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***Shb* deficiency in endothelium but not in leukocytes is responsible for impaired vascular performance during hindlimb ischemia**

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Short title: Hindlimb ischemia in *Shb* knockout mice

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Abstract

Aim: Myeloid cells have been suggested to participate in angiogenesis and regulation of vascular function. *Shb*-deficient mice display both vascular and myeloid cell abnormalities with possible consequences for recovery after hindlimb ischemia. This study was conducted in order to assess the contribution of *Shb*-deficiency in myeloid cells to impaired vascular function in ischemia. **Methods:** Wild type and *Shb*-deficient mice were subjected to peritoneal VEGFA followed by intraperitoneal lavage, after which blood and peritoneal cells were stained for myeloid markers. VEGFA-induced leukocyte recruitment to cremaster muscle was investigated using intravital microscopy of both mouse strains. Blood flow after femoral artery ligation was determined on chimeric mice after bone marrow transplantation. **Results:** No differences in neutrophil numbers or cell surface phenotypes were detected. Moreover, neutrophil extravasation in VEGFA-activated cremaster muscle was unaffected by *Shb* deficiency. However, blood and peritoneal CXCR4+ monocytes/macrophages were reduced in response to intraperitoneal VEGFA but not LPS in the absence of *Shb*. Furthermore, the macrophage population in ischemic muscle was unaffected by *Shb*-deficiency after two days but reduced seven days after injury. The bone marrow transplantation experiments revealed that mice with wild type vasculature showed better blood flow than those with *Shb*-deficient vasculature irrespective of leukocyte genotype. **Conclusion:** The observed aberrations in myeloid cell properties in *Shb*-deficient mice are likely consequences of an abnormal vascular compartment and are not responsible for reduced muscle blood flow. Structural vascular abnormalities seem to be the primary cause of poor vascular performance under provoked vascular stress in this genetic model.

Key words: *Shb*, VEGFA, hindlimb ischemia, myeloid cells, microvasculature, leukocyte extravasation

Introduction

The performance of the vascular system is of paramount importance for adequate oxygen supply to tissues and is thus regulated by numerous means. This includes affluence of arterial/arteriolar supply, the structure and density of the microvasculature, presence and function of perivascular cells and venal drainage (Dvorak, 2005, Ferrara, 2004). In addition, diffusion over endothelial cells and vascular permeability also play a role in vascular function (McDonald et al., 1999, Nagy et al., 2008). A dysfunctional vasculature is commonly seen in numerous diseases such as cancer, ischemia and inflammatory diseases (Folkman et al., 1989, Weis, 2008, Claesson-Welsh and Welsh, 2013). Besides the participation of endothelial and perivascular stromal cells in the function of the vasculature (Armulik et al., 2011), numerous studies have additionally implicated the participation of blood-derived myeloid cells (Nozawa et al., 2006, Christoffersson et al., 2010, Christoffersson et al., 2012a, Allavena et al., 2008, Cassetta et al., 2011, Rolny et al., 2011, Tugues et al., 2012, Capoccia et al., 2006).

Shb is a multi-domain adapter protein operating downstream of vascular endothelial growth factor receptor-2 (VEGFR-2) exerting pleiotropic cellular responses by generating multi-component signaling complexes (Anneren et al., 2003, Holmqvist et al., 2004). Examples of other signaling intermediates that may associate with Shb are Src-family kinases, phosphatidyl inositol-3' kinase, focal adhesion kinase (FAK) and the tyrosine kinase receptors platelet-derived growth factor receptors and fibroblast growth factor receptor-1 (Anneren et al., 2003, Holmqvist et al., 2003). In line with the complex *in vitro* effects of Shb, the *Shb* knockout mouse displays abnormalities in reproduction (Kriz et al., 2007, Calounova et al., 2010), glucose homeostasis (Akerblom et al., 2009), the vasculature (Funa et al., 2009, Christoffersson et al., 2012b, Akerblom et al., 2012) and hematopoietic cell function (Gustafsson et al., 2013, Gustafsson et al., 2011). Particularly the latter two alterations are prominent and multi-faceted. Absence of *Shb* causes impaired angiogenesis (both tumor and Matrigel), aberrant vascular permeability, changed endothelial cell morphology and altered vascular architecture (Christoffersson et al., 2012b, Funa et al., 2009). These differences result from an abnormal signaling signature showing elevated basal and reduced VEGFA-responsive activities of FAK, extracellular-signal regulated kinase (ERK), Akt and Rac1 (Funa et al., 2009, Zang et al., 2013). Furthermore, *Shb* knockout adherens junctions were morphologically different with

