was significantly down-regulated in Notch-AS-Tg mice. Quantitation of the results showed a >10-fold lower level of expression of these genes in Notch-AS-Tg mice than in control mice. These data indicate that the decrease in Notch-1 leads to a decreased transcription and synthesis of several key members of NF- $\kappa$ B family. This, in turn, may result in a reduced NF- $\kappa$ B activity.

#### Functional consequences of reduced NF- $\kappa$ B activity in Notch-AS-Tg mice

We asked whether decreased NF- $\kappa$ B activity in Notch-AS-Tg mice would be manifest in a decreased NF- $\kappa$ B-mediated function of

cells differentiated from HPC. LPS-induced B cell proliferation is tightly controlled by NF- $\kappa$ B (27, 28). B cells were purified from spleens of control and Notch-AS-Tg mice. Substantially lower activation of NF- $\kappa$ B in response to LPS was seen in B cells isolated from Notch-AS-Tg mice than in B cells from control mice (Fig. 5*A*). To evaluate cell proliferation, B cells were stimulated with different concentrations of LPS for 48 h. As expected, in control mice LPS induced a significant increase in B cell proliferation. In contrast, proliferation of B cells from transgenic mice was substantially (~2-fold) lower than that from the control mice (Fig. 5*B*).

Differentiation of myeloid DCs from HPC in the presence of GM-CSF is another process that is closely dependent on NF- $\kappa$ B (13, 21, 29). HPC were isolated from control and Notch-1-deficient mice and were cultured with GM-CSF and IL-4 for 5 days followed by 48 h incubation with TNF- $\alpha$ . This combination of cytokines and growth factors provides generation of mature fully functional DCs. To evaluate the presence of DCs, cells were labeled with anti-MHC class II Ab (IA<sup>b</sup>) and anti-CD86 (B7-2) Ab. The proportion of mature DCs (IA<sup>b</sup>+CD86<sup>+</sup>) in Notch-AS-Tg mice was significantly lower than in control mice (Fig. 6*A*). Decreased DC production was further confirmed in allogeneic MLR, a function specifically attributed to DCs. Cells generated from control mice demonstrated a 2-fold higher level of allogeneic T cell stimulation than cells generated from Notch-AS-Tg mice (Fig. 6*B*). To verify that defective DC differentiation was due to Notch-1 deficiency, HPC were transduced with retroviral construct encoding activated *Notch-1* gene and the *GFP* gene as a tag for transfection.



**FIGURE 5.** Decreased B cell response to LPS in Notch-AS-Tg mice. *A*, B cells were isolated from spleens of Notch-AS-Tg (*lanes 1 and 2*) and control (*lanes 3 and 4*) mice using B220 Ab and magnetic beads. B cells were incubated for 2 h in serum-free medium and then stimulated with 20  $\mu$ g/ml LPS for 2 h at 37°C. After that time, nuclear extracts were isolated, and EMSA was performed as described in *Materials and Methods*. *Lanes 1 and 3*, untreated cells; *lanes 2 and 4*, cells stimulated with LPS. *B*, B cells ( $1 \times 10^5$ /well) were plated into a 96-well plate and incubated with LPS for 48 h as described in *Materials and Methods*. One of two results is expressed as mean  $\pm$  SE. \*, Statistically significant differences from control ( $p < 0.05$ ).

