

Tumor necrosis factor- α and endothelial cells modulate Notch signaling in the bone marrow microenvironment during inflammation

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Objective. Homeostasis of the hematopoietic compartment is challenged and maintained during conditions of stress by mechanisms that are poorly defined. To understand how the bone marrow (BM) microenvironment influences hematopoiesis, we explored the role of Notch signaling and BM endothelial cells in providing microenvironmental cues to hematopoietic cells in the presence of inflammatory stimuli.

Materials and Methods. The human BM endothelial cell line (BMEC) and primary human BM endothelial cells were analyzed for expression of Notch ligands and the ability to expand hematopoietic progenitors in an in vitro coculture system. In vivo experiments were carried out to identify modulation of Notch signaling in BM endothelial and hematopoietic cells in mice challenged with tumor necrosis factor- α (TNF- α) or lipopolysaccharide (LPS), or in Tie2-tmTNF- α transgenic mice characterized by constitutive TNF- α activation.

Results. BM endothelial cells were found to express Jagged ligands and to greatly support progenitor's colony-forming ability. This effect was markedly decreased by Notch antagonists and augmented by increasing levels of Jagged2. Physiologic upregulation of Jagged2 expression on BMEC was observed upon TNF- α activation. Injection of TNF- α or LPS upregulated three- to fourfold Jagged2 expression on murine BM endothelial cells in vivo and resulted in increased Notch activation on murine hematopoietic stem/progenitor cells. Similarly, constitutive activation of endothelial cells in Tie2-tmTNF- α mice was characterized by increased expression of Jagged2 and by augmented Notch activation on hematopoietic stem/progenitor cells.

Conclusions. Our results provide the first evidence that BM endothelial cells promote expansion of hematopoietic progenitor cells by a Notch-dependent mechanism and that TNF- α and LPS can modulate the levels of Notch ligand expression and Notch activation in the BM microenvironment in vivo. © 2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

In the adult, hematopoietic cells of all lineages arise from self-renewing stem cells and expanding progenitors embedded in the stromal fabric of the bone marrow (BM). The

three-dimensional structure of the BM is constituted of the hematopoietic cells themselves; of extracellular matrix; and of stromal cells, which include fibroblasts, adipocytes, osteoblasts, and endothelial cells [1,2]. Despite the increasingly recognized role of the BM endothelial cells in supporting hematopoietic multipotential progenitors, the molecular mechanisms involved and their potential implication in providing homeostatic stimuli to resident hematopoietic cells have been little investigated. As an interface

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between blood and tissue, the endothelium is poised to respond quickly to local changes elicited by trauma or inflammation [3]. This response prevents physical disruption of the vessel wall by trauma, microbial organisms, or toxins, and elicits host defenses. For instance, acute and chronic infections trigger the release of the proinflammatory cytokines interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α), which stimulate the host innate immune response. When stimulated by IL-1 β or TNF- α , BM endothelial cells upregulate their cytokine production and adhesion molecules [4–6]. Physiologically, these events may facilitate recruitment of inflammatory cells to contain the infection and, at the same time, impact on steady-state hematopoiesis such that progenitor expansion and differentiation may be modulated to respond to stress.

During embryogenesis, cell-to-cell contact between endothelium and hematopoietic cells is necessary for development of the hematopoietic system [7,8]. Similarly, in adult life the close association of BM endothelial cells with BM hematopoietic cells suggests that the endothelium continues to be a critical regulator of hematopoiesis [2,9,10]. BM endothelial cells have an affinity for binding CD34⁺ progenitor cells [2,11] and recent observations reported selective localization of purified hematopoietic stem cells to the BM endothelium, suggesting that BM endothelial cells could provide a specific functional niche for the hematopoietic stem cells [12–14].

BM endothelial cells constitutively produce cytokines that regulate expansion and differentiation of hematopoietic progenitors, such as IL-6, Kit-ligand, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor. Several studies have shown that BM endothelial cell monolayers are a unique type of endothelium that can support long-term proliferation of multilineage hematopoietic progenitors [2,9,15]. Although this effect has been largely attributed to cytokine production by BM endothelial cells, cell-to-cell contact may have an important function that remains to be investigated. Cell-to-cell interactions between hematopoietic cells and their microenvironment play a critical role in the regulation of adult hematopoiesis. A class of molecules that govern cell differentiation and proliferation decisions through cell-to-cell contact is the Notch family of receptors and their ligands. Notch family members are highly conserved transmembrane receptors that play a critical role in regulating cell fate decisions in various organisms and in multiple tissues [16], including the hematopoietic tissues. Hematopoietic cells express Notch receptors and ligands and are surrounded, within the BM, by stromal cells expressing the Notch ligands Jagged1 (J1), Jagged2 (J2), Delta-like1 (Dll1), and Delta-like4 (Dll4) [17]. It is now well-documented that expression of Delta-like or Jagged family members on stromal feeder layers augment their ability to expand hematopoietic stem/progenitor cells *in vitro* [18–20]. Studies conducted by our group demonstrated that activation of Notch1 by J2

or Dll4 delays myeloid differentiation and preserves hematopoietic progenitors in a more immature phenotype [17,21,22]. Recently, J1 was found to be expressed by BM osteoblasts, a critical component of the BM stroma that elicits self-renewal of hematopoietic stem cells [23,24]. Here, we show that endothelial cells of the BM microvasculature express the Notch ligand Jagged2 and that its expression is upregulated by inflammatory stimuli, such as TNF- α and lipopolysaccharide (LPS). We demonstrated that the effects of BM endothelium on BM progenitors is mediated by Notch signaling and depend on Notch-ligand density, thus contributing to a fine-tuned regulation of the stem/progenitor cells pool as it is needed during conditions of inflammatory stress. We hypothesized that BM endothelial cells may represent a functional niche that regulates homeostasis of the BM progenitor pool during inflammation.

Materials and methods

Cells and cell cultures

BMEC cell line was generated as described previously [25] and provided by J. Ascensao and G. Almeida-Porada (University of Reno, Reno, NV, USA). Primary human BM endothelial cells (ECs; BM-ECs) were isolated from BM of healthy donors (according to the guidelines of the Human Investigation Committee; DFCI) by purification with CD105 microbeads (Miltenyi Biotec, Auburn, CA, USA) and selection in endothelial-supporting media, containing vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (EGM-2 media; Lonza, Walkersville Inc, Walkersville, MD, USA). BMEC and primary BM-ECs were maintained with EGM-2 medium and switched to Dulbecco's modified Eagle's medium 10% fetal bovine serum 2 days prior and during TNF- α or IL-1 β (Pepro Tech, Peprotech Inc, Rocky Hill, NJ, USA) stimulation. BM stroma from primary BM cells was generated using Dexter's culture conditions, as described previously [26]. Human CD34⁺ cells were derived from cord blood obtained from the Pediatric Research Institute, University of St Louis (St Louis, MO, USA) or the Saint Vincent's Hospital (Indianapolis, IN, USA) (according to guidelines of the Human Investigation Committee, Indiana University School of Medicine) and isolated by immunomagnetic beads (Miltenyi). Pools of cord blood CD34⁺ cells were grown in EX-VIVO-15 media (Lonza), stem cell factor (SCF; 50 ng/mL) and IL-3 (20 ng/mL; R&D Systems Inc, Minneapolis, MN, USA) in the presence or absence of BMEC. BMEC monolayers were formed in 24-well/plate or 6-well/plate wells in the presence of EGM-2 media; 70% to 80% confluent monolayers were rinsed twice with Iscove's modified Dulbecco's media prior to coculture with CD34⁺ cells. CD34⁺ cells were seeded on BMEC monolayers at the concentration of 1×10^5 (on 24 wells/plate wells) or 1×10^6 (on 6 wells/plate wells), spinoculated for 30 minutes at 1500 rpm and cultured at 37°C and 5% CO₂ for 1, 3, or 5 days. CD34⁺ and BMEC cocultures were grown for 5 days in the presence or absence of 2 μ M γ -secretase inhibitor (GSI X; Calbiochem). Prior to seeding, CD34⁺ cells were blocked for 10 minutes on ice with human immunoglobulins (Sigma, St Louis, MO, USA) and then incubated with saturating concentrations (25 μ g/mL) of soluble Dll4-Fc generated as described previously [22], the monoclonal antibody anti-N1

(Biomedica Corporation, Foster City, CA, USA) [27,28] or Fc fragment control (Sigma) for 1 hour on ice. The CD34⁺ fraction adherent to BMEC was harvested (using one rinse of phosphate-buffered saline [PBS] and one rinse of PBS/2 mM EDTA) and used for further analysis after 24 hours (for Western blot analysis) or 3 days (for colony assay). Harvested CD34⁺ were seeded in methylcellulose at 3000 to 5000 cells/mL and evaluated for colony-forming ability at days 10 to 14 as described previously [21].

3T3 and MS5-D114 cell lines were generated by retroviral transduction as described previously [21]. BMEC-green fluorescent protein (GFP) and BMEC-J2-GFP were generated by retroviral transduction with the MSCV-IRES-GFP vector alone or containing the full length cDNA of human Jagged2, following standard procedures [17,21,22].

Mice

Tie2-GFP reporter (FVB/N-TgN(TIE2GFP)287Sato), CD1 and C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Hes5-GFP mice were BAC transgenics from the National Institute of Neurological Disorders' Gene Expression Nervous System Atlas Project (Rockefeller University, www.gensat.org). Tie2-tmTNF- α transgenic mice and wild-type (WT) non-transgenic littermates were described in [29,30]. Mice were matched for sex and analyzed at 4 to 5 weeks of age. Recombinant human TNF- α (#410-MT) was from R&D Systems; LPS from *Pseudomonas aeruginosa* serotype 10 (#L8643) was from Sigma. Eight- to 12-week-old mice were each given 10 μ g TNF- α in PBS/0.1% bovine serum albumin intravenously or 500 μ g LPS in PBS intraperitoneally and sacrificed at different time points. BM was flushed from two femurs from each mouse with PBS/2 mM EDTA. All Animal Studies were approved by the MGH Subcommittee on Research Animal Care or by the Indiana University LARC Committee on Animal Research.

