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# IFNα-mediated remodeling of endothelial cells in the bone marrow niche

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EUROPEAN HEMATOLOGY ASSOCIATION

Ferrata Storti Foundation

**Haematologica** 2017
Volume 102(3):445-453

## ABSTRACT

In the bone marrow, endothelial cells are a major component of the hematopoietic stem cell vascular niche and are a first line of defense against inflammatory stress and infection. The primary response of an organism to infection involves the synthesis of immune-modulatory cytokines, including interferon alpha. In the bone marrow, interferon alpha induces rapid cell cycle entry of hematopoietic stem cells *in vivo*. However, the effect of interferon alpha on bone marrow endothelial cells has not been described. Here, we demonstrate that acute interferon alpha treatment leads to rapid stimulation of bone marrow endothelial cells *in vivo*, resulting in increased bone marrow vascularity and vascular leakage. We find that activation of bone marrow endothelial cells involves the expression of key inflammatory and endothelial cell-stimulatory markers. This interferon alpha-mediated activation of bone marrow endothelial cells is dependent in part on vascular endothelial growth factor signaling in bone marrow hematopoietic cell types, including hematopoietic stem cells. Thus, this implies a role for hematopoietic stem cells in remodeling of the bone marrow niche *in vivo* following inflammatory stress. These data increase our current understanding of the relationship between hematopoietic stem cells and the bone marrow niche under inflammatory stress and also clarify the response of bone marrow niche endothelial cells to acute interferon alpha treatment *in vivo*.

## Introduction

Tissue vasculature serves as a barrier between sites of inflammation or infection and immune cells.[1] Endothelial cells (ECs) are a diverse cell population which line the vasculature. These cells form a cell monolayer and are interconnected by junction molecules, including VE-cadherin and ESAM. This regulatory monolayer is ensheathed by pericytes and forms a selective, semi-permeable barrier that regulates tissue fluid homeostasis and migration of blood cells through the vessel wall.[2] Thus, ECs are primary responders to inflammation and infection. During an inflammatory response, ECs proliferate in order to maintain vessel integrity.[3] Immune cell loss, as well as interactions between immune cells and ECs, facilitates the emigration of circulating cells across the EC barrier to sites of inflammation. This process can, in turn, lead to EC activation.[4,5] Production of pro-inflammatory factors and the interaction between stimulatory cytokines and chemokines is a critical step in the inflammatory response. Interferon α (IFNα) is one of the most prominent immune-modulatory cytokines which is produced to facilitate the response to inflammation. Vascular endothelial growth factor (VEGF) regulates ECs during both homeostasis and inflammation. VEGF regulation is central to vascular dynamics, promoting EC survival, proliferation and migration.[4,6]

In the bone marrow (BM) microenvironment, the vascular system consists of a network of sinusoids, arterioles, and transition zones. Subtypes of BM vessels are

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Received: June 20, 2016.
Accepted: October 6, 2016.
Pre-published: October 14, 2016.

doi:10.3324/haematol.2016.151209

*Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/3/445*

heterogenous in both properties and functions.[7,8] BM ECs form a critical part of the hematopoietic stem cell (HSC) vascular niche. ECs have well-defined roles in HSC function and maintenance, retaining HSCs in culture and contributing to the creation of HSC niches.[9-13] *In vivo* ablation of ECs leads to hematopoietic failure.[14] In response to infection, hematopoietic cells mediate an altered expression of adherens molecules on the surface of ECs.[13,15] This suggests that HSCs may also directly affect ECs. However, in contrast to the defined roles for ECs on HSCs, the effect of HSCs on ECs in the BM niche remains unclear. In addition, little is known about the influence of inflammatory stress on ECs in the BM, or the interaction between IFNα and VEGF.

The stimulatory effect of IFNα on HSCs *in vivo* is not reflected *in vitro*. *In vitro*, IFNα has an inhibitory effect which leads to inhibition of HSC proliferation.[16] This suggests a role for the BM niche, including ECs, in inflammation-induced HSC stimulation. However, how crosstalk between HSCs and ECs in the BM is regulated under inflammatory stress remains unknown. To understand how inflammatory stress impacts on ECs in the BM niche, we investigated how BM ECs respond to IFNα *in vivo* and how the interaction between HSCs and ECs is regulated. We found that IFNα treatment of mice led to a rapid stimulation of BM ECs *in vivo*. IFNα stimulation of ECs was both direct and indirect. VEGF signaling, mediated by other BM hematopoietic cell types including HSCs, was a central mediator of the observed EC stimulation. This novel communication between activated hematopoietic cells and ECs in the BM suggests an acute 'emergency' response of the BM niche to primary inflammatory signaling from the hematopoietic system.

## Methods

### Animals

Eight- to 12-week old female wild-type (WT) mice [C57Bl/6J (CD45.2), Harlan Laboratories or B6.SJL-Ptprca Pepcb/BoyJ (CD45.1), Charles River Laboratories] and IFNAR$^{-/-}$ mice[17] were maintained in individually ventilated cages in the Deutsches Krebsforschungszentrum animal facility. All animal protocols were approved by the Animal Care and Use Committees of the German Regierungspräsidium Karlsruhe für Tierschutz und Arzneimittelüberwachung.

### In vivo *treatments*

Mice were injected intraperitoneally (i.p.) with PBS, 5 mg/kg polyinosinic-polycytidylic acid (pI:C) (Invitrogen), subcutaneously (s.c) with 5x10$^6$U/kg recombinant mouse IFNα (Miltenyi Biotech) or intravenously (i.v.) with 2.5 mg/kg Avastin (Roche).

### In vivo *vascular labeling*

*In vivo* labeling was carried out as described by Kunisaki *et al.*[18]

### *Evans blue assay*

Evans blue assay was carried out as described by Radu *et al.*[19]

### *Isolation of BM Cells and flow cytometry*

Mice were sacrificed and BM cells were subsequently prepared and analyzed as described in Haas *et al.*[20] In addition, ECs were stained using antibodies against CD4, CD8 CD11b, B220a, Gr-1 and TER119 as indicated,[20] and CD45 (30-F11), CD31 (390), VE-Cadherin (VECD1), VEGFR2 (Avas12), ESAM (1G8) (Biolegend), and Laminin (Sigma). Cells were stained with anti-VEGF antibody according to the manufacturer's instructions (Abcam).