fewer distinct gaps and more loose junctions (Christoffersson et al., 2012b). Such morphological changes paralleled less distinct VE-cadherin staining and increased VEGFR-2/VE-cadherin association in response to VEGFA (Zang et al., 2013).

The hematopoietic effects of *Shb* deficiency have also been characterized and involve both fully differentiated and primitive blood cells. For example, the T cell receptor response is altered in the absence of *Shb*, which promotes a Th2-skewing of activated naïve T helper cells and ultimately an augmented development of atopic dermatitis (Gustafsson et al., 2011, Gustafsson et al., 2014). In addition, the bone marrow of *Shb* knockout mice contains fewer phenotypically characterized long term-hematopoietic stem cells with lower capability of myeloid cell restitution over a long time period (Gustafsson et al., 2013).

We have recently found that the performance of the *Shb* knockout vasculature was inferior to the wild type counterpart in a model of hindlimb ischemia (Christoffersson et al., 2012b). Considering the numerous aberrations in hematopoietic cells in this genetic deficiency and that myeloid cells in many instances have been shown to participate in vascular responses, we decided to investigate to what extent abnormal properties of *Shb* deficient myeloid cells contribute to the impaired vascular function observed in this mouse.

Materials and methods

Mice:

Most experiments were done on Balb/c wild type and *Shb* knockout mice of 2 to 7 months of age (Gustafsson et al., 2013) except for macrophage infiltration on day 7 that was determined on mice with a mixed genetic background (FVB/C57Bl6/129Sv) as described (Christoffersson et al., 2012b). All animal experiments had been approved by the local animal ethics committee of Uppsala County Court.

Immunostaining:

Cryosections (8 µm) of the quadriceps muscle (normal or ischemic leg) were stained for the pan-macrophage marker CD68 using rat anti-mouse CD68 (AbD Serotec, Bio-Rad, Hercules, CA) followed by Alexa 594 donkey anti-rat secondary antibody (Invitrogen, Carlsbad, CA). Sections were mounted with Vectashield HardSet mounting medium (Vector Laboratories, Burlingame, CA) containing 4', 6-diamidino-2-phenylindole (DAPI) for staining of nuclei. A Nikon Eclipse TE2000-U

fluorescence inverted microscope, with a Nikon D Eclipse C1 camera was used to take photos using a 10x/0.30 objective.

Intraperitoneal lavage:

Mice were injected intraperitoneally with saline, VEGFA (80 ng) or lipopolysaccharide (LPS) (2 ng) as described previously (Christoffersson et al., 2012a, Shi et al., 2011). After 21 hours (saline or VEGFA) or 4 hours (LPS), blood was drawn through cardiac puncture and the animals were sacrificed. Five mL of PBS was subsequently injected intraperitoneally and the suspension of recruited cells was collected after abdominal palpation.

FACS analysis of myeloid cells:

The red blood cells were removed from the peripheral blood collected by cardiac puncture, by sedimentation in 6% (w/v) Dextran T500 (GE Healthcare, Hempstead, UK) followed by lysis in Red cell lysis buffer (Sigma Aldrich, St. Lois, MO). The blood leukocytes and the intraperitoneal cell suspension were thereafter stained with CD115-PE-Cy7, CXCR4-eFluor450, F4/80-APC-eFluor780, CD49d-PE (all from eBioscience), Ly6G-FITC (clone 1A8, BD Bioscience) and VEGFR-1 (APC-conjugated in house, R&D Systems). Flow cytometric analysis was carried out on an LSR II (BD Bioscience) and all data was processed with FlowJo (TreeStar, Ashland, OR) software.