Immunological reagents and procedures

Human BM endothelial cells were harvested, blocked with human immunoglobulins and incubated for 30 minutes on ice with the following antibodies: CD45, CD106 (vascular cell adhesion molecule), CD54 (intracellular adhesion molecule-1 [ICAM-1]), and CD144 (VE-Cadherin) from BD Pharmingen (BD Biosciences, San Jose, CA, USA); CD105 from Invitrogen; AC133/2-PE, Neuropilin-phycoerythrin (PE) (BDCA4) from Miltenyi Biotec; VEGFR 1/2/3 from R&D Systems; Von Willebrand (purified) from Serotec (Serotec Inc, Raleigh, NC, USA); GaM-PE from BioSource (BioSource International Inc, Camarillo, CA, USA). Murine BM mononuclear cells were flushed from femurs using PBS/2 mM EDTA. Prior to immunolabeling, cells were incubated with FC-receptor blocker (BD Pharmingen). BM cells were labeled with fluorescein isothiocyanate-, allophycocyanin- or percy-PE-conjugated control immunoglobulins or specific monoclonal antibodies directed to: Sca-1, c-Kit, CD31, FLK1, CD45, and the lineage markers cocktail (CD3, CD4, CD8, Gr1, CD19, NK, Ter119, Mac1, B220). Intracellular staining was performed using fixing and permeabilization solutions from Caltag (Caltag Laboratories, Burlingame, CA, USA). Antibodies against J2, N1, N2, Val1744N1, or IgGs control (Jackson ImmunoResearch) were added at the concentration of 5 μ g/mL for 30 minutes. Cells were washed and incubated with goat anti-rabbit antibody conjugated to PE (Sigma) (1 μ g/mL). Anti-J2 antibodies included the polyclonal antibody provided by J. Aster (Brigham and Woman

Hospital, Boston, MA, USA) [31], and the anti-J2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA; H-143). Anti-N1 antibodies included the polyclonal antibody provided by J. Aster [32], and the polyclonal from Santa Cruz Biotechnology (-20); anti-N2 was from Santa Cruz (25–255). The antibody recognizing activated N1 (Val 1744) was purchased from Cell Signaling (Danvers, MA, USA). Multicolor flow cytometric analysis was performed using the FACSCalibur instrument (Becton Dickinson).

Western blots were performed as described previously [33]. Antibody used for immunoblotting include anti-activated N1 (Cell Signaling; Val 1744), anti N1 (C-20) and anti- β -actin (I-19) from Santa Cruz. Signals were quantified using Molecular Dynamics scanner and ImageQuant analysis software (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Murine femurs were fixed in zinc-fixative (BD Pharmingen) and decalcified by formic acid prior embedding in paraffin. BM sections were stained by using standard techniques with anti-J2 (H-143), anti-CD144 and anti-Flk1 antibodies (R&D) followed by donkey anti-rabbit Alexa 488 and by donkey anti-goat Alexa 647 (Molecular Probes). Images were collected on an Olympus FluoView IX2 confocal microscope using a 40 \times 1.3-NA oil immersion objectives and the appropriate filters for simultaneous detection of the Alexa 488 and Alexa 647 dyes. Several Z-sections collected at 0.62- μ m intervals were combined into single-plane projections in Metamorph (Molecular Devices Corp, Sunnyvale, CA, USA) cropping and minimal level adjustments were done in Adobe Photoshop.

Reverse transcription polymerase chain reaction (RT-PCR) and PCR

Total RNA was isolated using RNA Trizol (Invitrogen) and reverse transcribed with AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN, USA) and random hexamers primers (Boehringer Mannheim). Forward and reverse primers used for PCR are described in Table 1 of the supplemental information. PCR products were amplified through 30 cycles at 95 $^{\circ}$ C for 60 seconds, 60 $^{\circ}$ C for 90 seconds, and 72 $^{\circ}$ C for 90 seconds on a GeneAmp 9600 thermal cycler (Perkin Elmer, Roche Molecular Systems, Shelton, CT, USA).

Statistical analysis

Equality of distributions for matched pairs of observations was tested using the *t*-test.

Results

Notch ligands Jagged are expressed by BM endothelial cells

Given the respective roles of BM endothelium and of Notch signaling in maintaining and preserving multipotential hematopoietic progenitors in an immature state, we determined the expression profile of Notch ligands on BM endothelial cells and evaluated whether they can regulate hematopoiesis through stimulation of Notch signaling. As a model, we used the human BM-derived endothelial cell line BMEC, which has been immortalized by the SV40 large T-antigen, but still retains phenotypic and functional

properties of endothelial cells [25]. In addition, we used primary endothelial cells (ECs) derived from BM of normal donors [34]. Immunophenotypic characterization of both BMEC and primary BM-ECs showed an endothelial-like phenotype. These cells are negative for CD45 and positive for CD105 (Fig. 1A). CD45⁻CD105⁺ cells uniformly express: Neuropilin, VEGF-Rs, CD133, CD106, CD146, CD144, ICAM-1 and von Willebrand Factor, a combination of molecules that characterize endothelial cells (Fig. 1B and C). Furthermore, these BMEC and BM-ECs can form capillary networks, bind UEA-1, incorporate acetylated-low density lipoproteins, inhibit smooth muscle cells, and respond to tumor stimuli with a signature similar to ECs from other vascular beds [34,35]. RT-PCR analysis of both BMEC cell line and primary BM-ECs showed expression of J1 and J2, but no detectable levels of Dll1 and Dll4 (Fig. 1D). The specific expression of J2 protein was further confirmed by flow cytometry analysis using antibodies that specially recognize J2 (Fig. 1E, left panel). Basal level of J2 expression was observed in the BMEC cell line (Fig. 1E, right panel) and in five of six primary BM-EC samples analyzed (four cases showed in Fig. 1F), whereas it was not detectable in BM-derived stroma that lacked of the endothelial component (Fig. 1F).

Upregulation of J2 on BM endothelial cells promotes expansion of hematopoietic progenitor cells by a Notch-dependent mechanism

BM endothelial cell monolayers have a distinct ability to support long-term proliferation of multilineage hematopoietic progenitors [2,9,15], generally attributed to the production of hematopoietic cytokines. We investigated whether the ability of BM endothelial cells to maintain multipotential progenitors was also dependent on Notch activation through cell-to-cell contact and whether it correlated to the levels of J2 expression. As anticipated, the colony-forming ability of human CD34⁺ progenitors was increased when cocultured in the presence of BMEC cell line, compared to CD34⁺ progenitors cultured with recombinant cytokines alone (Fig. 2A). However, this effect was markedly reduced when ligand-dependent Notch signaling was inhibited by the γ -secretase inhibitor GSI X, which selectively blocks the protease-dependent cleavage of Notch in response to ligand binding [36]. As shown in Figure 2A, CD34⁺ cells cocultured with BMEC cell monolayers in the presence of GSI generated a significantly lower number of colonies compared to CD34⁺ cells cocultured with BMEC monolayers in the absence of GSI, indicating a critical role of Notch signaling in this process. Next, to determine the role of ligand density, we engineered BMEC cells overexpressing J2 (BMEC-J2). Exposure of CD34⁺ progenitors to increased ligand density on BMEC-J2 monolayers furthered their ability to form colonies compared to CD34⁺ progenitors cocultured with BMEC cells control (BMEC-GFP; Fig. 2B). This effect was abrogated by inhibition of

Notch signaling by GSI treatment and by the presence of a transwell (data not shown). Thus, cell-to-cell contact dependent Notch signaling is critical for the greater effect induced by BM endothelial cells on hematopoietic progenitors. Notch activation was confirmed by transcriptional activation of its target gene *Hes1*, and by generation of the ligand-induced cleaved form of Notch (Notch^{ic}). *Hes1* transcription was higher in cells cultured in the presence of BMEC cell lines (BMEC and BMEC-J2) than in cells cultured in their absence (no coculture), and it was greatly reduced by GSI treatment (Fig. 2C). Accordingly, the cleaved activated form of Notch was visible only when CD34⁺ cells were cocultured in direct contact with BMEC cells (Fig. 2D, lanes 2 and 3). To determine the specificity of this interaction, Notch-Jagged contact between CD34⁺ and BMEC cells was inhibited by blocking Notch receptors with an antibody directed to the extracellular domain of Notch1 (N1ab) [27,28], or with the soluble form of Notch ligand Delta4 (Dll4-Fc) [22]. Both reagents can bind to N1 and N2; antibodies directed to ligand binding region of Notch receptors and soluble ligand-fusion proteins have been used as antagonist to inhibit the full physiologic ligand-receptor binding and activation [37–40]. Incubation of CD34⁺ cells with N1ab or Dll4-Fc prior seeding into BMEC monolayers diminished the robust Notch stimulation provided by the Jagged ligands on BMEC cells, as shown by the lower levels of cleaved Notch^{ic} generated in these conditions (Fig. 2D, left panel, lanes 3–5, and right panel). Similarly to the inhibition provided by GSI, decreased Notch signaling by preincubation with soluble Dll4-Fc or N1ab resulted in the decreased ability of cocultured CD34⁺ cells to generate colonies in a methylcellulose assay compared to CD34⁺ cells preincubated with control Fc (Fig. 2E), thus excluding potential indirect effects of GSI and confirming the specific role of Notch-ligand interactions.

Taken together, our results demonstrate that Notch signaling plays an important role in mediating the effect of BM endothelium on hematopoietic progenitor's expansion and significantly potentiates the effects induced by hematopoietic cytokines produced by the BMECs. Furthermore, our data provide evidence that the regulation of ligand density on BM endothelial cells and Notch signal intensity on hematopoietic progenitors can result in different degrees of progenitor expansion.