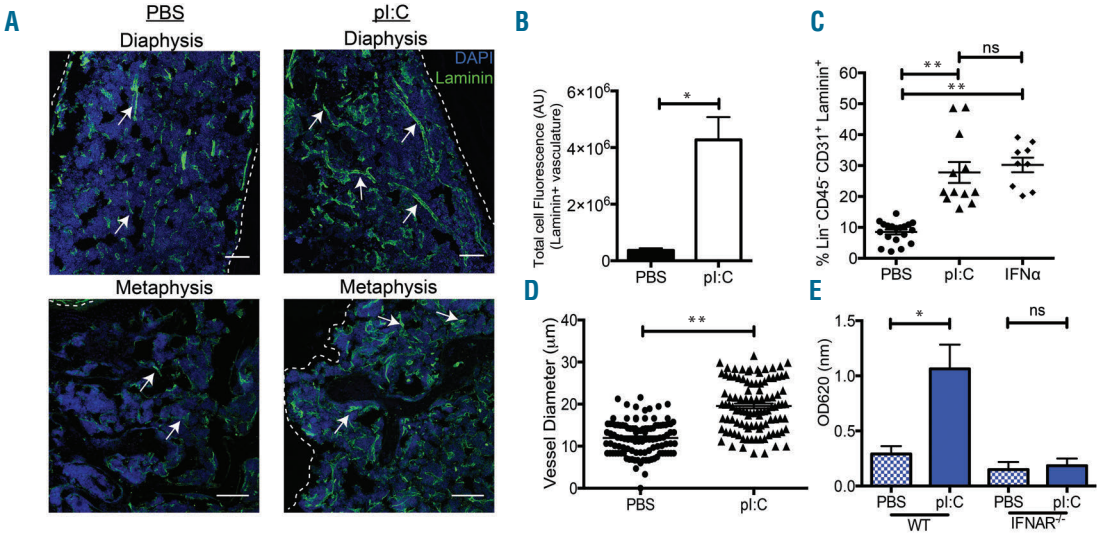


**Figure 1. Interferon α (IFNα) treatment leads to increased bone marrow (BM) vascularity and vascular permeability.** (A) Representative sections of murine femurs, with metaphysis and diaphysis regions indicated, from wild-type (WT) C57Bl/6 mice treated with either PBS or the IFN mimetic, pI:C, (5 mg/kg for 24 h). 8 μm sections of femurs were stained with Laminin (green) and mounted in DAPI containing mountant (blue). Scale bar represents 100 μm. (B) Quantification of Laminin positive vasculature in BM sections. Corrected total cell fluorescence is represented as Arbitrary Units (AU). (C) Laminin expression on ECs (Lin$^-$ CD45$^-$ CD31$^+$) from WT mice treated with either PBS, pI:C (5 mg/kg for 24 h) or IFNα (5x10$^6$U/kg for 24 h) was quantified by flow cytometry. (D) Graph representing the vessel diameter in BM from WT mice treated with either PBS or pI:C (5 mg/kg for 24 h) quantified following *in vivo* labeling with Alexa 633. (E) Evans blue assay to determine vessel leakiness in WT and IFNAR$^{-/-}$ mice treated with PBS (0 h) or pI:C (5 mg/kg for 24 h). Absorbance was measured at 620 nm. Data are representative of 3 or more independent experiments. Data are presented as mean±Standard Error of Mean (SEM) (n≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns: not significant, *$P$<0.001, **$P$<0.0001.

### Vascular endothelial growth factor ELISA

ELISA was carried out on BM supernatant from one crushed tibia and femur per mouse according to the manufacturer's instructions (BD Bioscience).

### BrdU incorporation assay

Mice were injected i.p. with BrdU (18 mg/kg, Sigma) 16 h prior to harvesting the BM. BM cells were stained as described and BrdU staining was carried out using the BD Pharmigen™ BrdU Flow Kit according to the manufacturer's instructions.

### Bone marrow transplantations

$3x10^6$ BM cells were diluted in 200 μl PBS and i.v. injected into lethally irradiated (2x500 rad) WT or IFNAR$^{-/-}$ mice.

### Immunofluorescence of bone sections

8 μm bone sections of frozen femurs were prepared using the Kawamoto tape method.[21] In brief, sections were stained overnight using anti-VEGFR2 (Avas12) and anti-Laminin antibodies, and subsequently with Alexa Fluor 488 secondary antibody (Jackson) for 1 h at room temperature. Images were acquired using an LSM710 microscope and were prepared using FIJI software. Corrected total cell fluorescence was calculated as: integrated density - (area of selected cell x mean fluorescence of background readings).

### Statistical analysis

GraphPad Prism® 6.0 was used for statistical analysis and graphical representation of data. Statistical analysis was per-

