Common and Distinctive Pathogenetic Features of Arteriovenous Malformations in Hereditary Hemorrhagic Telangiectasia 1 and Hereditary Hemorrhagic Telangiectasia 2 Animal Models—Brief Report

Eva M. Garrido-Martin, Ha-Long Nguyen, Tyler A. Cunningham, Se-woon Choe, Zhihua Jiang, Helen M. Arthur, Young-Jae Lee, S. Paul Oh

Objective—Hereditary hemorrhagic telangiectasia is a genetic disorder characterized by visceral and mucocutaneous arteriovenous malformations (AVMs). Clinically indistinguishable hereditary hemorrhagic telangiectasia 1 and hereditary hemorrhagic telangiectasia 2 are caused by mutations in ENG and ALK1, respectively. In this study, we have compared the development of visceral and mucocutaneous AVMs in adult stages between Eng- and Alk1-inducible knockout (iKO) models.

Approach and Results—Eng or Alk1 were deleted from either vascular endothelial cells (ECs) or smooth muscle cells in adult stages using Scl-CreER and Myh11-CreER lines, respectively. Latex perfusion and intravital spectral imaging in a dorsal skinfold window chamber system were used to visualize remodeling vasculature during AVM formation. Global Eng deletion resulted in lethality with visceral AVMs and wound-induced skin AVMs. Deletion of Alk1 or Eng in ECs, but not in smooth muscle cells, resulted in wound-induced skin AVMs. Visceral AVMs were observed in EC-specific Alk1-iKO but not in Eng-iKO. Intravital spectral imaging revealed that Eng-iKO model exhibited more dynamic processes for AVM development when compared with Alk1-iKO model.

Conclusions—Both Alk1- and Eng-deficient models require a secondary insult, such as wounding, and ECs are the primary cell type responsible for the pathogenesis. However, Alk1 but not Eng deletion in ECs results in visceral AVMs. (Arterioscler Thromb Vasc Biol. 2014;34:2232-2236.)

Key Words: Alk1 protein, mouse ■ arteriovenous malformations ■ endoglin protein, mouse ■ endothelial cells ■ myocytes, smooth muscle ■ telangiectasia, hereditary hemorrhagic

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular disorder with a prevalence of 1:5000.1 Several clinically indistinguishable forms of HHT are caused by mutations in ≥2 autosomal genes, among which endoglin (ENG; HHT1) and activin receptor-like kinase 1 (ACVRL1/ALK1; HHT2) constitute >80% of the cases.1 HHT is characterized by the presence of arteriovenous malformations (AVMs), abnormal direct connections between arteries and veins, in the skin, mucosa, and internal organs.1 It is generally thought that AVMs in the brain arise during developmental stages.2 But patients with HHT also develop small AVMs, called telangiectases in eyelids, lips, tongue, nasal cavity, and gastrointestinal tract, that are mostly acquired during postdevelopmental stages.3 The frequency and severity of intranasal and gastrointestinal telangiectases increase with the age, and their frequent ruptures can lead to severe anemia. Understanding the pathogenetic mechanisms that lead to the formation of telangiectases and identifying the cell types responsible for this process are essential for developing therapeutic strategies for epistaxis and gastrointestinal bleeding in patients with HHT.

We have previously shown that secondary events, such as wounding, are necessary for inducing de novo AVMs in adult stages of global Alk1-inducible knockout (iKO) model.4 Because both ALK1 and ENG are components of the transforming growth factor-β family signaling complex, it has been presumed that HHT1 and HHT2 share a common pathogenetic mechanism. However, it is not known whether Eng-deficient vessels also need an environmental insult in addition to the genetic predisposition to trigger the development of mucocutaneous AVMs in adult stages.