TNF- α upregulates J2 expression on BM endothelial cells in vitro and in vivo

Considering the importance of Notch ligand density on the cell surface, we asked whether microenvironmental cues could regulate their expression on BM endothelial cells. Because ECs play a critical role in inflammation and their function is highly regulated by proinflammatory cytokines such as TNF- α and IL-1 β , we determined whether J2 expression was modulated during cytokine-induced EC activation. The BMEC cell line and primary BM-ECs were

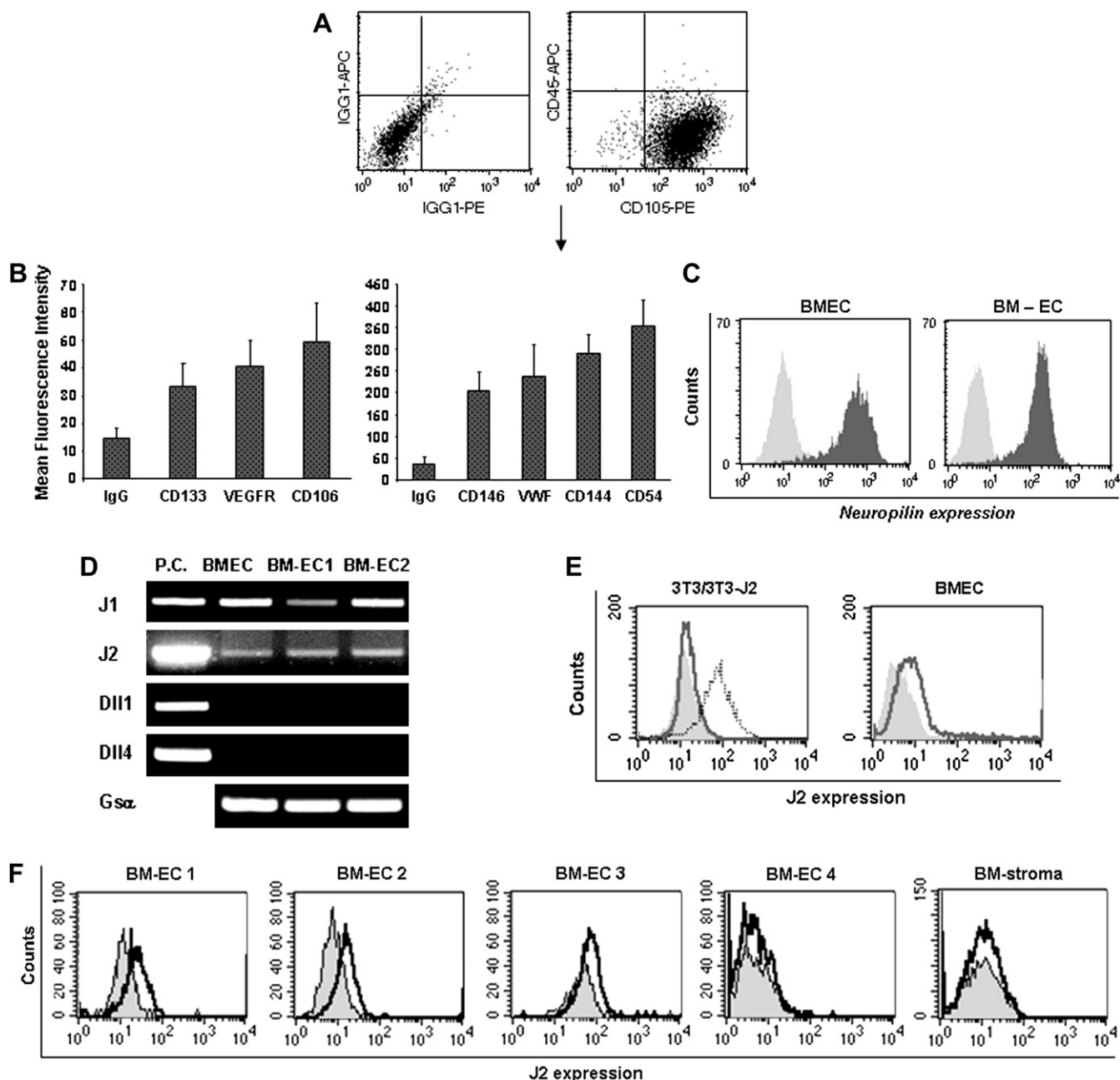


Figure 1. J2 is expressed by BM endothelial cells. (A-C) Characterization of human BM endothelial cells. BM-EC cell line clones (representing two different passages) and primary BM-ECs were harvested and labeled with the indicated antibodies in combination with CD45 and CD105 followed by multicolor analysis. (A) Dot plots show immunoglobulin controls (left) and expression of CD105 and CD45 in one representative sample. (B) CD45⁺CD105⁺ cells were analyzed for the expression of the indicated markers. Bar graphs represent one of three independent experiments. Numbers indicate average of mean fluorescence intensity of three samples for each antibody. Error bars represent standard deviation. (C) Histogram shows overlays of Neuropilin expression (dark gray filled curve) over IgG control (light gray filled curve) on: BM-EC (left panel) and primary BM-ECs (right panel). (D) Notch ligand expression profile in human BM-derived endothelial cells. RNAs obtained from BM-EC and BM-EC samples were used for PCR amplification. All samples were positive for the low molecular size PCR product of the housekeeping gene *Gsa*, confirming the absence of genomic DNA [22]. RNAs from MS-5 cells overexpressing human cDNA of each ligand were used as PCR positive control (PC). (E) Left panel: 3T3 cells were transduced with pBABE retrovirus alone or containing human J2 cDNA. Cells were selected by puromycin and J2 expression was confirmed by Western blot analysis. 3T3-vector and 3T3-J2 cells were labeled with polyclonal anti-J2 antibodies followed by anti-rabbit phycoerythrin-conjugated antibody. Histogram shows overlays of J2 expression on 3T3-vector (solid line) and on 3T3-J2 cells (dotted line); gray filled curve shows rabbit IgG control on 3T3-J2 cells. Right panel: Histogram shows overlays of J2 expression (solid line) and of control immunoglobulins (filled curve) on BM-EC cells. (F) Flow cytometric analysis of J2 on primary BM-ECs. Histograms show fluorescence intensity on the x-axis and cell count on the y-axis. Superimposed are fluorograms with anti-J2 antibody (solid lines) and control immunoglobulins (filled curves) on BM-EC samples derived from four different donors and on BM stroma derived from a donor.

stimulated with TNF- α (10 ng/mL; optimal dose) for 24, 48, and 72 hours and ECs activation was confirmed by detection of I κ B phosphorylation (data not shown) and upregulation of ICAM-1 (Fig. 3D). TNF- α induced a significant upregulation of J2 expression in the BMEC cell line, both at the level of protein and transcript (Fig. 3A and B). Similarly, three of four BM-EC samples upregulated J2 expression in response to TNF- α by 24 hours (Fig. 3C). In all cases, J2 expression returned to basal levels by 48 hours. Of note, sample 4, which did not show detectable J2 expression at basal condition, responded with a strong upregulation of J2 following TNF- α stimulation, whereas no upregulation was observed in the BM stroma (Fig. 3C). Stimulation of BMECs with IL-1 β induced a weaker effect on J2 expression compared to TNF- α as shown by a representative experiment in Figure 3E.

Next, we confirmed the ability of TNF- α to upregulate J2 expression on BM-ECs in vivo. CD1 or C57Bl/6 mice were injected with PBS or TNF- α (10 μ g) and their BM cells were analyzed by multicolor flow cytometer analysis at 6, 12, and 24 hours after injection. J2 expression was evaluated on primary murine BM endothelial cells, identified by a combinatorial analysis of their surface markers as a population of large cells negative for CD45 and positive for both Flk1 (VEGFR2) and CD31 (Fig. 4A, left panel). To further confirm the presence of ECs within this population, we analyzed the expression of the endothelial cell marker TIE2. CD45⁻FLK1⁺CD31⁺ cells were highly enriched in ECs coexpressing TIE2, as shown by analysis performed in TIE2-GFP reporter transgenic mice [41] (Fig. 4A, right panel), and showed high expression of ICAM-1 and J2 by real-time PCR on sorted populations (data not shown).

TNF- α administration resulted in a two- to threefold increase of J2 expression in BM endothelial cells over the baseline (Fig. 4B and C). Time course analysis showed that J2 upregulation was at its highest at 12 hours from TNF- α injection and declined over time (Fig. 4C). Overall, these results confirm the ability of TNF- α of upregulating J2 expression on BM endothelial cells in vivo and provide evidence for the modulation of Notch ligand expression by microenvironmental cues.

To investigate the physiological relevance of these findings, we analyzed the expression of J2 on BM endothelial cells following LPS inoculation in vivo. Bacterial LPS are the major triggers of TNF- α release and of inflammatory response. LPS binds to Toll-like receptor 4 on ECs resulting in their activation and intense production of TNF- α during bacterial infection [42]. In our experiments, in vivo induction of TNF- α by LPS was confirmed by evaluation of TNF- α serum levels by enzyme-linked immunosorbent assay after LPS inoculation (Fig. 4D). Similarly to TNF- α injection, LPS challenge resulted in a dramatic upregulation of J2 expression on BM endothelial cells, which was highest at 12 hours from the injection (Fig. 4B and C), suggesting that

J2 upregulation occurs in vivo in response to bacterial infection.

TNF and LPS stimulation results in increased expression and activation of Notch in BM progenitors

Next, we examined the expression and activation status of Notch on hematopoietic progenitors in response to TNF- α stimulation. Both TNF- α and LPS injection resulted in approximately twofold upregulation of N1 expression on Lin⁻Sca⁺ cells (Fig. 5A and B). N1 expression was analyzed on the larger population of Lin-Sca⁺ cells to allow a statistically significant number of events to be measured; however, a similar trend was observed in the smaller Lin⁻Sca⁺Kit⁺ subset. Treatment with TNF- α resulted also in an increase of N2 expression (Fig. 5C).

More importantly, increased expression of J2 and of N1 and N2 following TNF- α or LPS injection was accompanied by increased Notch activation, as determined by the levels of the active form of Notch1 (Notch^{ic}) on Lin⁻Sca⁺ cells detected by an antibody that specifically recognizes the cleaved intracellular form of Notch generated upon ligand binding [22] (Fig. 5D and E).

To complement this analysis and further demonstrate the activation of Notch signaling during inflammation in vivo, we injected LPS into transgenic mice expressing GFP under the control of the endogenous Hes5 promoter. Hes5 transcription is tightly and directly activated by Notch signaling in vivo [43]. Basal levels of HES5-driven GFP expression were barely detectable by flow cytometry in the BM cells of transgenic animals at steady-state conditions. In contrast, LPS challenge induced a significantly increase in Hes5-driven GFP expression in both Lin⁻Sca⁺ and Lin⁻Sca⁺Kit⁺ subsets (Fig. 5F).