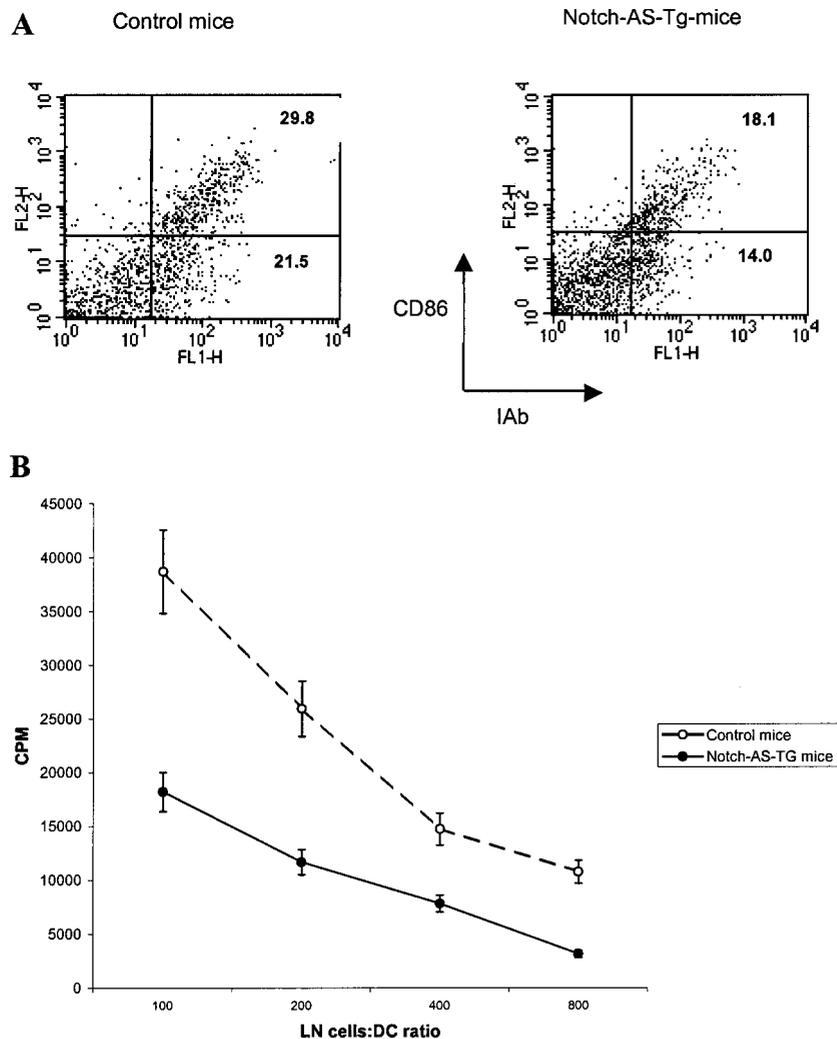
Retroviral vector containing only the *GFP* gene was used as a control. Only GFP-positive cells were analyzed. As shown in Fig. 6C, Notch-1-deficient mice had a 2-fold lower proportion of CD11c<sup>+</sup>IA<sup>b+</sup> or CD11c<sup>+</sup>B7-2<sup>+</sup> DCs than control mice. Expression of Notch-1 in HPC from Notch-AS-Tg mice with the use of a retroviral construct almost completely restored the proportion of DCs to the control level.

## Discussion

Here, we have demonstrated that NF- $\kappa$ B activity in HPC is strictly dependent on Notch-1 expression. This study was a result of our attempt to identify a possible role for Notch-1 in defective myeloid cell differentiation mediated by tumor-derived factors. During that study, we discovered that NF- $\kappa$ B activity in Notch-AS-Tg mice was significantly lower than that in control mice. Using EMSA and the luciferase reporter gene system, we have confirmed that NF- $\kappa$ B activity was indeed substantially reduced in Notch-AS-Tg mice. In light of the fact that Notch-1 expression is not completely abolished in these mice, we speculate that NF- $\kappa$ B function in HPC is

totally dependent on Notch-1. This hypothesis is supported by the fact that transduction of HPC with activated Notch-1 completely restored NF- $\kappa$ B activity. To confirm the functional significance of our findings, we have used two experimental systems in which the role of NF- $\kappa$ B is well established. LPS-inducible B cell proliferation is strictly dependent on NF- $\kappa$ B (27, 28). We hypothesized that in Notch-AS-Tg mice, decreased NF- $\kappa$ B activity would manifest itself in a decreased B cell response. Our experiments described in Figs. 5 and 6 confirmed the functional significance of decreased NF- $\kappa$ B activity in transgenic mice. It has been previously demonstrated that lack of Notch-1 did not affect B cell differentiation (30). Our study suggests that Notch-1 deficiency, although not affecting B cell differentiation, may interfere with B cell function. Another process closely dependent on NF- $\kappa$ B activity is the differentiation of myeloid DCs (13, 21, 29). We demonstrated here that HPC from Notch-1-deficient mice have significantly less ability to differentiate into DCs in presence of GM-CSF and IL-4 than HPC from control mice. Transduction of HPC with a constitutively active Notch-1 almost completely restored that