Intravital recordings of cremaster muscle:

Anesthetized mice (2% isoflurane, Abbott Scandinavia, Sweden) were placed on heating pads and the cremaster muscle was exposed on a transparent viewing pedestal as previously described (Phillipson et al., 2006, Massena et al., 2010). The muscle vasculature was visualized in an intravital microscope (Leica DM500B with 20 x 0.5W HCS Apo objective, Wetzlar, Germany) and images recorded via a high-sensitivity CCD-camera (Orca-R2, Hamamatsu, Japan) at high frame-rates using Volocity Acquisition 5.0 software (PerkinElmer, Waltham, MA, USA).

Bone marrow transplantation:

Iliac bones, femurs and tibias were collected from two months old wild type Balb/c or *Shb* knockout donor mice. The bones were crushed in 2% (v/v) fetal bovine serum in PBS followed by passage over a 40 µm cell strainer to obtain single cell suspension. Cell numbers were determined and 1.5×10^6 cells were transplanted into wild type or *Shb* knockout recipients by retro-orbital injection. Prior to the bone marrow transfer,

the recipients (two to three months old) were irradiated with a split dose of 9 Gy in a Nordion Gammacell 40 Exacto ¹³⁷Cs irradiator (MDS Nordion, Ottawa, ON) in order to ablate endogenous bone marrow. Peripheral blood chimerism was determined 6 weeks post transplantation by FACS analysis on blood for CD45.1 (wild type) and CD45.2 (*Shb* knockout). Chimerism proved to be more than 75% for donor bone marrow in all mice. Hindlimb ischemia was induced three to four months after transplantation to ensure complete restitution of the hematopoietic compartment.

Hindlimb ischemia and blood flow measurements:

Mice were anesthetized (Isoflurane, 2%, Abbott Scandinavia, Sweden) and the fur was removed from both legs (Veet, Recitt Benckiser, Valora Trade, Stockholm, Sweden). To induce hindlimb ischemia the left femoral artery was separated from the femoral vein, ligated and cut above the superficial epigastric artery branch.

Tissue perfusion was measured non-invasively in the knee region of anesthetized mice 2 days after artery ligation, using Laser Doppler flowmetry (PeriFlux 4001 Master, Perimed, Järfälla, Sweden) with 780 nm laser light applied directly onto the skin (Christoffersson et al., 2012b). Shifted backscattered light was collected via 0.5 mm fiber separated laser probes. Paw skin temperature was continuously recorded using an isolated thermistor transducer (MLT422/A) connected to a thermistor Pod (ML309) both AD Instruments, Oxford, United Kingdom.

Prior to recording blood flow, the probe was applied to three different spots over the quadriceps muscle in the region immediately proximal to the knee. This was to ensure reproducible and representative recordings of blood flow. Then, baseline tissue perfusion was recorded (30 min) in room temperature where after heat was applied to the entire limb for 30 min through tubing containing pre warmed water. Baseline and peak hyperemia blood flow were recorded after excluding breathing artifacts. Data was converted by PowerLab 4/3 and analyzed using LabChart 7 Pro software, both AD Instruments, Oxford, United Kingdom. Blood flow is expressed as perfusion units (PFU) and the functional blood flow increase as delta perfusion units.

Statistical analysis:

Means $\pm$ SEM are given. Comparisons for statistical significance were done by Students' t-test or when appropriate one-way ANOVA followed by Bonferroni's test.

Results

Infiltration of CD68-positive macrophages on day seven after arterial ligation in the hindlimb

In a previous study, blood flow restoration was significantly impaired by *Shb* deficiency at 7 days following arterial ligation (Christoffersson et al., 2012b). To assess the relevance of myeloid cells for the vascular dysfunction in the absence of *Shb*, infiltration of CD68-positive macrophages into the quadriceps muscle distally to arterial ligation was determined at seven days after ligation (Fig. 1). Increased macrophage infiltration as a consequence of ischemia was observed in the wild type situation only, demonstrating impaired recruitment of monocytes from circulation in the *Shb* deficient mice. Thus the data reveal aberrations in myeloid cell recruitment as a consequence of *Shb* deficiency in relation to ischemia.