In conclusion, these results provide evidence that proinflammatory stimuli, such as TNF- α and LPS, result in the activation of Notch signaling on hematopoietic progenitors in vivo.

Upregulation of Notch signaling in the BM microenvironment of Tie2- tmTNF- α mice

Systemic administration of recombinant TNF- α in vivo may affect multiple cellular types in a direct or indirect manner. To further corroborate the results obtained with soluble TNF- α we analyzed the levels of J2 expression and Notch signaling activation in an in vivo model of constitutive endothelial cell activation. In Tie2-tmTNF- α transgenic mice, a transmembrane, noncleavable form [mTNFD1-9,K(11)E] of TNF- α is expressed under the control of the Tie2 promoter in conjunction with an intronic enhancer element used to drive endothelial-specific expression [30]. In these mice, ECs exhibit a chronic activated phenotype as result of constitutive expression of the TNF- α membrane-bound form [29,30]. Analysis of J2 expression in Tie2-tmTNF- α mice showed increased levels of J2 on BM cells with endothelial immunophenotype CD45⁻CD31⁺FLK1⁺, compared to nontransgenic wild-type controls

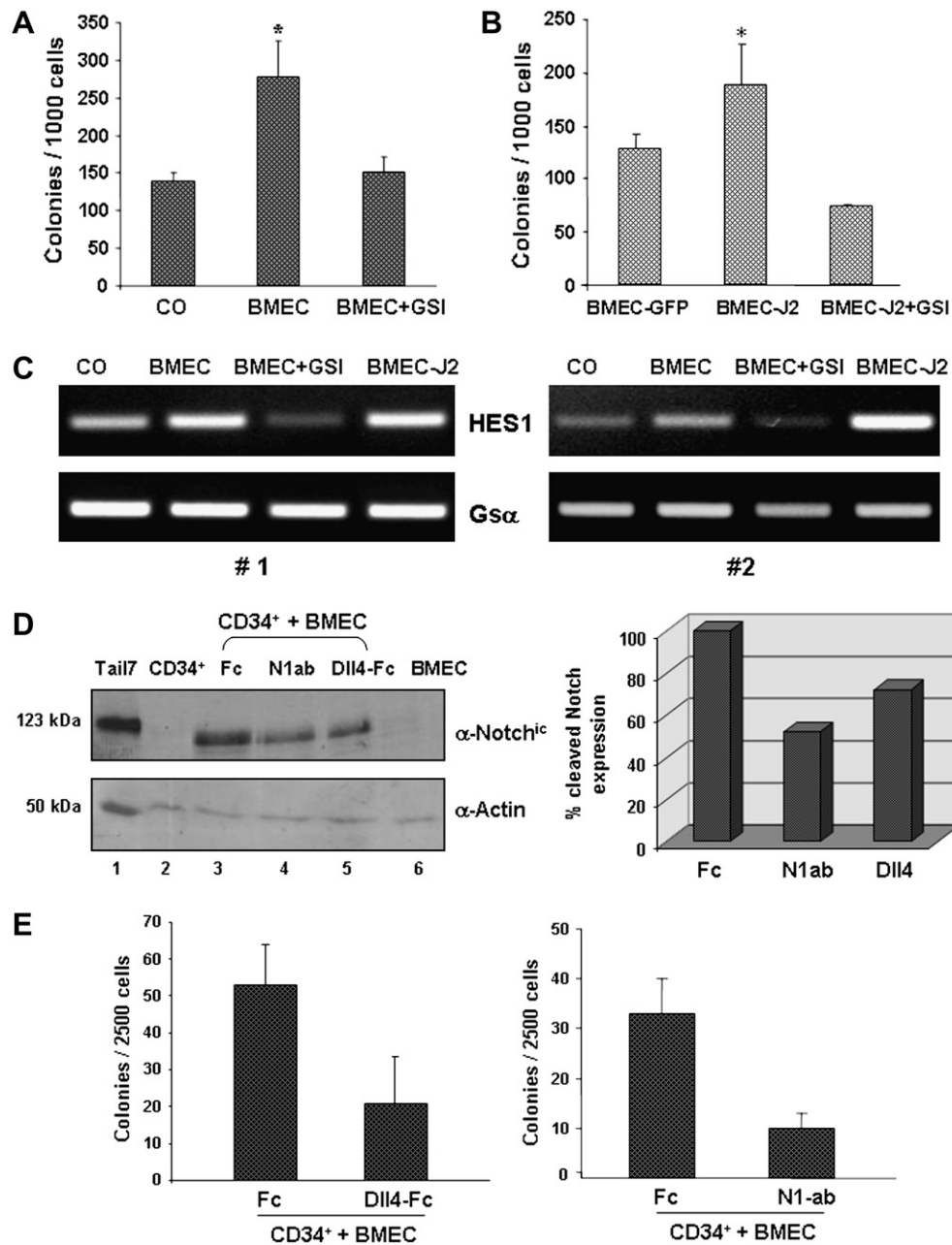


Figure 2. BM endothelial cells promote expansion of hematopoietic progenitor cells by a Notch dependent mechanism. (A) CD34⁺ cells were grown in liquid culture on plastic alone in the presence of cytokines (CO) or on BMEC monolayers, in the absence or presence of GSI (2 μ M). After 5 days, cells were harvested, washed, and plated in quadruplicate, at a density of 5000 cells/mL, in methylcellulose supplemented with interleukin-3, stem cell factor, and erythropoietin. Bar values indicate the average of three experiments. Colony-forming cells (CFCs) are expressed as number of colonies per 1000 cells seeded. Error bars represent standard error. * Differences between the populations (BMEC vs CO; BMEC vs BMEC+GSI) are statistically significant: $p < 0.02$. (B) CD34⁺ cells cocultured with BMEC-GFP (BMEC-J2) in the presence or absence of GSI (2 μ M) were harvested at day 7 of coculture and plated in triplicate at the density of 5000 cells/mL in methylcellulose, as described previously. Bars represent the average of four experiments. CFCs are expressed as number of colonies per 1000 cells seeded. Error bars represent standard error. * Differences between populations (BMEC-J2 vs BMEC-GFP and BMEC-J2+GSI) are statistically significant, $p < 0.001$; (C) RNAs from CD34⁺ cells cultured alone or in the presence of BMEC and BMEC-J2, in the presence or absence of GSI, were used for reverse transcription polymerase chain reaction amplification of the Hes1 gene. The two blots are representative of two independent experiments. (D, E) CD34⁺ cells were incubated with Fc fragment (control), DII4-Fc, or anti-N1 antibody for 1 hour prior to seeding into BMEC monolayers. (D) Expression of activated Notch in CD34⁺ progenitors. CD34⁺ cells in the different conditions were harvested after 24 hours of coculture. Cell extracts were analyzed by immunoblot by using a Notch antibody detecting the activated form of Notch. Bar graphs represent ImageQuant densitometric analysis of Notch^{ic} protein level. Values indicate percentage of Notch^{ic} detected following incubation with N1ab or DII4-Fc relative to the Fc control (100%). Notch densitometric values were normalized with β -actin levels. (E) CD34⁺ cells were harvested at 72 hours of coculture and seeded at 3000/mL in methylcellulose. Bar graphs represent two independent experiments. Bars values are average of four wells and represents number of colonies, CFCs, per cells seeded. Error bars represent standard error.