**FIGURE 6.** HPCs from Notch-1-deficient mice had reduced capacity to differentiate to dendritic cells. **A**, HPC were isolated from control or Notch-1-deficient mice and incubated with 20 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 days followed by 48 h incubation with TNF- $\alpha$ . Cells were labeled with PE-conjugated anti-CD86 and FITC-conjugated anti-IA<sup>b</sup> Ab and analyzed on a FACSCalibur flow cytometer. FL1-H, Fluorescence. Two experiments with the same results were performed. Gates were set around cells labeled with isotype control Ab. **B**, DCs were generated from HPC as described above, irradiated at 150 Gy, and cultured with lymph nodes (LN) isolated from control allogeneic BALB/c mice at different ratios (1:100–1:800). Cell proliferation was measured in triplicates as described in *Materials and Methods*. Values are the average  $\pm$  SE. Two experiments with the same results were performed. **C**, HPC were isolated from control or Notch-AS-Tg mice, and DCs were generated as described above (5 days with GM-CSF and IL-4 and 2 days with TNF- $\alpha$ ). On day 1, cells were infected with retroviruses containing either GFP gene alone (control virus) or *Notch-1* and *GFP* (Notch-1 virus). Infection was repeated three more times with 24-h intervals. Cells collected on day 7 were labeled with APC-conjugated anti-CD11c Ab and PE-conjugated anti-IA<sup>b</sup> or anti-B7-2 Ab. GFP-positive cells were gated using the fluorescence (FL-4H) channel of the flow cytometer (*top*). Isotype control level and the proportion of CD11c<sup>+</sup>IA<sup>b</sup><sup>+</sup> or CD11c<sup>+</sup>B7-2<sup>+</sup> DCs were evaluated among GFP-positive cells.



ability. These data do not contradict to recently published observation of the normal presence of thymic DCs in Notch-1 conditional knockout mice (30). Almost all thymic DCs are of lymphoid origin. In contrast to myeloid DCs, differentiation of lymphoid DCs is independent on NF- $\kappa$ B (29).

Both Notch-1 and NF- $\kappa$ B are closely involved in cell differentiation, and in particular in differentiation of cells of immune system. Activation of both of these factors can result in inhibition of apoptosis, a critical event for survival of T cells during their selection in thymus (3, 8, 31). Thus, it is not surprising that both Notch-1 and NF- $\kappa$ B were implicated in T cell differentiation. There are several conflicting reports about possible interactions between Notch-1 and NF- $\kappa$ B. One group demonstrated an ability of Notch-1 to mimic I $\kappa$ B and to sequester NF- $\kappa$ B dimers, thus preventing NF- $\kappa$ B-dependent activation of transcription (9). More recently, this inhibitory interaction has been shown to require nuclear migration of the intracellular subunit of Notch-1 and mapped to a region that partially overlaps the putative primary CBF<sub>1</sub>/RBP-J $\kappa$  interaction site (16). Another group reported stimulation of the p52 promoter by activated Notch-1, which overcomes transcriptional repression of p52 induced by CBF<sub>1</sub>/RBP-J $\kappa$  (17). A different mechanism of NF- $\kappa$ B activation by a transcriptional regulator Notch was proposed by the third group. These investigators recently have demonstrated that another Notch family member, Notch-3, induced phosphorylation and degradation of I $\kappa$ B, which in turn resulted in nuclear translocation of NF- $\kappa$ B dimers and ac-