Myeloid cell blood profiles after intraperitoneal injection of VEGFA

The importance of myeloid cells vis-à-vis *Shb* deficiency was further explored by subjecting the mice to VEGFA intraperitoneal injections followed by characterization of blood and intraperitoneal cell surface phenotypes. The cell surface markers investigated were Ly6G (neutrophils), CD115 (monocytes), F4/80 (macrophages), CD49d (VEGFA-extravasating neutrophils, Massena et al., submitted manuscript), CXCR4 (tissue leukocyte retention and hypoxia recruitment) and VEGFR1 (VEGFA-responsive myeloid cells). VEGFA intraperitoneal injections increased the percentage of blood neutrophils (Fig. 2), probably due to neutrophil mobilization from bone marrow. However, the percentage CXCR4⁺/CD49d⁺/VEGFR1⁺ cells was decreased upon VEGFA treatment, either due to increased recruitment of this neutrophil subset to the peritoneum or preferential recruitment of other neutrophil subsets from bone marrow. VEGFA treatment increased the percentage of peritoneal neutrophils in the wild type situation. The relative proportion of intraperitoneal CXCR4⁺/CD49d⁺/VEGFR1⁺ neutrophils was similar, regardless of treatment or genotype. Thus, neutrophil trafficking during VEGFA stimulation does not involve *Shb*.

Percentages blood monocytes were unaffected by *Shb* deficiency and VEGFA lavage (Fig. 3). However, CXCR4⁺ blood monocytes were relatively fewer in the VEGFA *Shb* knockout group, whereas no significant differences were observed among the VEGFR1⁺ monocytes. The percentages of monocytes plus macrophages in the lavage were unaffected by treatment (Fig 4). In line with the observations in blood, there were also relatively fewer CXCR4⁺ monocytes plus macrophages in the

Shb knockout lavage following VEGFA treatment (Fig. 4). CXCR4 and its ligand CXCL12 (chemokine C-X-C motif ligand) have been suggested to regulate retention of leukocytes in various tissues, including the bone marrow (Ceradini et al., 2004). The reduced proportions of CXCR4 expressing monocytes in the *Shb* knockout mice treated with VEGFA could thus merely reflect increased retention of these cells due to an aberrant activity of the CXCR4-CXCL12 signaling axis in the bone marrow.

In order to test whether the effects on CXCR4⁺ monocytes were VEGFA specific, the mice were subjected to a lavage with a low dose of LPS. This treatment has been demonstrated to result in a monocyte chemoattractant protein-1 (MCP-1) mediated release of monocytes from the bone marrow after 4 hours (Shi et al., 2011). Both wild type and *Shb* null mice responded promptly to the LPS challenge with increased proportions of circulation monocytes (Fig. 3). Interestingly, the LPS treatment did not result in a reduction of the CXCR4 expressing monocyte fraction in blood from the *Shb* deficient mice (Fig. 3). Moreover, no difference in the recruitment of CXCR4⁺ monocytes and/or macrophages to the peritoneal cavity was detected (Fig. 4), implying that the effect seen on this subpopulation of cells following VEGFA treatment indeed was VEGFA specific.

Cremaster neutrophil adherence and extravasation in response to VEGFA

Intravital examination of cremaster venules and leukocyte adherence and extravasation was studied in wild type and *Shb* knockout mice upon superfusion with a solution containing 60 ng mL⁻¹ VEGFA. During the time period studied, almost exclusively neutrophils are recorded (Massena et al, submitted manuscript). In Fig. 5 it can be seen that VEGFA increased adherence and extravasation of leukocytes in both wild type and *Shb* knockout mice. These data reinforce the view suggested by Fig. 2 that *Shb* deficient neutrophils show no major functional difference in response to VEGFA.