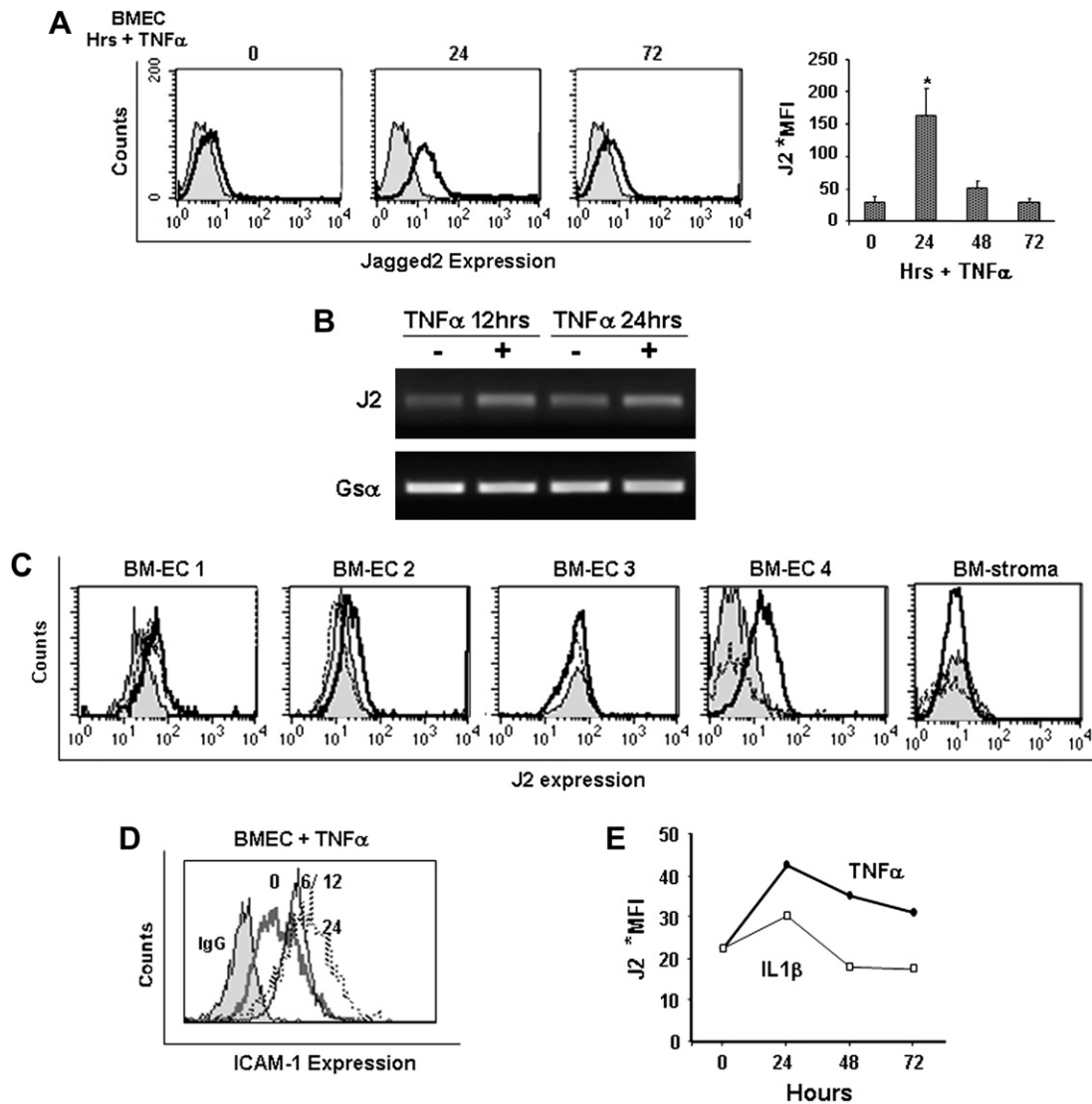


Figure 3. J2 expression on BM endothelial cells is upregulated by TNF- α in vitro. (A) BMEC cell line was stimulated with TNF- α (10 ng/mL), and cells harvested and labeled with anti-J2 antibody at the indicated time points. Histograms on the left show flow cytometric analysis of a representative experiment. Superimposed are fluorograms with anti-J2 antibody (solid line) and control immunoglobulin (filled curve). Bar graph on the right represents summary of multiple independent experiments ($n = 4$). Numbers indicate average values of normalized median fluorescence intensity (*MFI) of J2 expression relative to IgG controls (*MFI = MFI J2 - MFI IgG1). * Difference between populations is statistically significant: TNF- α at 12 hours vs 0 hours $p \leq 0.01$. (B) RNAs obtained from BMEC samples stimulated with TNF- α (10 ng/mL) were used for reverse transcription polymerase chain reaction amplification of J2 and G α . (C) Primary BM-ECs were stimulated with TNF- α (10 ng/mL), harvested and labeled with anti-J2 antibody. Histograms show intensity of J2 expression. Superimposed are fluorograms with anti-J2 antibody on cells stimulated with TNF- α for 24 hours (solid lines) and 48 hours (dotted lines) and on cells not stimulated (basal expression, solid filled curve). (D) BMEC stimulated by TNF- α (10 ng/mL) were harvested at the indicated time points and labeled with anti-CD54 (intracellular adhesion molecule-1 [ICAM-1]) monoclonal antibody. Histogram shows intensity of ICAM-1 expression. Superimposed are fluorograms with anti-ICAM-1 on cells stimulated with TNF- α 6 hours (solid thin line), 12 hours (overlap with the 6 hours) and 24 hours (dotted lines), and on cells not stimulated (solid thick line) and immunoglobulins control (solid filled curve). (E) The line graph shows a representative case of three (BM-EC1) stimulated with interleukin (IL)-1 β (10 ng/mL) or with TNF- α (10 ng/mL) for 3 days. Values in the graph represent values of normalized J2- *MFI during time.

(Fig. 6A and B). In both, wild-type and Tie2-tmTNF- α expression of J2 was specific for CD45⁻CD31⁺FLK1⁺ cells and was absent in the CD45⁻CD31⁺FLK1⁻ population (Fig. 6A). Similarly to what observed during recombinant TNF- α injection, increased levels of J2 on Tie2-tmTNF- α BM-ECs were associated with increased expression of Notch^{ic} on Lin⁻Sca⁺Kit⁺ progenitors (Fig. 6C). As physiologic

Notch signaling can be activated only by its cognate ligand, these results suggest a direct link between augmented J2 on BMEC cells and Notch activation on hematopoietic progenitors. Finally, given the robust expression of J2 in the BM endothelial cells of Tie2-tmTNF- α transgenic animals (Fig. 6A and B), we utilized this model to demonstrate endothelial J2 expression in the BM based on its colocalization

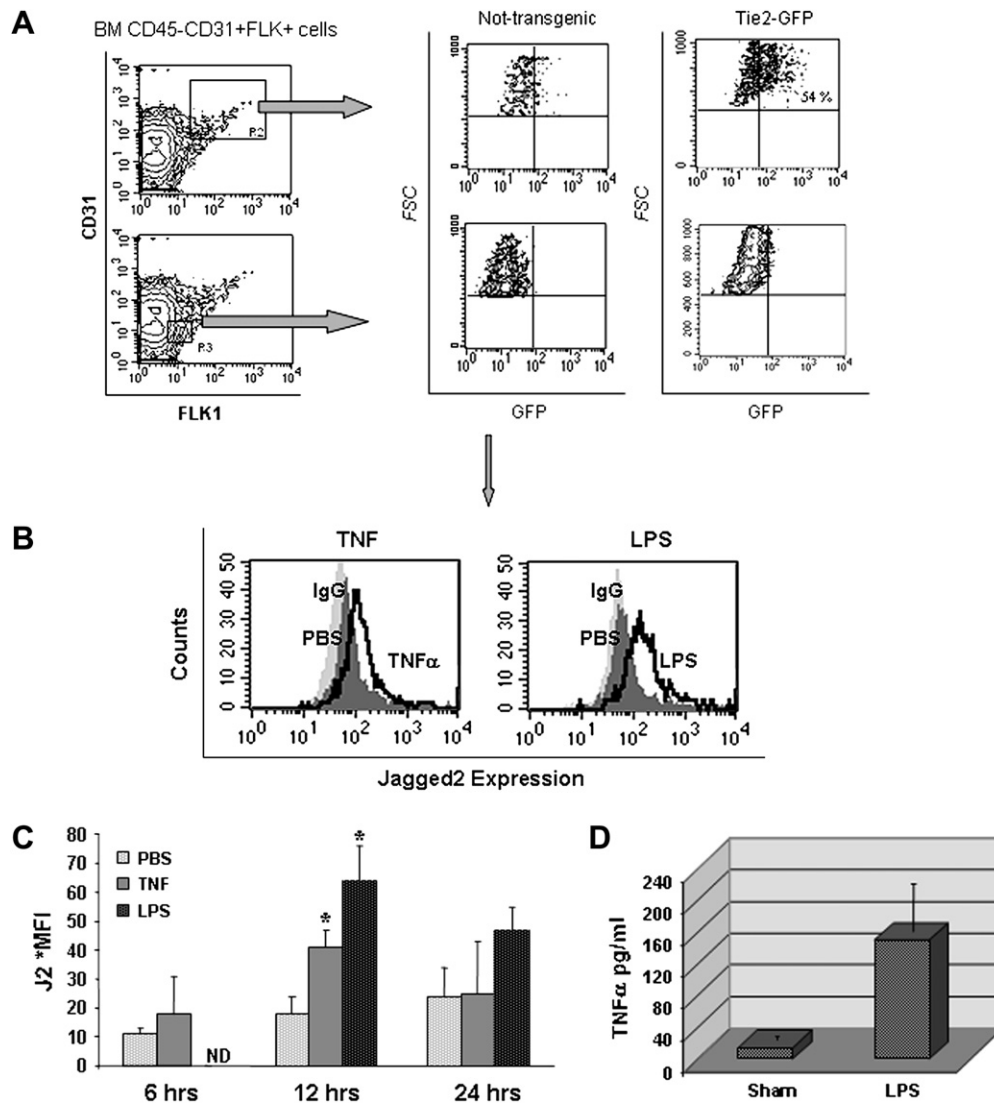


Figure 4. TNF- α and LPS upregulate J2 expression on BM endothelial cells in vivo. (A) Identification of endothelial cell population in the BM. BM cells from TIE2-GFP and not transgenic control mice were labeled with antibody directed to CD45, CD31, and FLK1. Dot blots on the left show expression of CD31 and FLK1 on gated CD45-negative cells (R2). Dot blots on the right show autofluorescence in the FL-1 channel and TIE2 promoter-driven GFP on gated CD45⁻CD31⁺FLK1⁺ population from a not transgenic mouse and a TIE2-GFP reporter mouse, respectively. CD31⁺FLK1⁻ cells (R3, bottom dot blot) were used as internal negative control for GFP. (B) Histograms show intensity of J2 expression on BM endothelial cells derived from mice injected with TNF- α (10 μ g) or LPS (500 μ g) at 12 hours from injection compared to phosphate-buffered saline (PBS). Superimposed are fluorograms with IgG control (IgG, solid filled curve, light gray) and anti-J2 antibody on cells derived from mice control treated with PBS (solid filled curve, dark gray) or stimulated with TNF- α for 12 hours (solid line, black). (C) Bar graph represents summary of J2 upregulation by TNF- α or LPS over time in multiple independent experiments ($n = 4$). Numbers indicate average of normalized median fluorescence intensity (MFI) (*MFI) values for J2 expression on BM endothelial cells. Error bars represent standard deviation. * Difference between populations is statistically significant: TNF- α vs PBS, $p = 0.03$; LPS vs PBS, $p < 0.01$ (PBS, $n = 10$; TNF, $n = 9$; LPS, $n = 5$). (D) Bar graph represents average values of TNF- α (pg/mL) in the serum of control mice or mice injected with LPS, at 12 hours. Serum was collected from three animals/group and triplicate samples for each mouse were analyzed by enzyme-linked immunosorbent assay. Error bars represent standard deviation. *Difference between populations is statistically significant: $p = 0.0012$.

with VE-Cadherin/ Flk1 expression by confocal microscopy (Fig. 6D).

Discussion

The results described here provide the first demonstration that primary BM endothelial cells express the Jagged family of Notch ligands and that such expression can be regulated by

TNF- α and LPS in the bone marrow microenvironment in vivo. Furthermore, we showed that the distinct ability of BM endothelial cells to support hematopoietic multipotential progenitor cells in vitro is not exclusively related to cytokines production, but is also mediated by Notch. Our results demonstrate that BM endothelial cells express the Notch ligand J2 and promote a greater expansion of hematopoietic progenitor cells through a Notch-mediated mechanism.