tivation of transcription (18). We have obtained independent evidence that soluble Notch ligands induce rapid I $\kappa$ B kinase  $\alpha$  activation and NF- $\kappa$ B transcriptional activity in human keratinocytes. This is followed by peroxisome proliferator activated receptor  $\gamma$  induction and eventually by NF- $\kappa$ B inhibition.<sup>4</sup> Together with our observations, these data suggest that Notch and NF- $\kappa$ B may be involved in a complex feedback mechanism that affects cell differentiation. NF- $\kappa$ B expression and/or activation may be induced by Notch-1, at least in some cells, whereas nuclear accumulation of Notch-1 may eventually result in NF- $\kappa$ B inhibition, providing a mechanism for signal termination. This model predicts that the effects of Notch-1 activation on NF- $\kappa$ B are likely to be time and dose dependent, as we have observed in keratinocytes.<sup>4</sup> In this study, we asked whether physiological NF- $\kappa$ B activity is dependent on the presence of Notch-1. Our current data strongly suggest that Notch-1 is a critical factor that controls NF- $\kappa$ B activity in HPC and NF- $\kappa$ B-dependent processes such as B cell activation and DC maturation.

Based on published observations, several mechanisms potentially may explain the effect of Notch-1 on NF- $\kappa$ B activity. These include transcriptional effects, physical binding between NF- $\kappa$ B and Notch-1, and indirect effects on I $\kappa$ B phosphorylation. Our data

<sup>4</sup> B. Nicholoff, J.-Z. Qin, V. Chaturvedi, M. Denning, B. Bonish, and L. Miele. Activation of Notch signaling is necessary and sufficient to create mature human epidermis. *Submitted for publication.*