The cellular origin of the AVMs remains unresolved. We have previously shown that Alk1 deletion using an endothelial...
cell (EC)–specific Cre driver (L1Cre) resulted in AVMs in the brain, lungs, and gastrointestinal tract at embryonic and neonatal stages. However, it is unknown whether ECs are the primary cellular source of the wound-induced skin AVMs and visceral AVMs in adult Alk1-iKO model as well. Alk1 expression was detected beyond vascular ECs, as the lymphatic ECs and monocytes/macrophages (Y.H. Kim, PhD, et al, 2014, unpublished data), and the Cre activity was detected in macrophages of L1Cre mice. These data raise a question whether ECs are the sole source of Alk1-deficient effect for the AVM development in L1Cre/Alk12f/2f mice. In the case of Endoglin, the origin of the AVMs could reside in several cell sources, considering that it is expressed in a variety of cell types relevant for vascular functions, including ECs, smooth muscle cells (SMCs), endothelial precursors, and macrophages. Interestingly, deletion of either \(\text{Eng}^+\) or \(\text{Eng}^-\) by SM22α-Cre resulted in brain AVMs, implicating potential functions of ALK1 and ENG in vascular SMCs for establishing the proper arteriovenous network. In this study, we examined the effect of wounding in the development of mucocutaneous telangiectases and investigated the cellular origin of de novo AVMs in both HHT1 and HHT2 mouse models.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

To investigate the role of ENG in adult mice, \(\text{Eng}^+\) was globally deleted using the tamoxifen-inducible ROSA26CreERT mouse strain (R26CreERT). We found that 3 consecutive day injection of tamoxifen at 2.5 mg/25 g body weight was the most effective regimen for R26CreERT;\(\text{Eng}^{2f/2f}\) mice (Figure 1 in the online-only Data Supplement). In 3 to 4 days after the first tamoxifen injection, the R26CreERT;\(\text{Eng}^{2f/2f}\) mutant mice displayed signs of illness, such as slow movement, diarrhea, and dehydration, and died around day 4 to 10 (n=30). To analyze the subdermal vessels in the back skin, vascular casting with blue latex was performed at days 5 to 8 by infusing it into the left ventricle. Subdermal vessels were unaffected in the intact back skins of tamoxifen-injected adult R26CreERT;\(\text{Eng}^{2f/2f}\) mice (data not shown) and also wounded skin of tamoxifen-injected adult R26CreERT;\(\text{Eng}^{2f/2f}\) mice (Figure 1A in the online-only Data Supplement; n=6). However, areas of wounds in mid-dorsum and ear of tamoxifen-injected R26CreERT;\(\text{Eng}^{2f/2f}\) mice showed dilated and tortuous vessels, and the latex dye was found in both arteries and veins, indicating the presence of AV shunts (Figure 1B in the online-only Data Supplement; n=13). However, blood vessels away from the wound in \(\text{Eng}^+\)KOs had normal morphology and latex only in arterial branches.

To determine the vascular cell type where ENG plays a critical role for the development of the vascular network at adult stages, we used 2 cell-type–specific inducible Cre lines: Scl-CreERT \(\text{Eng}^+\) for ECs and Myh11-CreERT \(\text{Eng}^+\) for SMCs. Whole mount X-Gal staining of back skin tissues collected from tamoxifen-injected Scl-CreERT(+)R26CreERT(+) mice displayed Cre activity in the vascular network (Figure 1C). The Cre activity was found in the vascular SMCs of both arteries and veins of tamoxifen-treated Scl-CreERT(+) mice as expected (Figure 1D and 1F). \(\text{Eng}^-\) deletion induced in Myh11-CreERT(+) \(\text{Eng}^{2f/2f}\) mice by tamoxifen treatment in adult stages did not result in AV shunts in any areas of the skin including the wound areas (Figure 1C).

We have previously demonstrated that induced global deletion of Alk1 in adult stages (tamoxifen-treated R26CreERT;Alk12f/2f) resulted in gastrointestinal AVMs and skin AVMs only in the wound area. We also investigated the cellular source of these malformations with the same inducible approaches used for \(\text{Eng}^+\)KOs. Tamoxifen-treated Scl-CreERT(+) \(\text{Eng}^{2f/2f}\) mice displayed multiple AV shunts in the ear and skin wound areas (Figure 1D). The vessels associated with AV shunts were tortuous, enlarged, and presented characteristic loops recapitulating those previously reported in R26CreERT;Alk12f/2f mice. These Alk1-iKO mice exhibited AVMs in gastrointestinal tract and severe hemorrhages in the cecum (Figure 1E). The survival rate of the mutant mice was \(\approx 2\) weeks on average after first tamoxifen injection, and lethality is most likely associated with gastrointestinal hemorrhages. However, tamoxifen-treated Myh11-CreERT(+) \(\text{Eng}^{2f/2f}\) mice did not display AV shunts in any areas of the skin, including the wound areas (Figure 1F).