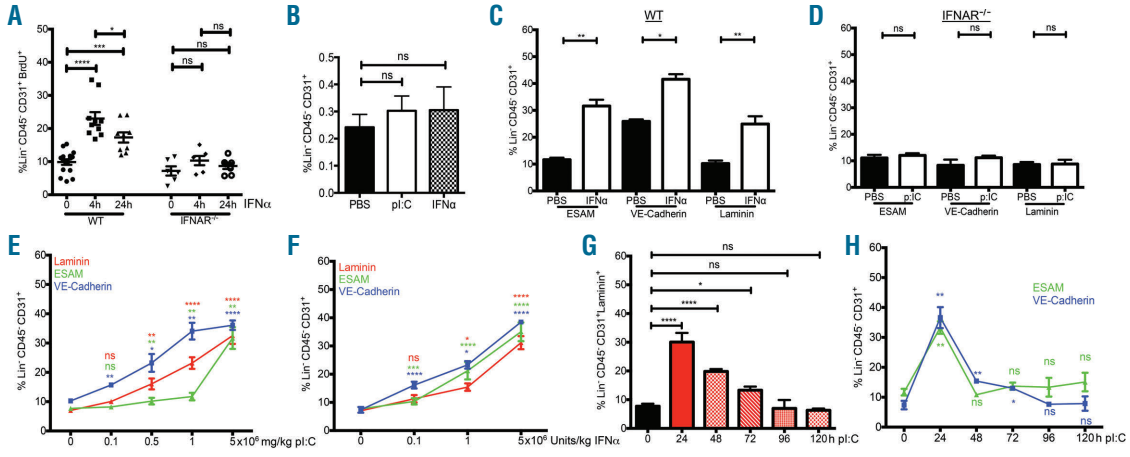


**Figure 2. Bone marrow (BM) endothelial cells (EC) are stimulated following interferon α (IFNα) treatment *in vivo*.** (A) FACS analysis of percentage of BrdU positive ECs (Lin$^-$ CD45$^-$ CD31$^+$) from wild-type (WT) or IFNAR$^{-/-}$ mice treated with either phosphate-buffered saline (PBS) (0 h) or IFNα ($5x10^6$U/kg for up to 24 h) and BrdU (18 mg/kg, 16 h). (B) Percentage of Lin- CD45- CD31$^+$ BM cells in BM from WT mice treated with either PBS, the interferon mimetic, pI:C, (5 mg/kg for 24 h), or IFNα ($5x10^6$U/kg for 24 h). (C and D) FACS analysis of the expression of ESAM, VE-Cadherin or Laminin on ECs (Lin- CD45- CD31$^+$) from (C) WT mice treated with either PBS or IFNα ($5x10^6$U/kg for 24 h) or (D) IFNAR$^{-/-}$ mice treated with either PBS or pI:C (5 mg/kg for 24 h). (E and F) FACS analysis of the expression of ESAM, VE-Cadherin or Laminin on ECs (Lin$^-$ CD45$^-$ CD31$^+$) from WT mice treated with either (E) pI:C (0-5 mg/kg for 24 h) or (F) IFNα (0-$5x10^6$U/kg for 24 h). (G) FACS analysis of the expression of Laminin on ECs (Lin$^-$ CD45$^-$ CD31$^+$) from WT mice treated with either PBS (0 h) or pI:C (5 mg/kg for 0-120 h). (H) FACS analysis of the expression of VE-Cadherin and ESAM on ECs (Lin$^-$ CD45$^-$ CD31$^+$) from WT mice treated with either PBS (0 h) or pI:C (5 mg/kg for 0-120 h). Data are representative of 3 or more (A-C) or 2 or more (D-H) independent experiments. Data are presented as mean±Standard Error of Mean (SEM) (n≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

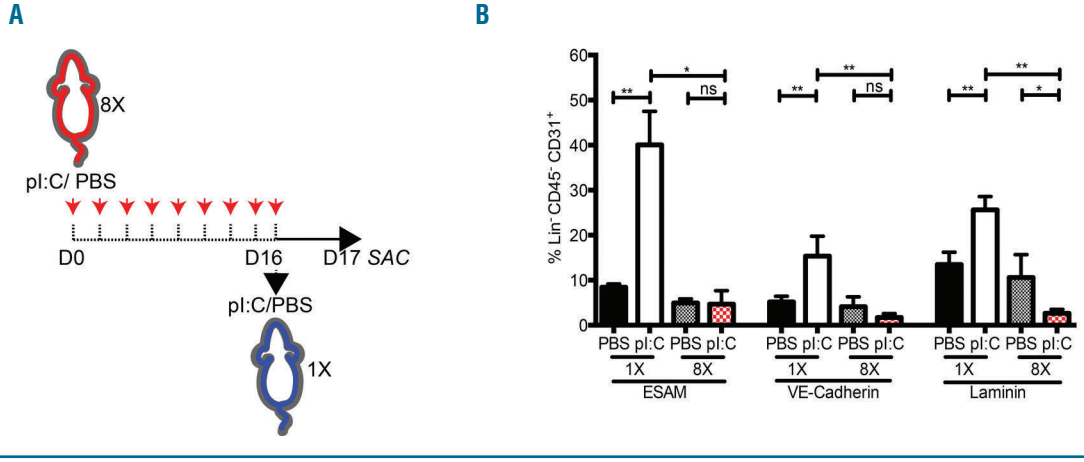


**Figure 3. Bone marrow (BM) endothelial cells (EC) are not activated by multiple rounds of treatment with the interferon mimetic, pI:C.** (A) Experimental design. 1x: treatment with PBS or interferon, pI:C, for 24 h. 8x: treatment with PBS or pI:C every second day for 16 days. Mice were sacrificed 24 h after final treatment (on day 17). (B) FACS analysis of the expression of ESAM, VE-Cadherin and Laminin on ECs (Lin$^-$ CD45$^-$ CD31$^+$) from wild-type (WT) mice treated with either 1 round or 8 rounds of PBS or pI:C (5 mg/kg). (C) Data are representative of 2 or more independent experiments and data are presented as mean±Standard Error of Mean (SEM) (n≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns (not significant), **P*<0.01, ***P*<0.0001

formed using unpaired Student *t*-test (two-tailed). All data are expressed as mean±Standard Error of Mean (SEM) unless otherwise indicated. Statistical significance is indicated in the individual figures.

## Results

### *Acute inflammatory stress induces increased BM vascularity and vessel permeability*

To monitor the response of the BM vasculature to inflammatory stress, we treated WT C57Bl/6 mice with a single dose of the IFNα mimetic, pI:C, to mimic an acute inflammatory response. After 24 h, there was a significant increase in BM vasculature in both the diaphysis and metaphysis regions of the BM in pI:C-treated WT mice in comparison to mice treated with PBS, as visualized and quantified by anti-Laminin staining in frozen BM sections (Figure 1A and B). Increased expression of Laminin on ECs upon injection of either pI:C or IFNα was confirmed by FACS analysis (Figure 1C). To quantify the IFNα-mediated increase in vasculature, BM vessels were directly labeled *in vivo* by i.v. injection of Alexa Fluor 633 phalloidin[18] (Figure 1D). Quantification of BM vessel diameter based on Alexa 633 labeling showed that the BM vasculature became enlarged 24 h following pI:C treatment. The integrity of the BM vasculature was quantified using an Evans blue assay, as previously described.[19] Evans blue staining in the BM of PBS-treated mice showed basal efflux of macromolecules over the EC vasculature under homeostasis (0 h, Figure 1E). However, 24 h after pI:C treatment, BM Evans blue staining increased 2-fold in WT mice, but not in mice lacking the IFNα receptor (IFNAR$^{-/-}$) (Figure 1E). This indicated that increased vessel leakage was the result of IFNα signaling. Taken together, the observed increase in BM vascularity, Laminin expression on ECs and compromised vessel integrity suggests that acute inflammatory signaling stimulates the vasculature within the BM.