Hindlimb blood flow after bone marrow transplantation

The ultimate test for assessing the relative contribution of the leukocyte genotype to the blood flow response in the absence of a conditional *Shb* gene knockout is to obtain chimeric mice with mixed wild type and *Shb* knockout populations after bone marrow transplantation. This requires an inbred strain of mice and since the *Shb* knockout is embryonically lethal on the C57Bl6 background (Kriz et al., 2007), Balb/c mice were used instead. Ligation of the femoral artery generates a much more severe phenotype on this genetic background with necrosis in the lower

hindlimb after 2 days (results not shown) and thus the experiments could not be extended for more than two days unlike the seven day protocol previously adopted. Differences between mouse strains in their sensitivity to hindlimb ischemia have previously been reported (Limbourg et al., 2009). On day two (Fig. 6, upper graph), basal blood flow was significantly higher in the two groups of chimeric mice carrying the wild type recipient genotype (wt to wt and ko to wt) than in those carrying the *Shb* knockout recipient genotype (wt to ko and ko to ko) indicating that the vascular genotype is the primary determinant deciding the overall performance of the vasculature with little or no contribution of the myeloid cell genotype. The heat-induced hyperemia was small and failed to reach significance in all groups, indicating true ischemia in the legs.

Incidentally, the same differences in blood flow were seen in the healthy legs (Fig. 6 lower graph), *i.e.*, higher blood flow in wt recipients (wt to wt and ko to wt) again indicating the genotype of the recipient vasculature as the primary determinant for vascular function in this setting.

On day 2, no differences in quadriceps macrophage infiltration in the ischemic or normal legs were detected (Fig 7).

Discussion

The data suggest that the *Shb*-gene genotype of neutrophils or monocytes/macrophages has no major bearing on the vascular performance of both ischemic and healthy hindlimbs. In addition, we obtained no evidence for neutrophil dysfunction in blood or peritoneum due to absence of *Shb*. However, a difference in the cell surface phenotype of *Shb* deficient monocytes and/or macrophages was noted with decreased expression of the chemokine receptor CXCR4 after intraperitoneal VEGFA injections and lavage in both blood and peritoneum. CXCR4 responds to CXCL12 and the CXCL12-CXCR4 signaling axis has previously been reported to regulate leukocyte recruitment to sites of hypoxia (Ceradini et al., 2004, Grunewald et al., 2006). In addition, CXCR4 is thought to mediate retention of leukocytes in the bone marrow and/or peripheral tissues (Ceradini et al., 2004, Grunewald et al., 2006). The finding that the impaired VEGFA response was not observed with LPS, which recruits monocytes/macrophages in an MCP-1-dependent manner (Shi et al., 2011), indeed suggests that the effect was VEGFA specific. Furthermore, VEGFR1 expressing monocytes/macrophages were similar in both genotypes, supporting the

notion that VEGFR1 signaling *per se* is not much affected by absence of *Shb*. Regardless, the relevance of *Shb* deficiency for macrophage function becomes clear when investigating muscle macrophage infiltration on day seven after induction of ischemia showing an inability to increase tissue accumulation under such conditions. Based on the time course of vascular performance determined as blood flow and on the data on chimeric mice receiving bone marrow transplantation the presently observed evidence of macrophage dysfunction seems to have little or no relevance for vascular function under these conditions. An alternative possibility is that macrophage infiltration participates in tissue repair after damage due to ischemia.

The changes in blood flow seem primarily dependent on inherent features of the vasculature as evidenced by the bone marrow transplantation experiments. The reduced functionality due to absence of *Shb* was also observed in the healthy leg. This defect was apparent at an early time point (day two in present study and day one in previous study (Christoffersson et al., 2012b)) and appears to be the consequence of abnormal structural features of the *Shb* knockout vasculature. These include increased number of small arterioles, fewer branch points, vascular irregularities and increased vessel tortuosity (Christoffersson et al., 2012b). Together, these aberrations provide a microvasculature that is less functional, especially under conditions of provoked stress. Many of these changes could be attributed to defective angiogenesis in the absence of *Shb* followed by functional adaptation under steady state conditions.