Defects in lymphangiogenesis have been reported under global Alk1 deletion. To investigate the association between vascular malformation and lymphangiogenesis defects, lymphatic vessels neighboring to the AVMs were analyzed. As shown in Figure 1V in the online-only Data Supplement, deletion of Alk1 specifically in the vascular ECs did not affect the morphology, branch density, and caliber of lymphatic vessels. These data would indicate that the lymphatic phenotype

Nonstandard Abbreviations and Acronyms

| AVM | arteriovenous malformation |
| EC | endothelial cells |
| HHT | hereditary hemorrhagic telangiectasia |
| iKO | inducible knockout |
| SMC | smooth muscle cells |

Role of Endothelial ALK1 and ENG in AVM Development

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previously observed in tamoxifen-treated R26<sup>CreER</sup>+/+;Alk1<sup>2f/2f</sup> mice<sup>13</sup> is likely a cell-autonomous effect on lymphatic vasculature, and that vascular malformation occurs independent from lymphatic dysplasia. A dorsal skinfold window chamber system coupled with hyperspectral imaging<sup>4,14</sup> allows us to monitor the vascular remodeling and the AVM shunts intravitaly by mapping the oxygen saturation content in the blood vessels. We have tracked the process of wound-induced AVM formation in Scl-CreER<sup>+(+)/Eng<sup>2f/2f</sup> mice in parallel with Scl-CreER<sup>+(+)/Alk1<sup>2f/2f</sup> mice. Consistent with the results shown in Figure 1, AVMs developed near the wound in both models (Figure 2; Figure V in the online-only Data Supplement). AVM shunts began to appear around the day 3 as veins get dilated and arterial blood flows into veins, and characteristic looping shunts became apparent by days 8 to 9. There were individual variations in the process of AVM development in this wound-induced model. Nonetheless, the process of AVM formation in Eng-iKO (HHT1 model) and Alk1-iKO (HHT2 model) at adult
stages share common features: both require environmental stresses, such as wounding, and the vascular EC is the primary cell type pertinent to the development of AVMs. Either ENG or ALK1 in SMCs is dispensable for maintaining normal vasculature and for the formation of a normal vascular network during wound healing in adult stages. We also found some differences in these 2 HHT models: EC-specific Alk1-iKO, but not Eng-iKO, exhibited gastrointestinal bleeding and visceral AVMs. This result may indicate that pathogenetic mechanisms for gastrointestinal AVM in HHT1 may involve non-ECs. In addition, intravital imaging of wound-induced AVM development guided by hyperspectral system revealed that Eng-iKO model involves more dynamic processes for de novo AVM development when compared with Alk1-iKO model.

This result further emphasizes the importance of secondary factors, such as wound, inflammation, infections, or trauma, in the development of mucocutaneous AVMs in adult stages for both HHT1 and HHT2 models. The wound-induced mucocutaneous AVM model shown here closely mimics telangiectases forming in the nasal mucosa of patients with HHT. Identification of ECs as the primary cell type pertinent to the development of AVMs provides an important platform for studying pathogenetic mechanisms of HHT and determining the cellular target of therapy. Further molecular and cellular mechanisms by which ALK1- or ENG-deficient ECs respond to various environmental cues would provide important insights for developing therapies directed to epistaxis in patients with HHT.

Acknowledgments
We thank Drs Su and Weiser-Evans for providing the Scl-CreER and Myh11-CreER transgenic mice, respectively. We thank Haeja Choi and Mi Jung Kim for technical assistance in histological analysis and Southern blot analysis, respectively.