### *Acute inflammatory stress induces transient BM EC proliferation and activation* in vivo

To investigate whether the observed vascular expansion was due to an increased activation of ECs, we next analyzed the proliferative and activation status of ECs following IFNα treatment. BrdU incorporation was increased after 4 h in BM ECs (Lin$^-$ CD45$^-$ CD31$^+$) from mice treated with IFNα in comparison to PBS-treated mice (Figure 2A and B). This suggested an increase in cells which were in S-phase of the cell cycle. IFNα treatment of IFNAR$^{-/-}$ mice confirmed that the increased BrdU incorporation was due to IFN signaling. To determine the activation status of BM ECs, we analyzed the expression of the key cellular junction proteins ESAM, VE-cadherin and Laminin.[22] Twenty-four hours after either IFNα or pI:C treatment of mice, expression of ESAM, VE-cadherin and Laminin were up-regulated on the surface of WT BM ECs (Figure 2C) but not on IFNAR$^{-/-}$ BM ECs (Figure 2D). Indeed, increased BM EC activation was detectable even from low-dose treatment. Exposure of mice to low-dose pI:C (0.5 mg/kg) (Figure 2E) or IFNα (0.1 Units/kg) (Figure 2F) led to increased expression of activation markers. These data indicated that BM ECs were activated by IFNα or pI:C treatment in an IFNα-dependent manner, and that BM ECs were activated even in response to low doses of treatment. When mice were allowed to recover after treatment, upregulation of Laminin (Figure 2G), VE-Cadherin

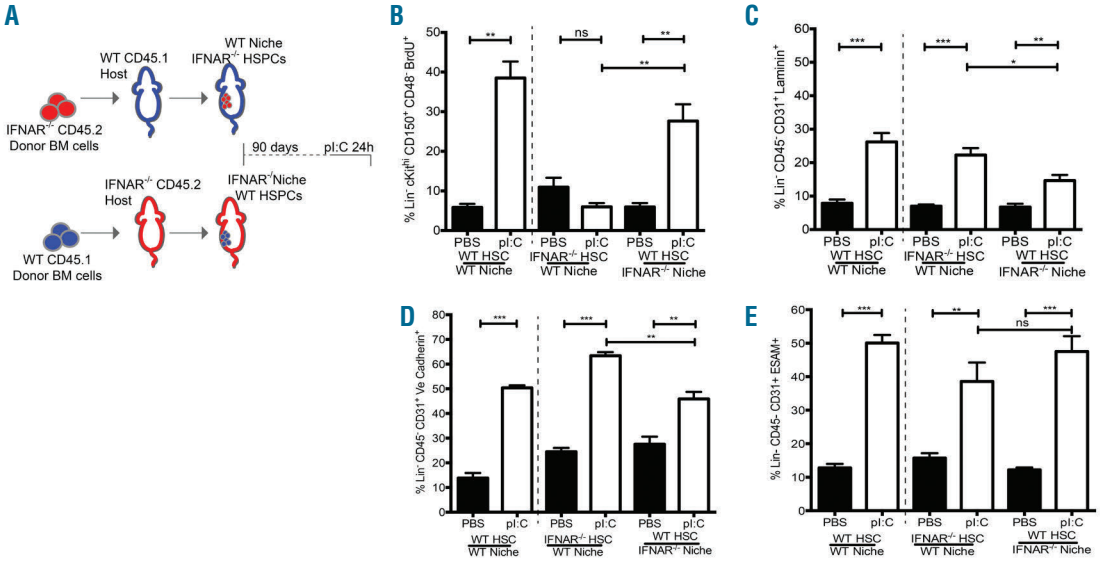


**Figure 4. Bone marrow (BM) endothelial cell (EC) activation can be mediated by the interferon (IFN) mimetic, pI:C, stimulation of hematopoietic or non-hematopoietic cells.** (A) Experimental design: 3x10$^6$ BM cells from either wild-type (WT) (CD45.1) or IFNAR$^{-/-}$ (CD45.2) mice was transplanted into lethally irradiated IIFNAR$^{-/-}$ or WT mice, respectively. Mice were allowed to recover for 90 days (d) prior to treatment with either PBS or pI:C (5 mg/kg for 24 h). (B) FACS analysis of percentage of BrdU positive HSCs (Lin$^-$ ckit$^{hi}$ CD150$^+$CD48$^-$) from chimeric mice, as described in (A) following treatment with either PBS or pI:C (5 mg/kg for 24 h) and BrdU (18 mg/kg, 16 h). (C-E) FACS analysis of the expression of (C) Laminin (D) VE-Cadherin and (E) ESAM on ECs (Lin$^-$ CD45$^-$ CD31$^+$) from chimeric mice, as described in (A) following treatment with either PBS or pI:C (5 mg/kg for 24 h). Data are representative of 3 or more (B) and 2 or more (C-E) independent experiments, and data are presented as mean±Standard Error of Mean (SEM) (n=≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.0001.

and ESAM (Figure 2H) returned to homeostatic levels after 96 h. This indicated that, similar to the response of HSCs,[16] the rapid response of ECs to IFNα treatment is transient. Thus, EC proliferation and activation is modulated following acute IFNα treatment. Proliferation and activation are dependent on expression on the IFNα receptor. Taken together with increased vascularity and compromised BM vessel integrity (Figure 1), EC proliferation and activation indicate enhanced BM vessel remodeling occurs.

To test whether chronic IFNα treatment could lead to an accumulation or an exhaustion of BM EC activation, as previously described,[23] mice were treated with pI:C every second day for a total of 16 days (Figure 3A). In contrast to the increase in activation markers upon 1 injection (1x), BM ECs expressed homeostatic levels of ESAM, VE-Cadherin and Laminin after multiple injections with pI:C (8x) (Figure 3B). Thus, BM ECs were not continually activated by multiple treatments of pI:C. These data are indicative of the contrast in the response of BM ECs, as well as HSCs, to acute and chronic IFNα treatment.[23] This supports the hypothesis of a rapid, acute stimulation of BM ECs following inflammation.