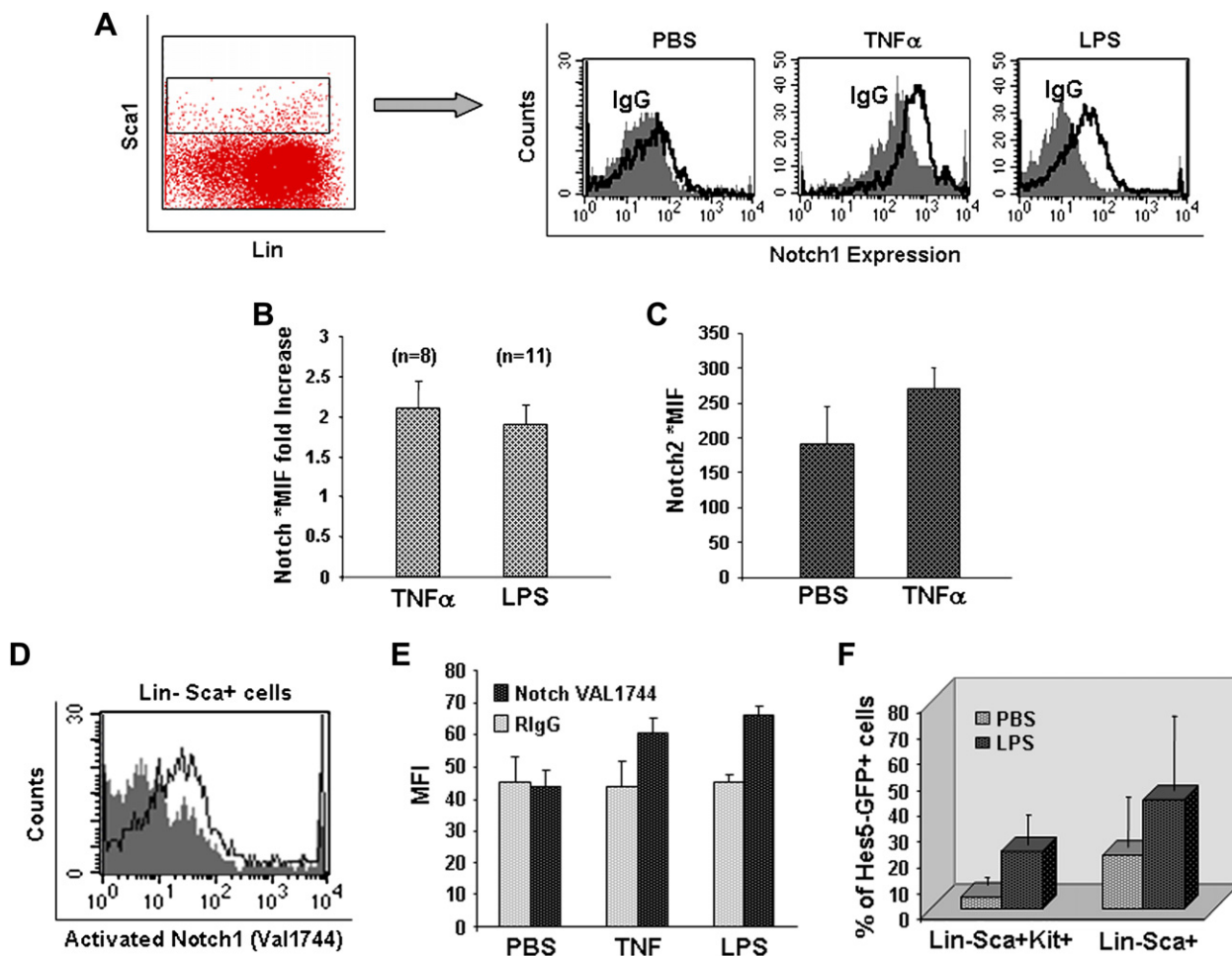


Figure 5. TNF- α and LPS modulate N1 expression and activation on BM progenitors in vivo. (A–F) BM cells were harvested from mice inoculated with PBS, TNF- α (10 μ g), or LPS (500 μ g) at 12 hours from injection and were labeled with antibodies directed to lineage markers (Lin), Sca1, c-Kit and than with anti-N1 antibody or antiN1Val1744. (A) Histograms show N1 expression on gated Lin⁻Sca1⁺ cells, as shown in the dot blot on the left panel. Superimposed are fluorograms with IgG control (solid filled curve) and with anti-N1 antibody (solid line, black) on cells derived from mice inoculated with PBS (controls) or stimulated with either TNF- α or LPS. (B) Bar graph represents summary of N1 expression on Lin⁻Sca1⁺ cells in multiple independent experiments (n = 4). Values represent average of fold increase of N1 expression (*MFI) in mice that showed upregulation. Number in parenthesis indicates the total number of mice analyzed. Error bars represent standard deviation. To avoid bias due to random variation of fluorescence of intensity, each median fluorescence intensity (MFI) value for N1 was normalized toward its IgG control (*MFI); in each experiment, the ratio between N1 *MFI in animals challenged with TNF- α or LPS and N1 *MFI in mice that received PBS was calculated and expressed as fold difference. (C) Bar graph represents summary of N2 expression on Lin⁻Sca1⁺ cells. Values represent average of *MFI in control or TNF- α challenged mice (n = 4). Error bars represent standard deviation. (D) Lin⁻Sca1⁺ gated BM cells were analyzed for expression of activated N1 using the monoclonal antibody against N1^{ic} (Val 1744). Superimposed are fluorograms with N1^{ic} (Val 1744) antibody on cells harvested from TNF- α -stimulated (solid line) or control mice (filled curve). (E) Bar graph represents summary of N1^{ic} (Val 1744) expression on Lin⁻Sca1⁺ cells in independent experiments (n = 4). For each condition, values represent average of mean intensity of fluorescence of samples labeled with IgG control (gray bars) or with N Val1744 antibody (black bars). (F) Hes5-GFP transgenic mice were injected with PBS or LPS. BM cells were harvested 12 hours after injection, labeled with the indicated antibodies (x-axis) and analyzed by flow cytometry for expression of GFP. Graph shows average percent GFP expression in Lin⁻Sca1⁺ Kit⁺ and Lin⁻Sca1⁺ subsets in LPS stimulated and in control mice. Error bars represent standard deviation.

It is known that Notch signaling by itself can not bypass growth factor requirement, particularly in primary cells, and necessitates the presence of serum or cytokines to manifest its effects [22]. Indeed, there is substantial evidence that Notch signaling and hematopoietic cytokines synergize in promoting multipotential progenitor expansion. Independent studies have demonstrated synergism between Notch signaling and various hematopoietic cytokines (i.e., SCF, IL-6, IL-11, FLT-3L, IL-7, IL-3, and thrombopoietin) in

promoting multipotential progenitor expansion [44,45]. BMEC cells express hematopoietic cytokines (GM-CSF, SCF, IL-6) [25] and express Notch ligands. These two functions could be distinguished in vitro through inhibition of Notch-Jagged binding by Notch antagonists, such as the γ -secretase inhibitor, soluble Dll4-Fc or anti-Notch monoclonal antibody, all of which significantly decreased the capability of BMEC to promote CD34⁺ cell colony-forming ability and revealed the role of cytokines alone.