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most patients with HHT experience recurrent epistaxis and gastrointestinal bleeding that are a heavy burden to them because these inhibit social activities, cause anemia, and occur throughout their lifetime (it gets worse as one ages). This is the first side-by-side comparison data of HHT1 and HHT2 models for the development of gastrointestinal and mucocutaneous AVMs in adult stages. We demonstrate that for both models, a secondary factor represented by wound is required for Eng- or Alk1-deficient adult mice to develop de novo skin AVMs.

These data infer 2 therapeutic axes for inhibiting de novo AVMs: overcoming ALK1 or ENG deficiency and blocking the secondary factor (eg, angiogenesis). Our results delineate endothelial cells as the primary cell type to scrutinize molecular and cellular mechanism of ALK1 or ENG deficiency further that leads to the development of abnormal arteriovenous connections in response to secondary factors.
Common and Distinctive Pathogenetic Features of Arteriovenous Malformations in Hereditary Hemorrhagic Telangiectasia 1 and Hereditary Hemorrhagic Telangiectasia 2

Animal Models—Brief Report

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Supplementary Figures and Legends

For

Common and distinctive pathogenetic features of arteriovenous malformations in HHT1 and HHT2 animal models

Brief report

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Running title: Role of endothelial ALK1 and ENG in AVM development
Supplementary Figure I. Determination of appropriate regimen for tamoxifen-inducible Eng deletion using R26\textsuperscript{CreER} mice. A. Kaplan-Meier survival plot of R26\textsuperscript{CreER/+};Eng\textsuperscript{2f/2f} mice in response to different tamoxifen (TM) administration regimens. TM in two different dosages (2.5 mg/25 g bw and 2.5 mg/40 g bw) was injected i.p. once or 2 and 3 consecutive days into R26\textsuperscript{CreER/+};Eng\textsuperscript{2f/2f} mice. The control represent 3 days of TM (2.5 mg/25 g bw) injection into R26\textsuperscript{+/+};Eng\textsuperscript{2f/2f} mice. Day 1 represent one day after the first TM injection. B and C. Genomic Southern blot analyses demonstrating induced Alk1 (B) and Eng (C) deletion by TM administration. Numbers shown below each lane indicate consecutive days of TM injection i.p. at 2.5 mg/25g or 2.5 mg/25g body weight. The ‘1f’ band (null) indicates the band resulted from Cre-mediated recombination of the conditional ‘2f’ allele.

Supplementary Figure II. De novo skin AVMs in Eng-deficient mice are formed in response to a wound. Blood vessels visualized by latex dye injection in the back skin, ear, and cecum of TM-injected control (R26\textsuperscript{CreER/+};Eng\textsuperscript{2f/+};A) and mutant (R26\textsuperscript{CreER/+};Eng\textsuperscript{2f/2f};B) mice. Back skin and ears were subjected to wounding (indicated by asterisks). AV shunts formed around the wounds are indicated by the presence of latex in both arteries (A) and veins (V). Sites of AV shunt are indicated by arrowheads Scale bars indicate 3 mm (back skin top) and 1 mm (back skin bottom and ear).

Supplementary Figure III. Expression of Cre-recombinase in Scl-CreER and Myh11-CreER mice. A. Whole mount X-Gal staining and immunohistochemical analysis of wounded back skin of Scl-CreER(+);R26R(+), showing EC-specific TM-induced Cre recombinase activity. Panel (ii) is a magnified view of panel (i), showing X-gal-positive staining in both veins (V) and arteries (A). Immunostaining with Mucin14 (iii) and \textalpha-SMA (iv) antibodies demonstrate X-gal-positive ECs in veins and arteries, respectively, Capillaries are also X-gal-positive (iv-b). B. Whole mount X-Gal staining and immunohistochemical analysis of wounded back skin of Myh11-CreER(+);R26R(+), showing SMC-specific TM-induced Cre recombinase activity. C. Immunostaining of whole mount X-gal stained Scl-CreER(+);R26R(+) skin with Lyve1 antibodies demonstrate that Cre activity is not detected in Lyve1-positive lymphatic ECs. (arrowheads=blood vessels, arrows=lymphatic vessels). D. Peritoneal macrophages from Scl-CreER(+);R26R(+) mice showing absence of Cre activity (left panel), compared to presence of Cre activity (arrows) in macrophages from Lyz2\textsuperscript{Cre/+};R26R(+) mice used as a positive control (right panel). E and F. Histological sections of whole mount X-gal stained intestines (Ilium) from Scl-CreER(+);R26R(+) (E) and Myh11CreER(+);R26R(+) (F) mice treated with 5 dosages of TM (2.5mg/25g BW) injected i.p. Staining was performed at day 8 after first injection. Arrows in E indicate ECs in submucosal and intravillus blood vessels, while arrows in F indicate visceral and vascular SMCs. Scale bars in A-i and B-i represent 4 mm, and those in A-ii and B-ii 1 mm.