### BM EC stimulation by IFNα occurs via both hematopoietic and non-hematopoietic pathways

IFNα has been reported to have heterogenous effects on ECs.[24-28] We tested whether the observed stimulatory effect of IFNα on BM ECs was directly or indirectly mediated by IFNα.[16] BM chimeras were created where either WT or IFNAR$^{-/-}$ BM cells were transplanted into lethally irradiated IFNAR$^{-/-}$ or WT host mice, respectively. Thus, either the BM (IFNAR$^{-/-}$ BM into a WT niche) or the niche (WT BM into an IFNAR$^{-/-}$ niche) can no longer directly respond directly to IFNα in these mice (Figure 4A). In agreement with our previous data,[16] WT HSCs in recipient IFNAR$^{-/-}$ mice (WT BM into an IFNAR$^{-/-}$ niche) proliferated in response to pI:C treatment; IFNAR$^{-/-}$ HSCs in WT recipient mice (IFNAR$^{-/-}$ BM into a WT niche) did not (Figure 4B). This indicated that the response of HSCs to pI:C was dependent on the expression of the IFNα receptor (IFNAR) on HSCs, not on niche cells. In contrast, Laminin (Figure 4C), VE-Cadherin (Figure 4D) and ESAM (Figure 4F) expression was increased on IFNAR$^{-/-}$ ECs with a WT hematopoietic system present (WT BM into an IFNAR$^{-/-}$ niche) and on WT ECs with a hematopoietic system lacking the IFNα receptor (IFNAR$^{-/-}$ BM into a WT niche). These data indicated that BM ECs can be stimulated by IFNα via a non-hematopoietic effect of IFNα directly on the BM ECs as well as an indirect effect of IFNα via signaling from IFNα-stimulated hematopoietic cells in the BM.

### pI:C treatment induces VEGF production and signaling in the BM

Bone marrow chimera experiments suggested that IFNα-stimulated hematopoietic cells may produce factors which can stimulate BM ECs (Figure 4C and E). Thus, we next tested the activity of known mediators of EC activation in this setting. Platelet activation and VEGF signaling are fundamental mediators of EC activation during inflam-

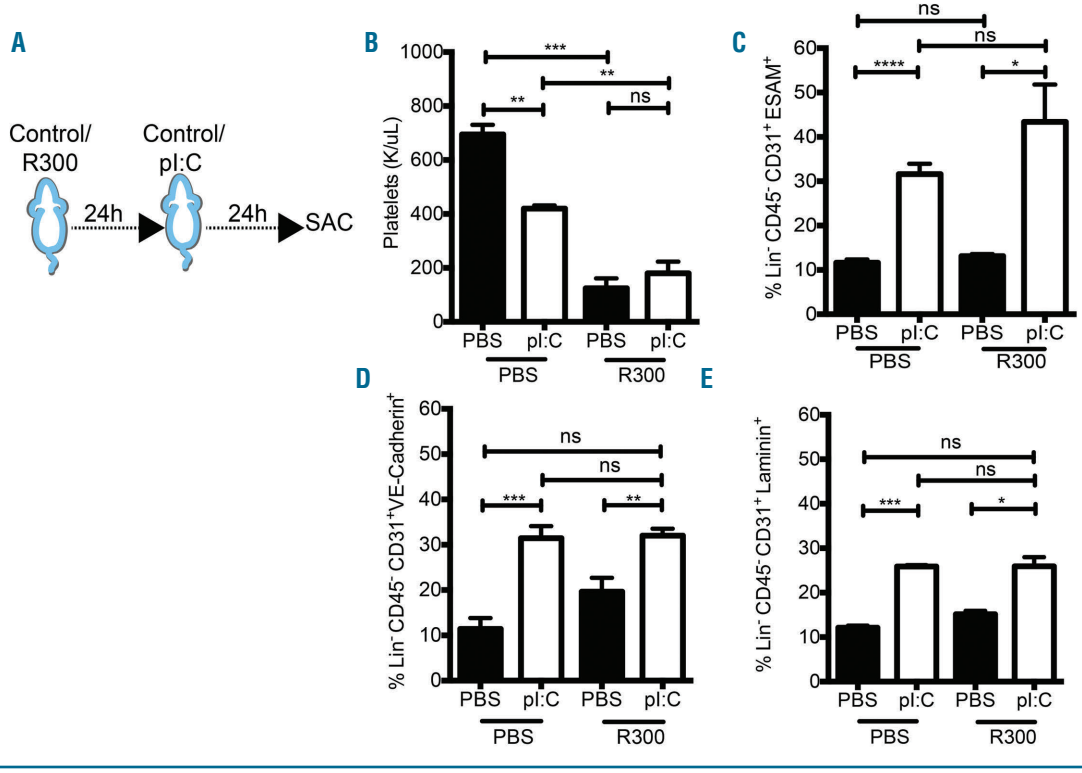


**Figure 5. The IFN mimetic-, pI:C, mediated bone marrow (BM) endothelial cell (EC) stimulation is not affected by platelet abrogation.** (A) Experimental design: mice were treated with the anti-platelet antibody R300 (2 μg/g) and either PBS or the IFN mimetic, pI:C, (5 mg/kg) for 24 h. (B) Platelet counts in the peripheral blood of wild-type (WT) mice following treatment as outlined in (A). (C-E) FACS analysis of the expression of (C) ESAM (D) VE-Cadherin and (E) Laminin on ECs (Lin- CD45$^-$ CD31$^+$) from WT mice treated as outlined in (A). Data are representative of 3 or more independent experiments, and are presented as mean±Standard Error of Mean (SEM) (n≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

mation,[29,30] and megakaryocytes, which give rise to platelets, regulate BM HSC quiescence.[31] To test pI:C-mediated EC activation in the absence of platelets, mice were treated with a platelet depletion antibody, anti-GPIbα (CD42b) antibody (R300), prior to pI:C administration (Figure 5A). Platelet levels were reduced upon R300 treatment (Figure 5B); however, EC activation markers were not altered following platelet depletion (Figure 5C-E). This suggested that platelet activation was not central to IFNα-induced BM EC stimulation. To investigate whether VEGF was regulated by acute pI:C treatment, we carried out a BM ELISA and intracellular staining for VEGF in BM cell types following pI:C treatment. After 24 h there was a significant increase in secreted VEGF in the BM supernatant of pI:C treated mice (Figure 6A). Intracellular VEGF did not increase in BM ECs (Figure 6B and C). However, there was a significant increase in intracellular VEGF in hematopoietic cells, including progenitors and HSCs ($Lin^-$ $ckit^{hi}$ $CD150^+$ $CD48^-$ $CD34^-$), both after pI:C (Figure 6B) and IFNα (Figure 6C) treatment. Consistent with the transient increase in activation of BM ECs (Figure 2G and H), the increase in intracellular VEGF levels in HSCs upon pI:C treatment was also transient. VEGF production peaked after 24 h and returned to homeostatic levels 72 h after treatment (Figure 6D). In addition, VEGF production was increased in WT HSCs in recipient $IFNAR^{-/-}$ mice (WT BM into an $IFNAR^{-/-}$ niche) and in $IFNAR^{-/-}$ HSCs in WT recipient mice ($IFNAR^{-/-}$ BM into a WT niche) following pI:C treatment (Figure 6E). These data suggested that production of VEGF production in HSCs was stimulated both directly and indirectly by pI:C treatment. To assess whether VEGF signaling was consequently active in the BM,[6] the expression of the VEGF receptor, VEGFR2, was analyzed in pI:C treated mice as an indicator of VEGF signaling. VEGFR2 expression was increased in BM sections (Figure 6F). In addition, VEGFR2 was up-regulated on the surface of ECs, but not on HSCs, in response to both pI:C and IFNα (Figure 6G-I). This suggested that VEGF signaling was active in ECs but differs between ECs and HSCs at this time point. As with VEGF production (Figure 6D), the increase in VEGFR2 expression on BM ECs was transient, peaking 24 h after pI:C treatment (Figure 6J). This time point correlated with the peak of increased expression of activation markers on BM ECs (Figure 2G and H). Taken together, these data indicate that pI:C and IFNα treatment leads to an increase in VEGF production and signaling in the BM. In addition, they suggest that, upon pI:C treatment of mice, BM ECs responded to VEGF, which is produced by other BM cell types including HSCs in response to pI:C. Thus, VEGF may be a mediating factor in the activation of BM ECs by IFNα-stimulated hematopoietic cells.