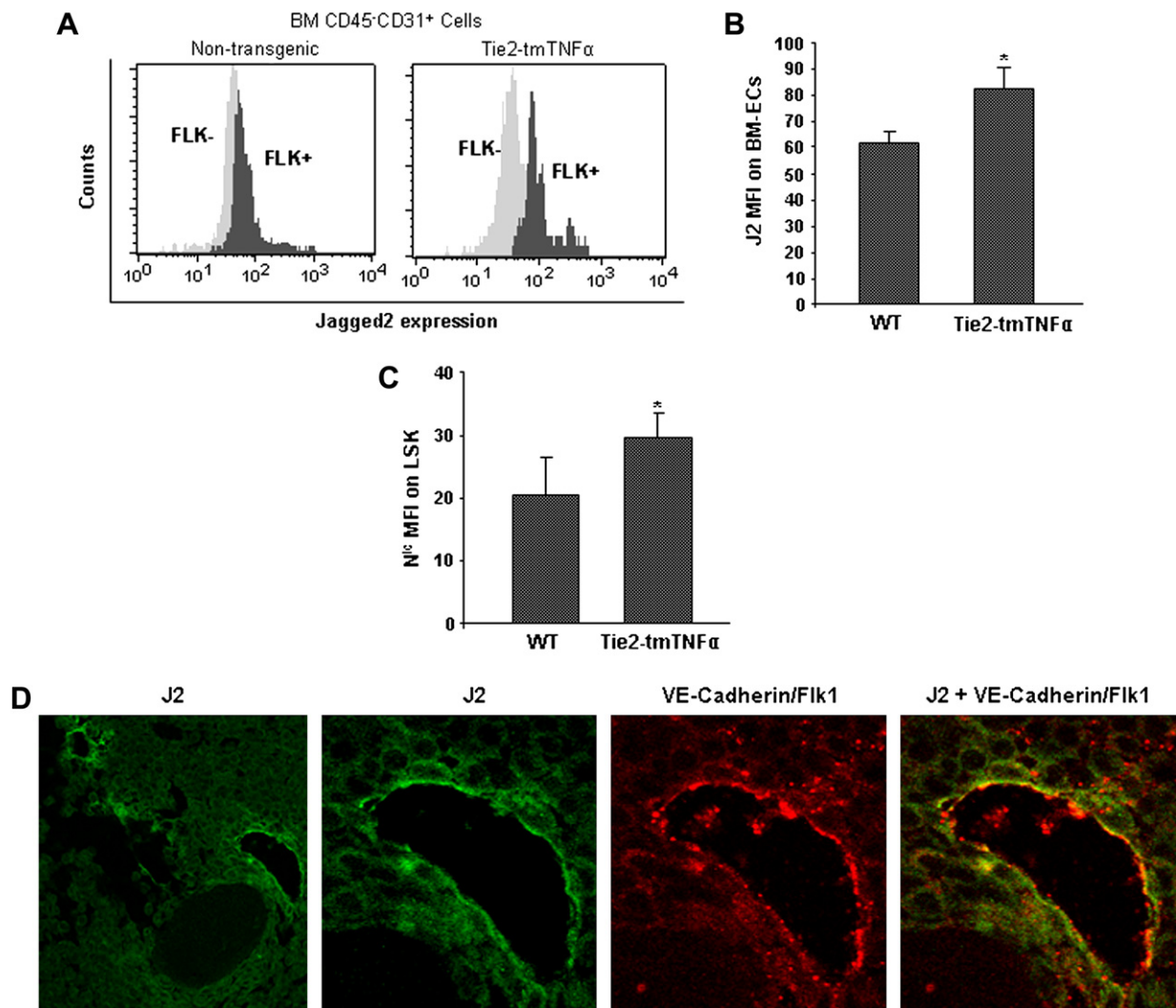


Figure 6. J2 expression and Notch signaling are upregulated in Tie2-tm-TNF- α mice bone marrow. Bone marrow (BM) cells from Tie2-tmTNF- α and control, not transgenic, mice were labeled with antibody combination directed to CD45, CD31 and Flk1, and directed to lineage markers (Lin), Sca1, c-Kit. (A) J2 expression on CD45⁻CD31⁺Flk1⁺ BM cells. CD45/CD31/Flk1 staining was followed by intracytoplasmic labeling of J2. Histograms show intensity of J2 expression on gated populations of nontransgenic and Tie2-tmTNF- α mice. Superimposed are fluorograms with anti-J2 antibody on CD45⁻CD31⁺Flk1⁺ cells (solid filled curve, dark gray) and anti-J2 antibody on CD45⁻CD31⁺Flk1⁻ cells (solid filled curve, light gray); anti-J2 antibody fluorescence on CD45⁻CD31⁺Flk1⁻ coincided with IgG controls (not shown). (B) Bar graph represents summary of J2 upregulation in CD45⁻CD31⁺Flk1⁺ cells from non-transgenic and Tie2-tmTNF- α mice ($n = 6$). Numbers indicate average of normalized median fluorescence intensity (*MFI) values for J2 expression on BM endothelial cells. Error bars represent standard deviation. *Difference between populations is statistically significant: $p < 0.01$. (C) Notch^{1c} expression on LSK cells. Lin⁻Sca1⁺c-Kit⁺ staining was followed by intracytoplasmic labeling of cleaved Notch using the monoclonal antibody against N1^{1c}. Bar graph represents summary of Notch^{1c} upregulation in Lin⁻Sca1⁺c-Kit⁺ (LSK) cells from nontransgenic and Tie2-tmTNF- α mice ($n = 6$). Numbers indicate average of normalized MFI (*MFI) values for Notch^{1c} expression on LSK cells. Error bars represent standard deviation. *Difference between populations is statistically significant: $p = 0.01$. (D) Colocalization of J2 and endothelial markers on endothelial cells of BM microvasculature. Images show a microvessel within the BM of a Tie2-tmTNF- α mouse. Fixed and decalcified BM sections were stained with rabbit anti-J2 and a mixture of goat anti-VE-cadherin and goat anti-Flk1 antibodies. Donkey anti-rabbit antibody conjugated with Alexa 488 and donkey anti-goat antibody conjugated with Alexa 647 were used as secondary antibody. BM sections were imaged by an Olympus FluoView IX2 confocal microscope. Images show 40 \times magnification of the BM section stained with J2 antibody (green; far left panel); 80 \times magnification of BM section with J2 (second panel, green) and VE-Cadherin+Flk1 staining (red; third panel), merging in an endothelial cell lining in a BM microvessel (yellow; far right panel).

We have also demonstrated that the efficiency of BMEC cells in supporting hematopoietic progenitors increases with the increased expression of J2. As shown by a recent study, Notch-ligand density upregulation by as much as twofold has quantitative and qualitative impact on hematopoietic progenitor cell differentiation and proliferation [46].

Thus, it is relevant to investigate whether microenvironmental cues could regulate Notch ligands expression on endothelial cells. We found that known regulators of EC activation, such as TNF- α , and to a lesser extent IL-1 β , upregulate J2 expression on BMECs. In this perspective, activation of BMEC by TNF- α has a potent effect, resulting

both in the induction of hematopoietic cytokines [4–6], as well as in the upregulation of Notch ligands, which together can promote progenitor expansion more efficiently than cytokines alone [44,45]. Furthermore, we provide evidence that J2 expression on BM endothelial cells can be upregulated three- to fourfold by TNF- α and LPS in vivo. Overall, our findings link for the first time Notch, an intrinsic regulator of hematopoiesis, with TNF- α , a critical regulator of inflammation, and suggest a partnership between Notch signaling and BM endothelial cells in the regulation of hematopoiesis during inflammation.

A role for Notch in inflammation is supported by previous studies showing the interplay between Notch signaling and the TNF- α /nuclear factor κ B (NF κ B) pathway. Overexpression of the NF κ B members c-Rel or p65 has been shown to induce J1 expression on HELA cells, whereas a NF κ B dominant negative form suppresses it [47]. Similarly, another group reported the upregulation of N1, N4, and J2 by TNF- α on rheumatoid synovial fibroblasts [48]. On the other hand, numerous in vitro and in vivo studies have demonstrated the ability of Notch to expand stem/multipotential progenitors [49]. Recently, Calvi et al. [24] have shown that osteoblasts within the osteoblastic stem cell niche support hematopoietic stem cells through expression of the Notch ligand J1. The observation that BM endothelial cells express the Notch ligands J1 and J2 and are also in tight contact with hematopoietic stem/progenitor cells [12] poses the possibility that multiple stem cell niches exist in the BM, sensing different microenvironmental cues and providing different functions. Because BM endothelial cells are finely regulated by inflammatory stimuli, our results suggest the potential role of the “endothelial stem cell niche” to regulate stem/progenitor cells homeostasis during condition of inflammatory stress. Stimulation of endogenous Notch with the physiologic ligands delays myeloid differentiation and preserves hematopoietic progenitors in a more immature phenotype [18,19,21,50,51]; moreover, activation of Notch in murine stem cells promotes stem cell expansion in vivo [52]. However, conditional knockout mice of N1, N2, or J1 do not exhibit significant alterations in the stem cells and progenitor compartment [53,54], suggesting compensatory mechanisms by other Notch receptors and Notch ligands, and perhaps a limited role of Notch signaling in stem cell regulation at steady-state conditions. Indeed, many studies have shown that in vitro exposure of hematopoietic stem/progenitor cells to Notch ligands promotes their expansion while opposing their differentiation [18–21], indicating that hyperactivation of Notch signaling may play a physiologic role in response to conditions of stress.

The physiologic relevance of our findings is confirmed by the demonstration that TNF- α or LPS, which is a major trigger to bacterial response, upregulate J2 expression on BM endothelial cells in vivo and correlate with increased Notch activation. Of note, J2 upregulation appeared to be

more robust when induced by LPS than when induced by TNF- α alone. One explanation for this effect is that LPS stimulation may induce a more constant release of TNF- α in vivo or/and that Toll-like receptor 4 activation by LPS may directly contribute to J2 regulation, as shown recently for the induction of Dll4 by LPS in macrophages [55]; however, further studies are required to assess the distinct role of Toll receptors in modulating Notch signaling in hematopoietic cells during infections. In response to inflammatory stimuli, Notch1 and Notch2 expression is increased on hematopoietic cells, likely due to a direct effect of TNF- α [48]. Expression of Notch receptors does not correlate necessarily with their activation status, which is dependent, instead, on the presence and abundance of the ligand on neighboring cells. Thus, our observation that J2 upregulation on BMECs is associated with the increase of Notch activation on hematopoietic progenitor cells following TNF- α or LPS stimulation is most relevant. Because TNF- α and LPS do not have a direct effect on Notch-receptor intracytoplasmic cleavage and activation, which can occur only upon binding of Notch to its ligand on a neighboring cells, our data suggest the existence of endothelial-hematopoietic cell-to-cell interaction similar to what we have observed in the coculture model in vitro. This notion is further supported by analysis of the Tie2-tmTNF- α mice. In these mice, endothelial cells exhibit a chronic activated phenotype as result of endothelial-specific constitutive expression of the TNF- α membrane-bound form [29,30], and not in response to exogenous TNF- α . As a result of endothelial cell activation, we found that J2 expression is upregulated in BM-ECs and that it correlates with increased Notch activation on Lin⁻Sca⁺Kit⁺ hematopoietic progenitors.

Increases of Jagged may have an impact on endothelial cells themselves following TNF- α stimulation. Studies conducted during mouse development showed an inhibitory effect of Dll4 on arterial formation [56]. We have investigated the effects of increased Notch activation on BMEC cells by overexpressing the Notch ligands (Carlesso, unpublished observations). Although ligand overexpression increased Notch signaling in BMEC cells and resulted in some alteration of their proliferation, we did not observe inhibitory effects on cocultured hematopoietic cells, but rather stimulatory effects as the ligand density on the BMEC surface was higher. Further in vivo studies are necessary to determine the vascular biology of adult BM endothelial cells during inflammation.

Overall, our data suggest that conditions associated with TNF- α release and endothelial cell activation, such as infection and inflammation, result in increased Notch-ligand density on BM-EC surface, and in the increase of Notch signaling on hematopoietic progenitors. Given the proximity of endothelium and hematopoietic progenitors in the BM, it is conceivable that the endothelial niche plays a role in hematopoietic progenitor activation and expansion in vivo, in particular under conditions of inflammatory

stress. The *in vitro* coculture experiments of BMEC and hematopoietic cells and the *in vivo* studies demonstrating selective localization of hematopoietic stem cells to the BM endothelium [14] support this view; however, additional studies employing genetic models and *in vivo* imaging will be necessary to demonstrate *in vivo* specific Notch receptor-ligand interactions between BM endothelium and stem cells.

In conclusion, we propose a working model in which the BM endothelium represents a functional niche capable of regulating hematopoietic progenitor homeostasis in response to inflammatory stimuli through a mechanism that involves Notch signaling.

Acknowledgments

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References

- Suda T, Arai F, Hirao A. Hematopoietic stem cells and their niche. *Trends Immunol.* 2005;26:426–433.
- Rafii S, Shapiro F, Pettengell R, et al. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood.* 1995;86:3353–3363.
- Cines DB, Pollak ES, Buck CA, et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood.* 1998;91:3527–3561.
- Sieff CA, Tsai S, Faller DV. Interleukin 1 induces cultured human endothelial cell production of granulocyte-macrophage colony-stimulating factor. *J Clin Invest.* 1987;79:48–51.
- Warner SJ, Auger KR, Libby P. Human interleukin 1 induces interleukin 1 gene expression in human vascular smooth muscle cells. *J Exp Med.* 1987;165:1316–1331.
- Broudy VC, Kaushansky K, Segal GM, Harlan JM, Adamson JW. Tumor necrosis factor type alpha stimulates human endothelial cells to produce granulocyte/macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A.* 1986;83:7467–7471.
- Zambidis ET, Oberlin E, Tavian M, Peault B. Blood-forming endothelium in human ontogeny: lessons from *in utero* development and embryonic stem cell culture. *Trends Cardiovasc Med.* 2006;16:95–101.
- Tavian M, Coulombel L, Luton D, Clemente HS, Dieterlen-Lievre F, Peault B. Aorta-associated CD34+ hematopoietic cells in the early human embryo. *Blood.* 1996;87:67–72.
- Rafii S, Mohle R, Shapiro F, Frey BM, Moore MA. Regulation of hematopoiesis by microvascular endothelium. *Leuk Lymphoma.* 1997;27:375–386.
- Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med.* 2004;10:64–71.
- Mohle R, Moore MA, Nachman RL, Rafii S. Transendothelial migration of CD34+ and mature hematopoietic cells: an *in vitro* study using a human bone marrow endothelial cell line. *Blood.* 1997;89:72–80.
- Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell.* 2005;121:1109–1121.
- Moore KA, Lemischka IR. Stem cells and their niches. *Science.* 2006;311:1880–1885.
- Sipkins DA, Wei X, Wu JW, et al. *In vivo* imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature.* 2005;435:969–973.
- Li W, Johnson SA, Shelley WC, Yoder MC. Hematopoietic stem cell repopulating ability can be maintained *in vitro* by some primary endothelial cells. *Exp Hematol.* 2004;32:1226–1237.
- Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science.* 1999;284:770–776.
- Milner LA, Bigas A. Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood.* 1999;93:2431–2448.
- Han W, Ye Q, Moore MA. A soluble form of human Delta-like-1 inhibits differentiation of hematopoietic progenitor cells. *Blood.* 2000;95:1616–1625.
- Karanu FN, Murdoch B, Miyabayashi T, et al. Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood.* 2001;97:1960–1967.
- Varnum-Finney B, Purton LE, Yu M, et al. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood.* 1998;91:4084–4091.
- Carlesso N, Aster JC, Sklar J, Scadden DT. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood.* 1999;93:838–848.
- Sarmiento LM, Huang H, Limon A, et al. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27Kip1 degradation. *J Exp Med.* 2005;202:157–168.
- Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature.* 2003;425:836–841.
- Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature.* 2003;425:841–846.
- Almeida-Porada G, Ascensao JL. Isolation, characterization, and biologic features of bone marrow endothelial cells. *J Lab Clin Med.* 1996;128:399–407.
- de Wynter E, Allen T, Coutinho L, Flavell D, Flavell SU, Dexter TM. Localisation of granulocyte macrophage colony-stimulating factor in human long-term bone marrow cultures. Biological and immunocytochemical characterisation. *J Cell Sci.* 1993;106(Pt 3):761–769.
- Garces C, Ruiz-Hidalgo MJ, Font de Mora J, et al. Notch-1 controls the expression of fatty acid-activated transcription factors and is required for adipogenesis. *J Biol Chem.* 1997;272:29729–29734.
- Zlobin A, Jang M, Miele L. Toward the rational design of cell fate modifiers: notch signaling as a target for novel biopharmaceuticals. *Curr Pharm Biotechnol.* 2000;1:83–106.
- Rajashekhar G, Willuweit A, Patterson CE, et al. Continuous endothelial cell activation increases angiogenesis: evidence for the direct role of endothelium linking angiogenesis and inflammation. *J Vasc Res.* 2006;43:193–204.
- Willuweit A, Sass G, Schoneberg A, Eisel U, Tiegs G, Clauss M. Chronic inflammation and protection from acute hepatitis in transgenic mice expressing TNF in endothelial cells. *J Immunol.* 2001;167:3944–3952.
- Luo B, Aster JC, Hasserjian RP, Kuo F, Sklar J. Isolation and functional analysis of a cDNA for human Jagged2, a gene encoding a ligand for the Notch1 receptor. *Mol Cell Biol.* 1997;17:6057–6067.
- Hasserjian RP, Aster JC, Davi F, Weinberg DS, Sklar J. Modulated expression of notch1 during thymocyte development. *Blood.* 1996;88:970–976.

33. Classon M, Salama S, Gorka C, Mulloy R, Braun P, Harlow E. Combinatorial roles for pRB, p107, and p130 in E2F-mediated cell cycle control. *Proc Natl Acad Sci U S A*. 2000;97:10820–10825.
34. Costa LF, Balcells M, Edelman ER, Nadler LM, Cardoso AA. Proangiogenic stimulation of bone marrow endothelium engages mTOR and is inhibited by simultaneous blockade of mTOR and NF-kappaB. *Blood*. 2006;107:285–292.
35. Veiga JP, Costa LF, Sallan SE, Nadler LM, Cardoso AA. Leukemia-stimulated bone marrow endothelium promotes leukemia cell survival. *Exp Hematol*. 2006;34:610–621.
36. Berezovska O, Jack C, McLean P, et al. Aspartate mutations in presenilin and gamma-secretase inhibitors both impair notch1 proteolysis and nuclear translocation with relative preservation of notch1 signaling. *J Neurochem*. 2000;75:583–593.
37. Conboy IM, Conboy MJ, Smythe GM, Rando TA. Notch-mediated restoration of regenerative potential to aged muscle. *Science*. 2003;302:1575–1577.
38. Shimizu K, Chiba S, Saito T, et al. Integrity of intracellular domain of Notch ligand is indispensable for cleavage required for release of the Notch2 intracellular domain. *EMBO J*. 2002;21:294–302.
39. Sun H, Li L, Vercherat C, et al. Stra13 regulates satellite cell activation by antagonizing Notch signaling. *J Cell Biol*. 2007;177:647–657.
40. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res*. 2004;6:R605–R615.
41. Motoike T, Loughna S, Perens E, et al. Universal GFP reporter for the study of vascular development. *Genesis*. 2000;28:75–81.
42. Rietschel ET, Kirikae T, Schade FU, et al. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J*. 1994;8:217–225.
43. Pissarra L, Henrique D, Duarte A. Expression of hes6, a new member of the Hairy/Enhancer-of-split family, in mouse development. *Mech Dev*. 2000;95:275–278.
44. Varnum-Finney B, Brashem-Stein C, Bernstein ID. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood*. 2003;101:1784–1789.
45. Suzuki T, Yokoyama Y, Kumano K, et al. Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein. *Stem Cells*. 2006;24:2456–2465.
46. Dallas MH, Varnum-Finney B, Delaney C, Kato K, Bernstein ID. Density of the Notch ligand Delta1 determines generation of B and T cell precursors from hematopoietic stem cells. *J Exp Med*. 2005;201:1361–1366.
47. Bash J, Zong WX, Banga S, et al. Rel/NF-kappaB can trigger the Notch signaling pathway by inducing the expression of Jagged1, a ligand for Notch receptors. *EMBO J*. 1999;18:2803–2811.
48. Ando K, Kanazawa S, Tetsuka T, et al. Induction of Notch signaling by tumor necrosis factor in rheumatoid synovial fibroblasts. *Oncogene*. 2003;22:7796–7803.
49. Ohishi K, Katayama N, Shiku H, Varnum-Finney B, Bernstein ID. Notch signalling in hematopoiesis. *Semin Cell Dev Biol*. 2003;14:143–150.
50. Ohishi K, Varnum-Finney B, Bernstein ID. Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. *J Clin Invest*. 2002;110:1165–1174.
51. Varnum-Finney B, Wu L, Yu M, et al. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J Cell Sci*. 2000;113 Pt 23:4313–4318.
52. Stier S, Cheng T, Dombkowski D, Carlesso N, Scadden DT. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood*. 2002;99:2369–2378.
53. Mancini SJ, Mantei N, Dumortier A, Suter U, Macdonald HR, Radtke F. Jagged1 dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood*. 2005;105:2340–2342.
54. Saito T, Chiba S, Ichikawa M, et al. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity*. 2003;18:675–685.
55. Fung E, Tang SM, Canner JP, et al. Delta-like 4 induces notch signaling in macrophages: implications for inflammation. *Circulation*. 2007;115:2948–2956.
56. Duarte A, Hirashima M, Benedito R, et al. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev*. 2004;18:2474–2478.

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.exphem.2007.12.012](https://doi.org/10.1016/j.exphem.2007.12.012).

Table 1. RT-PCR Primer sequences

Gene name	Primers	Production length (bp)	Genebank Seq#
Jaggad1	Forward: 5'-tgaccagaatggcaacaaaa-3' Reverse: 5'-gttggctcctgaatacccct-3'	361	NM_000214
Jaggad2	Forward: 5'-tctctgtgaggtggatgctcg-3' Reverse: 5'-ggcagtcgtcaatgttctca-3'	329	NM_002226
Delta-like1	Forward: 5'-ttgctgtcaggtctggag-3' Reverse: 5'-ttctgttcgaggtcatcag-3'	372	NM_005618
Delta-like4	Forward: 5'-gaggcagctgtaaggaccag-3' Reverse: 5'-acagtaggtcccgtgaatc-3'	305	NM_019074
Hes1	Forward: 5'-ccaaagacagcatctgagca-3' Reverse: 5'-cattgatctgggtcatgcag-3'	373	NM_005524
GS-alpha	Forward: 5'-gctgctggccaccacgaagatgat-3' Reverse: 5'-gtgatcaagcaggctgactatgtg-3'	200	