Supplementary Figure IV. Lymphatic vessels are not affected by Alk1-deletion in vascular endothelium. Whole mount immunodetection of lymphatic and blood vessels in the area surrounding a wound in the ear of Scl-CreER(+);Alk1\textsuperscript{2f/+} control (left) and
Scl-CreER(+);Alk1^{2f/2f} mutant (right) mice on day 8 after the first tamoxifen injection and wounding. Lymphatic vessels are detected with anti-Lyve-1 (green) and blood vessels are detected with anti-iB4 (red). Veins (V) and arteries (A) in the control mouse are clearly distinguished. Tortuous and dilated vessels due to AVM were found in the mutant mouse in the area around the wound (indicated by arrows with an asterisk). No apparent defects in lymphatic vessels were observed: the caliber and number of lymphatic vessels in the mutants were similar with those in controls. Scale bar represents 200 µm.

Supplementary Figure V. Real time hyperspectral imaging of de novo AVM formation in subdermal vessels of EC-specific Eng-iKO and Alk1-iKO in response to wound. Bright field intravital images (left) and corresponding hyperspectral images (right) of the wounded area of the skin located inside of a dorsal window chamber of representative Scl-CreER(+);Eng^{2f/2f} (A, n=5) and Scl-CreER(+);Alk1^{2f/2f} (B, n=5) mutants. Images show days 1-9 after the first TM administration and wounding. Arterial and venous bloods are distinguished by spectral imaging detecting oxygen content. Dotted lines are superimposed at days 1 and 9 to point out the location of the shunts (blue=veins, red=arteries, yellow=shunts). EC-Alk1-iKO vessels form AV shunts that consolidate with the time, whereas EC-Eng-iKO vessels form AV shunts that subsequently regress in a highly dynamic process during AVM formation. All images are in the same magnification, and the scale bar represents 1 mm.
Supplementary Figure I
Supplementary Figure II
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Supplementary Figure IV
Supplementary Figure V

A: Scl-CreER(+) \( \times \) Eng\(^{2f/2f} \)

B: Scl-CreER(+) \( \times \) Alk\(^{2f/2f} \)

Day 1 - Day 9
Supplementary Methods:

For

Common and distinctive pathogenetic features of arteriovenous malformations in HHT1 and HHT2 animal models

Brief report

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Running title: Role of endothelial ALK1 and ENG in AVM development
Supplementary methods

Mice
All in vivo procedures were conducted in accordance with a protocol approved by the University of Florida Institutional Animal Care and Use Committee (IACUC). The inducible smooth muscle-specific Cre transgenic mouse line Myh11-CreER\(^1\) and the inducible endothelial-specific Cre transgenic mouse line Scl-CreER\(^2\) were generously provided by Dr. Mary C.M. Weiser-Evans and Dr. Yunchao Su, respectively. Generation of Alk1\(^{2\text{loxP}}\) (Alk1\(^{2f}\)) and Eng\(^{2\text{loxP}}\) (Eng\(^{2f}\)) mice was described previously\(^3, 4\). ROSA26-CreER, R26R and Lyz2-Cre mice were acquired from The Jackson Laboratory\(^5-7\).