### *IFNα-mediated stimulation of ECs* in vivo *is facilitated by VEGF*

To test whether VEGF signaling was involved in BM EC activation, mice were co-treated with pI:C and the VEGF binding antibody, Avastin (Figure 7A). Avastin treatment did not affect the expression of VEGF or VEGFR2 in comparison to PBS-treated mice (Figure 7B-D). While the expression level of VEGF in ECs was unchanged (Figure 7B), pI:C-induced VEGF expression in HSCs (LK SLAM $CD34^-$) was significantly reduced by co-treatment with Avastin (Figure 7C). In addition, the pI:C-induced expression of VEGFR2 on BM ECs was reduced upon Avastin co-treatment (Figure 7D). In contrast, Avastin treatment did not affect pI:C-mediated proliferation of HSCs (Figure 7E). This suggests that co-treatment with Avastin leads to reduced pI:C-mediated VEGF signaling in the BM. To assess the effect of diminished VEGF signaling on pI:C-mediated EC

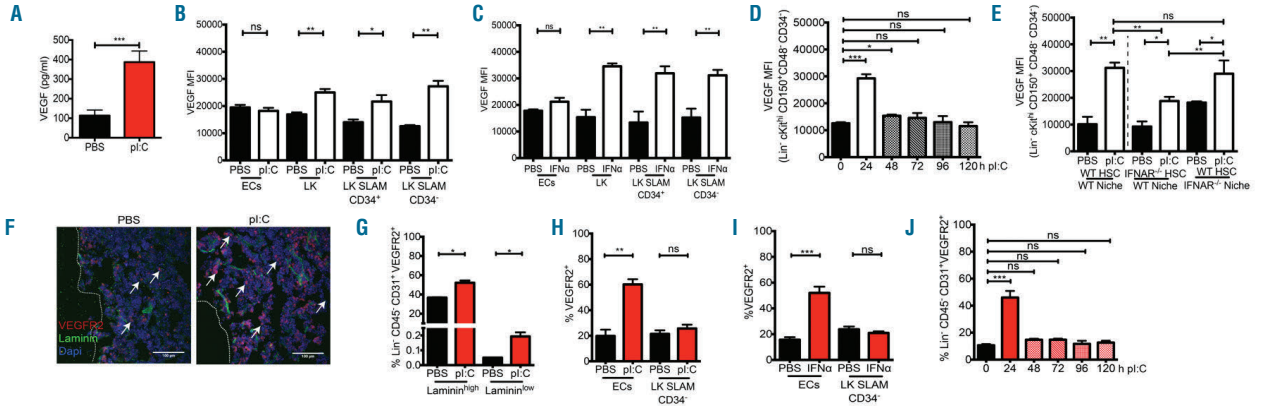


**Figure 6. Bone marrow (BM) vascular endothelial growth factor (VEGF) is modulated by the interferon mimetic, pI:C.** (A) ELISA of BM VEGF from wild-type (WT) mice treated with PBS or the IFN mimetic, pI:C, (5 mg/kg for 24 h). (B and C) FACS analysis of intra-cellular staining of VEGF in indicated BM cell types after treatment with either PBS, (B) pI:C (5 mg/kg for 24 h), or (C) IFNα ($5x10^6$U/kg for 24 h). Data are presented as fold change in mean fluorescence intensity (MFI). (D) FACS analysis of intra-cellular staining of VEGF in hematopoietic stem cells (HSCs) ($Lin^-ckit^{hi}$ $CD150^+CD48^-$ $CD34^-$) after treatment with either PBS (0 h) or pI:C (5 mg/kg, 0-120 h). Data are presented as fold change in MFI. (E) FACS analysis of intra-cellular staining of VEGF in HSCs ($Lin^-$ $ckit^{hi}$ $CD150^+CD48^-CD34^-$) from chimeric mice, as described in Figure 4A, following treatment with either PBS or pI:C (5 mg/kg for 24 h). Data are presented as fold change in MFI. (F) Representative bone sections of VEGFR2 expression (Red) and Laminin (Green) after treatment with either PBS or pI:C (5 mg/kg for 24 h). (G) FACS analysis of VEGFR2 expression on (F) $Laminin^{high/low}$ ECs ($Lin^-$ $CD45^-$ $CD31^+$, $Laminin^{high/low}$) treated with PBS or pI:C (5 mg/kg for 24 h). (H and I) FACS analysis of VEGFR2 expression on ECs (Lin- CD45-CD31+) and HSCs ($Lin^-$ $ckit^{hi}$ $CD150^+CD48^-$ $CD34^-$) from mice treated with either (H) PBS or pI:C (mg/kg for 24 h) or (I) PBS or IFNα ($5x10^6$U/kg for 24 h). (J) FACS analysis of the expression of VEGFR2 on ECs from WT mice treated with either PBS or pI:C (5 mg/kg for up to 120 h). Data are representative of 3 or more independent experiments (A, B, D-G) and 2 or more (C and H) independent experiments, and are presented as mean±Standard Error of Mean (SEM) (n≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.001.

activation, the expression of EC activation markers following Avastin treatment was analyzed. While the increased expression of ESAM was not affected, the pI:C-induced expression of both VE-Cadherin and Laminin was significantly diminished upon co-treatment with Avastin (Figure 7F and G). This indicated that VEGF was involved, in part, in pI:C-mediated BM EC activation. Taken together, these data demonstrate that VEGF signaling is important for the stimulation of BM ECs following pI:C treatment.

## Discussion

Bone marrow ECs are a primary defense against infection, so understanding the effect of inflammation on the BM vasculature is critical. We demonstrate for the first time a rapid, transient activation of the BM vasculature in response to acute inflammatory signaling. We find that there is a direct and indirect effect of IFNα signaling in the BM on ECs, mediated by an activated hematopoietic sys-

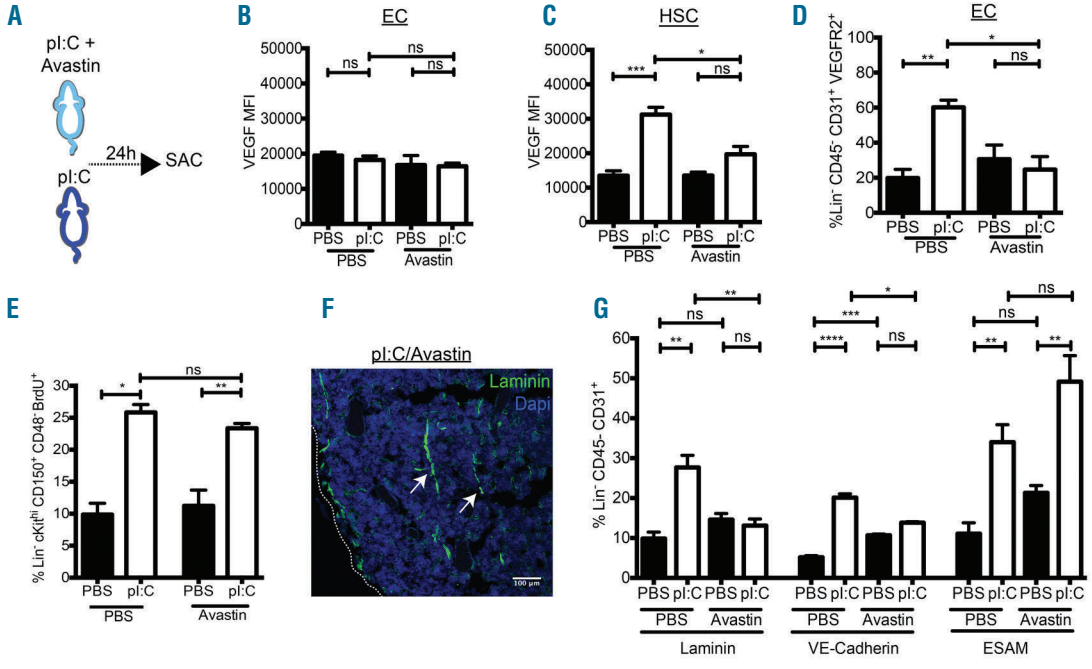


**Figure 7. The IFN mimetic-, pI:C, mediated stimulation of bone marrow (BM) endothelial cells (ECs) is mediated by vascular endothelial growth factor (VEGF) signaling.** (A) Experimental design: mice were treated with Avastin (2.5 mg/kg) and either PBS or the interferon mimetic, pI:C, (5 mg/kg) for 24 h. (B and C) FACS analysis of intra-cellular staining of VEGF in (B) ECs ($Lin^-$ $CD45^-$ $CD31^+$) and (C) HSCs ($Lin^-$ $ckit^{hi}$ $CD150^+CD48^-$ $CD34^-$) from wild-type (WT) mice treated as described in (A). Data are presented as fold change in mean fluorescence intensity (MFI). (D) FACS analysis of VEGFR2 expression on ECs ($Lin^-$ $CD45^-CD31^+$) from WT mice treated as described in (A). (E) FACS analysis of percentage of BrdU positive HSCs (Lin- $ckit^{hi}$ $CD150^+$ $CD48^-$) from WT mice treated as described in (A) and BrdU (18 mg/kg, 16 h). (F) Representative bone marrow section (Laminin in green, DAPI in blue) from WT mice treated as described in (A). (G) FACS analysis of the expression of Laminin, VE-Cadherin and ESAM on ECs ($Lin^-$ $CD45^-$ $CD31^+$) from WT mice treated as described in (A). Data are representative of 3 or more (A-D, E-G) and 2 or more (E) independent experiments and are presented as mean±Standard Error of Mean (SEM) (n≥3). Statistical analysis was performed using unpaired Student *t*-test (two-tailed). ns (not significant), **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

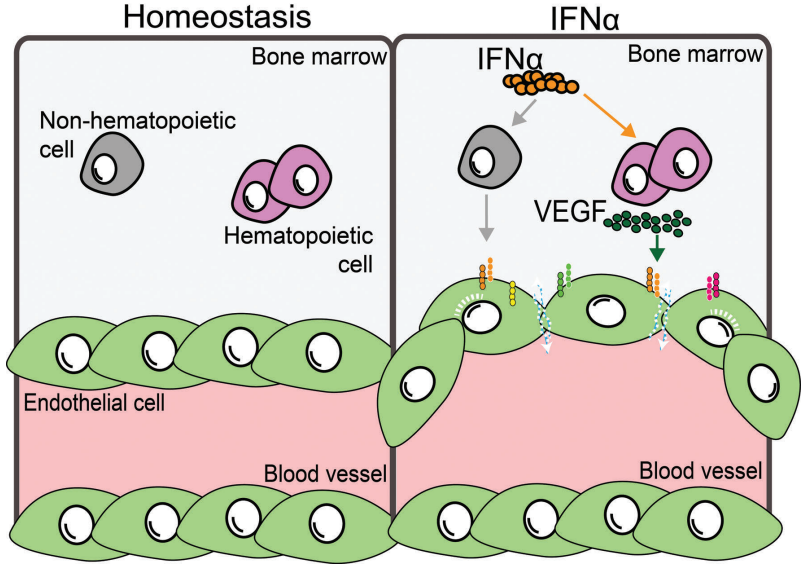


**Figure 8. Acute IFNα-stimulation of BM ECs is mediated by VEGF signaling in both hematopoietic and non-hematopoietic cells.** Model depicting BM vasculature remodeling following stimulation of BM hematopoietic or non-hematopoietic cells by acute interferon α (IFNα) treatment.

tem. Our data suggest a role for VEGF signaling in the BM in IFNα-mediated BM EC activation. This rapid, transient effect may be an emergency response to inflammatory signaling, coming from the hematopoietic system and affecting BM EC niche cells. This response may in turn facilitate the maintenance of BM homeostasis. In this acute setting, the vasculature may be rapidly 'primed' or activated, likely in anticipation of greater insult. In contrast to treatment with an isolated cytokine, initial inflammatory signaling during a full infection is followed by production of other cytokines, and stimulation of additional signaling.[32] It is, therefore, likely that the response elicited by an isolated cytokine differs to that elicited by a full infection, particularly with regard to continuation of signaling and recovery from the initial stimulation.

We have found that acute pI:C exposure results in a transient expansion of the vasculature in the BM after 24 h. The integrity of this expanded vasculature was compromised (Figure 1). Increased BM vascular permeability is in keeping with an increase in the transit of immune cells or leakage of plasma during an inflammatory response.[33] Acute pI:C treatment induces production of IFNα and mimics an acute inflammatory response.[16] Acute IFNα treatment *in vitro* may reduce apoptosis of endothelial cell lines.[27] Whether IFNα has a similar effect on apoptosis of ECs *in vivo* is unknown. Reduced BM EC apoptosis, mediated by IFNα during inflammatory stress, may influence vessel integrity. Permeability of different types of BM vasculature is distinct.[7] Therefore, the integrity of specific BM vessels following an acute inflammatory response is likely influenced by vessel permeability under homeostasis. This may be a mechanism to maintain BM homeostasis during the early stages of an inflammatory response.

The effects of IFNα on ECs and other hematopoietic cell types *in vivo* are in contrast to the *in vitro* situation[26,28,34-36] where cells are isolated from their niche environment. In the BM niche, IFNα treatment rapidly and efficiently stimulates HSC proliferation *in vivo*, whereas *in vitro*, HSCs do not undergo increased proliferation.[16] We have shown BM EC activation in response to acute IFNα exposure *in vivo* (Figures 2 and 3), in contrast to the described effect of IFNα on ECs *in vitro*. In addition to the differential effect of IFNα *in vitro* and *in vivo*, IFNα has also been described as being both stimulatory and inhibitory with regard to VEGF signaling.[37-39] We find that VEGF production was increased in the BM in response to pI:C (Figure 6). BM EC activation following acute pI:C treatment was dependent in part on VEGF signaling (Figure 7). pI:C mediated HSC proliferation was not affected by inhibition of VEGF, using Avastin treatment (Figure 7E). This corresponds with previous data showing that HSCs are directly activated by IFNα.[16] An IFNα-mediated increase of VEGF is in contrast to previous studies, which suggest that VEGF is suppressed by long-term IFN treatment or in combination with chemotherapy.[38-41] Together, these data highlight the contrast between chronic *versus* acute IFN treatments, and between *in vitro* and *in vivo* cytokine responses. As inflammatory stress is a complex signaling cascade, the *in vivo* cytokine response in mice is, therefore, more reflective of the inflammatory response than the *in vitro* situation.

In the BM niche, signaling between different cell types is imperative for maintenance of cellular homeostasis and a rapid response to inflammation. ECs and the vasculature itself have been ascribed many functions in the BM as regulators of HSCs.[8,10,13,18,42-45] Furthermore, there are distinct vessel subtypes within the BM which differentially regulate hematopoiesis.[7] In addition, Notch signaling in BM ECs has been shown to expand the HSC niche *in vivo*.[8] As Notch signaling is involved in the inflammatory response of ECs,[46] these data may further support a role for inflammatory signaling in BM niche remodeling. Furthermore, IFNα does not lead to mobilization of hematopoietic stem progenitor cells HSPCs that are not in the BM.[16,35] However, the percentage of HSCs found within 20 μm of arterioles in sternal BM is significantly reduced following treatment with pI:C in comparison to the control.[18] These data suggest that the location of HSCs in the BM is affected by pI:C. Relocating HSPCs can potentially affect many different BM cell types, and the BM vasculature, following treatment with pI:C. Whether pI:C-stimulated BM vasculature affects the location of HSPCs within the BM remains unclear. Many cytokines produced by hematopoietic cells, such as EPO and GCSF, have been shown to affect specific EC functions in isolation.[47,48] However, signaling from the hematopoietic system to the ECs in the BM niche has not been examined in detail. To address this question, we have created BM chimeras in which only either hematopoietic cells or niche cells respond directly to IFNα. Using this system, we have demonstrated that inflammatory signaling from an activated hematopoietic system can affect the BM vasculature (Figure 4). Platelets, which can induce EC stimulation,[30] do not play a major role in BM EC stimulation in this setting (Figure 5). As the inflammatory response is complex, these data do not exclude the possibility that IFNα-mediated signaling from other cell types within the BM, in addition to hematopoietic cells, may be involved in this response. Further, these data cannot discriminate within the heterogeneous population of BM ECs. Whether there is crosstalk between pI:C-stimulated BM ECs and BM HSPCs within this context, remains to be elucidated. Importantly, we demonstrate that BM hematopoietic cells, including HSCs, are implicated in the pI:C-mediated BM EC stimulation, and thus in vasculature remodeling. This provides evidence for crosstalk between BM HSPCs and ECs under inflammatory stress conditions (Figure 8).

Our findings demonstrate a novel response of the BM vasculature to primary inflammatory signaling. We have revealed potential crosstalk between the hematopoietic system and the BM vasculature under inflammatory stress. The transient activation of the BM vasculature represents a novel, emergency response of the BM stem cell niche to inflammation. Future studies will likely uncover other emergency situations in which HSCs influence the BM niche. Understanding this critical cellular relationship under stress conditions such as infection, but also chemotherapy, may reveal new mechanisms for the maintenance and recovery of BM homeostasis.

***Acknowledgments***

*The authors would like to thank Drs M. Milsom, H. Augustin and A. Trumpp for helpful discussions, A. Atzberger and Dr S. Schmitt from the DKFZ Flow Cytometry Facility, M. Brom, Dr F. Bestvater and Dr D. Krunic from the DKFZ Imaging Core Facility, the DKFZ Animal Laboratory Services for their expertise and assistance, and L. Prendergast for critical reading of the manuscript.*

***Funding***

*This work was supported by FOR2033 NicHem and SFB873, both funded by the Deutsche Forschungsgemeinschaft, and the Dietmar Hopp Foundation.*

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