Impaired VEGFA-induced vascular permeability is yet another feature of the *Shb* knockout mouse (Funa et al., 2009). Such an impairment was not seen in response to the inflammatory agent histamine (Gustafsson et al., 2014). Although the physiological role of vascular permeability has not yet been established it may contribute to leukocyte extravasation (Schulte et al., 2011) and thus play a role tissue recovery. Macrophage infiltration was not influenced by genotype on day two after ischemia but increased in the wild type situation on day seven. Due to the severity of the ischemic phenotype in the Balb/c mouse background we could not directly establish by transplantation experiments whether the difference on day seven was due to the vascular genotype promoting vascular permeability and macrophage infiltration or the altered properties of the *Shb* knockout monocytic/macrophagic population. However, the reduced VEGFA-stimulated (and not LPS) recruitment to the peritoneal cavity of *Shb* knockout CXCR4-positive monocytes and/or macrophages irrespective of their VEGFR1 cell surface expression is compatible with the view that this

primarily reflects an impairment of the endothelial component. Consequently, the reduced macrophage infiltration in response to ischemia may mirror the same fundamental mechanism.

In summary, the reduced hindlimb blood flow is a consequence of the *Shb* knockout phenotype of vascular cells and not that of myeloid cells. Despite this, myeloid cells may contribute to tissue repair and neo-angiogenesis in the later stages of recovery.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure legends:

Figure 1: Macrophage infiltration into hindlimb muscle on day seven after ligation. Wild type (n=5) or *Shb* knockout (4) mice were subject to femoral artery ligation or not and macrophage infiltration was determined by staining for CD68. Values are number of macrophages per mm². Means $\pm$ SEM are given. * indicates $p < 0.05$ using a paired Students' t-test.

Figure 2: Effects of VEGFA lavage on blood and peritoneal neutrophils. Wild type (n=4) and *Shb* knockout (n=4) mice were subjected to VEGFA intraperitoneal injections. Blood and lavage were collected 21 hours later and analysed by flow cytometry after staining for myeloid cell markers. Neutrophils were defined as Ly6G+ cells. Values are percent of parent populations given as means $\pm$ SEM. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively, using one-way ANOVA followed by Bonferroni post-hoc test.

Fig 3: Blood monocytes after intraperitoneal VEGFA injections and LPS lavage. Wild type (n=4) and *Shb* knockout (n=4) mice were injected intraperitoneally with VEGFA or saline, where after blood and peritoneal lavage were collected 21 h later and analysed by flow cytometry. For LPS lavage, cells were collected after 4 h. Monocytes were defined as CD115+ cells. Values are percent of parent cell population and given as means $\pm$ SEM. * indicates $p < 0.05$ using one-way ANOVA followed by Bonferroni post-hoc test.

Figure 4: Intraperitoneal monocytes plus macrophages after intraperitoneal VEGFA injections and lavage. Wild type (n=4) and *Shb* knockout (n=4) mice were injected intraperitoneally with VEGFA or saline. Macrophages and/or monocytes (CD115+ and F4/80+ cells) were collected 21 h later by lavage and analysed by flow cytometry. Alternatively, mice were injected intraperitoneally with LPS and analysed after 4 h. Values are percent of parent population given as means $\pm$ SEM. ** indicates $p < 0.01$ by a Students' t-test.

Figure 5: Neutrophil adherence and emigration in cremaster muscle after VEGFA addition. Cremaster muscles from wild type and *Shb* knockout mice (n= 5 each) were

subjected to intravital microscopy. VEGFA (60 ng/ml) was added to the superfusion medium (time 0) and neutrophil adherence and extravasation was recoded at the given time points. Values are means $\pm$ SEM. * indicates $p < 0.05$ when compared with 0 time point value using a paired Students' t-test.

Figure 6: Hindlimb blood flow after bone marrow transplantation. Mice were subject to lethal irradiation and bone marrow transplantation according to the schemes indicated in the figure. Three to four months later, the femoral artery was ligated and blood flow determined two days later in ischemic (upper graph) and contralateral healthy legs (lower graph). Blood flow in means $\pm$ SEM are give for three mice wild type (wt) to wt, five mice *Shb* knockout (ko) to wt, seven mice wt to ko and three mice ko to ko where first genotype is donor bone marrow and the second is that of recipient. * indicates $p < 0.05$ difference by Students' t-test.

Figure 7: Macrophage infiltration after bone marrow transplantation and hindlimb ischemia. Bone marrow transplantations and femoral artery ligations were done as in figure 6. Excised quadriceps muscles distally to the site of ligation were stained for CD68 on day 2 after ligation. Values are means $\pm$ SEM for 3-5 mice.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7