Tamoxifen (2.5mg in 100μL of corn oil, Sigma, St Louis, MO) was intraperitoneally injected once every five consecutive days at a dosage of 2.5mg/25g body weight (BW) for induction of CreER activity in Scl-CreER and Myh11-CreER mouse lines, and once every three consecutive days at the same dosage in the case of R26\(^{\text{CreER/++;Eng^{2f/2f}}}\) mice.

Southern blot analysis
For Southern blot analysis to distinguish the 1f allele from the 2f allele of Alk1, genomic DNAs isolated from lungs were digested with EcoRV, run on a 0.8% agarose gel, blotted onto Hybond-XL membrane (GE Healthcare Life Sciences), and then hybridized with the radiolabeled probe (chr15:101,139,523-101,140,420, GRCm38/mm10). The probe detected 9.5-kb and 8.2-kb bands for 2f and 1f alleles, respectively. For Eng Southern blot analysis, genomic DNAs isolated from lungs were digested with BamHI. The membrane was hybridized with the radiolabeled probe (chr2:32,673,062-32,673,821, GRCm38/mm10). The probe detected 4.1-kb and 3.4-kb bands for 2f and 1f alleles, respectively.

Whole mount tissue X-Gal staining
The Cre reporter mice R26R were intercrossed with Scl-Cre-ER, Myh11-Cre-ER or Lyz2\(^{\text{Cre}}\) mice to analyze Cre activity, where Cre recombination induces lacZ expression. Fresh tissues were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl\(_2\), 5 mM EGTA, and 0.02% NP-40 in phosphate buffered saline (PBS) for 10 min with gentle agitation. After washes with PBS, the tissues were incubated in 5 mM K3[Fe(CN)]6, 5 mM K4[Fe(CN)]6, 2 mM MgCl\(_2\), 0.01% sodium deoxycholate, 0.02% NP-40 and 0.75 mg/mL X-gal in PBS at 37°C overnight with gentle agitation. After washes and postfixation with 10% formalin, the samples were cleared in organic solvent (benzyl alcohol and benzyl benzoate 1:1, Sigma) before taking photographs.

Skin wound generation
Adult mice (about 2-4 months old, males and females) were anesthetized by placing the animal in an induction chamber with a flow of isoflurane gas in O\(_2\) and anesthesia was maintained at 2-3% isoflurane rate during the procedure. The back of the mouse was shaved and one 4 mm-diameter wound was inflicted on the mid-dorsum and another one on the untagged ear using a 4 mm-biopsy-punch. Povidone iodine was applied to the wounds that remained unsutured for 8 days. Tamoxifen was injected i.p. at 2.5mg/25g BW on the day of wounding and during the four following days for the
complete deletion of the floxed allele mediated by Scl-CreER and Myh11-CreER transgenes.

**Latex injection**
Eight days after wound generation, the mice were anesthetized with a mixture of ketamine/xylazine (100 mg/15 mg per kg body weight, i.p.). Abdominal and thoracic cavities were opened. Right atrium was sniped, and a solution of heparin and vasodilators (sodium nitroprusside and papaverine, Sigma) was infused into the left ventricle. Latex blue liquid rubber (Connecticut Valley Supply Co) was slowly and steadily injected into the left ventricle with a 26-gauge needle 1-ml syringe. The mice were washed and the hair of the back skin was shaved. After overnight fixation of the mice with formalin, internal organs were washed in PBS. The dorsal skin was peeled off, and stretched, flattened and post fixed again overnight in formalin, followed by brief washes with PBS. The skin hair was completely removed before dehydration in methanol series and clearing with organic solvents (benzyl alcohol and benzyl benzoate 1:1, Sigma) and images of the blood vessels containing blue latex were taken. Organs were also dehydrated and cleared in the same way.