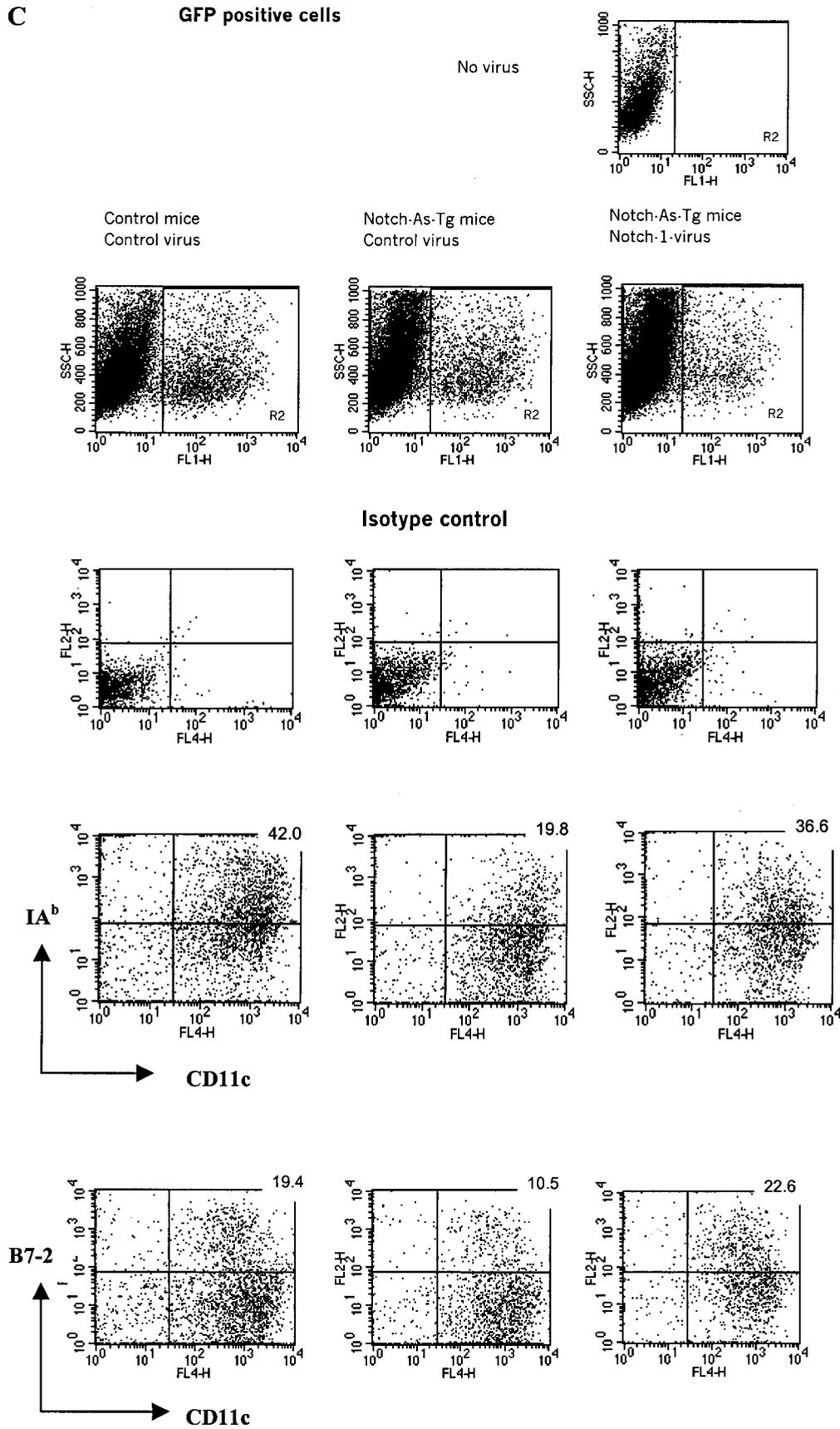


FIGURE 6. (Continued)

show that HPC from Notch-AS-Tg mice have substantially lower levels of p65 (RelA), c-Rel, RelB, and p50 than HPC isolated from control mice. These data suggest that Notch-1 may be required for basal synthesis of NF- $\kappa$ B subunits. This hypothesis is further supported by the fact that expression of *p65* and *c-Rel* mRNA was strongly reduced in transgenic mice. Thus, it appears that Notch-1 is required for basal transcription of several NF- $\kappa$ B subunits. There are several possible mechanisms of Notch-1-mediated regulation of NF- $\kappa$ B transcription. Notch-1 may bind one or more NF- $\kappa$ B repressors, blocking their activity and thus *trans* activating NF- $\kappa$ B. This mechanism is consistent with the data of Oswald et al. (17). Because NF- $\kappa$ B can be positively autoregulated after cell stimulation (32, 33), increased nuclear translocation of NF- $\kappa$ B dimers may result in increased synthesis of NF- $\kappa$ B, which would explain differences in protein levels of several NF- $\kappa$ B subunits observed in Notch-AS-Tg mice. Another mechanism is suggested by the work of Bellavia et al. (18). Notch-1 may induce phosphorylation and degradation of I $\kappa$ B, inducing nuclear translocation of NF- $\kappa$ B with subsequent increased transcription of NF- $\kappa$ B subunits. However, our data (Fig. 4B) argue against this hypothesis. All these possible mechanisms currently are under investigation.

Our data indicate that the interaction between Notch-1 and NF- $\kappa$ B may be a factor in normal cell differentiation and function. During the normal process of cell differentiation, Notch-1 becomes activated via interaction with its specific ligands. Two Notch ligands, Jagged-1 and Jagged-2, are expressed on bone marrow stromal cells and on HPC themselves (2). Notch signaling is an extremely conserved and highly pleiotropic mechanism that controls cell fate determination in organisms from invertebrates to humans (2). A hallmark of Notch-dependent effects is their striking context dependence, which suggests that numerous target genes may be affected by Notch signaling, depending on cell type and other variables. This effect of Notch-1 on NF- $\kappa$ B activity suggests that the >150 genes that are known to be controlled by NF- $\kappa$ B may be added to other well-known Notch targets, such as inhibitory helix-loop-helix transcription factors. This may help explain the great variety of Notch effects observed in diverse cell types. Whether Notch-1 regulation of NF- $\kappa$ B activity is affected in cancer or other pathological conditions remains to be investigated.

In this study, we have demonstrated that Notch-1 is required for normal expression and function of NF- $\kappa$ B in HPC. It appears that Notch-1 exerts its effect via transcriptional regulation of several members of NF- $\kappa$ B family. This may contribute to a complex network that regulates the differentiation and function of hemopoietic cells.

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