**Immunohistochemistry**
Macrophages and skin tissues were stained with X-gal as described. For immunohistochemical staining, whole-mount X-gal-stained samples were fixed with formalin followed by paraffin embedding and sectioning. Monoclonal mouse ascites anti-α-SMA (1:800, clone 1A4, Sigma), monoclonal rat IgG2A anti-Mucin14 (1:200, clone V.7C7, Santa Cruz Biotechnology), and polyclonal rabbit anti-Lyve1 (1:500, Novus Biologicals) were used as primary antibodies for identification of smooth muscle, venous endothelial and lymphatic endothelial cells, respectively. Primary antibodies were coupled to Alkaline phosphatase-labelled secondary systems Mouse on mouse AP-Polymer (Biocare Medical), Rat on mouse AP-Polymer (Biocare Medical), and MACH2 Rabbit AP-Polymer (Biocare Medical) respectively, and detection was performed with Vulcan Fast Red Chromogen kit 2 (Biocare Medical). Sections were counterstained with hematoxylin before dehydration and later mounted with Permout.

**Macrophage extraction and culture conditions**
Peritoneal cells were harvested from the peritoneal cavity of Lyz2Cre;R26R(+) or Scl-CreER(+)R26R(+) mice. The cells were washed out with cold sterile PBS and placed in 6-well plates in 10% FBS-containing Dulbecco’s Modified Eagle Medium (DMEM). After culturing the plates 2 hours at 37°C and 5% CO₂, the cells were washed out to remove non-adherent cells. Next day, the peritoneal macrophages were subjected to brief fixation and X-gal staining before being photographed.

**Whole mount immunofluorescence**
Hair was removed from the ear and the back skin of mice and skin was peeled off and fixed in 4% paraformaldehyde (PFA) at 4°C overnight (O/N), followed by washes with PBS and permeabilization with 0.5% Triton X100, 1% bovine serum albumin (BSA) in PBS at 4°C O/N. After washing, samples were incubated with biotinylated Isolectin B4 (Sigma) in 1% Triton X100/100µM CaCl₂/100µM MgCl₂/100µM MnCl₂ in PBS at 4°C
O/N. After washing, samples were incubated with streptavidin-Alexa 594 (Invitrogen) in 0.25% Triton X100, 0.5% BSA during 6 hours at 4°C. Counterstaining was performed with rabbit polyclonal anti-Lyve1 (Novus Biologicals) and goat anti-rabbit-Alexa488 (Invitrogen) and samples were mounted with Vectashield Mounting Medium (Vector laboratories). Vascular and lymphatic vessels around the wound were visualized using an Olympus DSU-IX81 Spinning Disc Confocal microscope.

**Window chamber installation**
Scl-CreER(+) ;Alk12f/2f, Scl-CreER(+) ;Eng2f/2f, and control mice weighing more than 30g (about 4-6 months old) were subjected to window chamber installation. Tamoxifen (2.5mg/25g BW) was administered by oral gavage prior to window chamber installation. Four more dosages of TM were administered orally in the four days following to the window chamber installation for a complete deletion of the genes of interest. Mice were anesthetized with a mixture of ketamine/xylazine (100 mg/10mg per kg BW), intraperitoneally. One layer of dorsal skin was cut in a 12 mm-diameter circular area for window chamber installation. A wound was made by a 16-gauge needle at an avascular region in the centre of the window chamber and a coverslip was placed on it. Animals were housed in an environmental chamber maintained at 33°C and 50% humidity with free access to food and water and standard 12 hour light/dark cycles. All mice were monitored up to 13 days post-TM injection and sacrificed.

**Hyperspectral image acquisition and processing**
A Zeiss microscope was used as the imaging platform. A 100W tungsten halogen lamp was used for transillumination of the window chamber. Spectral image datasets were acquired with a monochrome scientific grade CCD camera thermoelectrically cooled to 20°C (DVC company). A 2.5X objective (Carl Zeiss Inc.) was utilized for imaging. Customized LabView software (National Instruments) was used to automatically acquire spectral images. Spectral image data were collected using a liquid crystal tunable filter (CRI) with a 400-720 nm pass band and 10 nm nominal bandwidth. Band-limited images were acquired from 500 nm to 575 nm at 5 nm intervals to create haemoglobin saturation pseudocolor maps from pure oxy- and deoxy-hemoglobin reference spectra as described previously. Real-time spectral / bright field images were acquired everyday up to 13 days after TM injection. All image processing was performed by MATLAB software (The Mathworks Inc.).
References:


