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Investigating how VCAM-1$^+$ sinusoidal endothelial cells regulate hematopoietic stem cell trafficking

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Susan E. Calhoun

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The thesis of Susan E. Calhoun
is approved:

Professor Glenn Millhauser, Chair

Professor Camilla Forsberg

Professor Carrie Partch

Tyrus Miller
Vice Provost and Dean of Graduate Studies

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ABSTRACT

Susan E. Calhoun

Investigating how VCAM-1$^+$ sinusoidal endothelial cells regulate hematopoietic stem cell trafficking

Although hematopoietic stem cells (HSCs) have been successfully used in transplantation therapies for more than 50 years, we still do not have a complete mechanistic understanding of the factors that regulate HSC trafficking and engraftment. Recent findings in the Forsberg lab indicate that the vascular endothelium plays important roles in regulating HSC trafficking into and out of bone marrow niches. We hypothesized that sinusoidal endothelial cells marked by the adhesion molecule VCAM-1 mediate the trafficking of HSCs from blood to bone marrow and from bone marrow to blood. Using *in vitro* transendothelial migration assays, I show that blocking VCAM-1 on HSCs, using blocking antibodies, significantly impairs the ability of HSCs to migrate through endothelial cell layers. These results suggest that VCAM-1 on HSCs is important for HSC extravasation and provide insight into what may be happening *in vivo* when HSCs must interact with endothelial cells to migrate into and out of the bone marrow.

DEDICATION

This thesis is dedicated to the people in my life whose daily support and encouragement made this all possible: Karthik Ramalingam, my parents, my family and all of my amazing lab mates. I couldn't have done this with you!

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FIGURE/EXPERIMENTAL CONTRIBUTIONS

Figure 1. (A) Schematic was adapted from the Forsberg lab group lineage tree; (B) Schematic was published in Mendelson et al., 2014

Figure 2. Schematic was adapted from Wendt et al., 2015

Figure 3. (A-D) Published in Smith-Berdan et al., 2015; (E) Schematic created by Stephanie Smith-Berdan

Figure 4. (A-G) Susan Calhoun

Figure 5. (A-D) Susan Calhoun

Figure 6. (A) Susan Calhoun; (B) Schematic adapted from Boyer et al., 2012; (C-E) Susan Calhoun

Figure 7. (A) Schematic published in Smith-Berdan et al., 2015; (B-C) Susan Calhoun

Figure 8. (A-B) Susan Calhoun

Figure 9. (A) Susan Calhoun; (B) published in Smith-Berdan et al., 2015; (C) Susan Calhoun

Figure 10. (A-B) Susan Calhoun

Figure 11. (A-B) Susan Calhoun

KEY TERMS

Nomenclature for Cell Types

HSC: Hematopoietic Stem Cell

MPP: Multipotent Progenitor

CMP: Common Myeloid Progenitor

CLP: Common Lymphoid Progenitor

MEP: Myeloid-Erythroid Progenitor

GMP: Granulocyte-Macrophage Progenitor

GM: Granulocytes/ Macrophages

RBC: Red Blood Cell

HSPC: Hematopoietic Stem and Progenitor Cells

EC: Endothelial Cell

SEC: Sinusoidal Endothelial Cell

HUVECS: Human Umbilical Vein Endothelial Cells

Molecules

VCAM-1: Vascular Cell Adhesion Molecule 1

ITGα4: Integrin alpha 4

General Terms:

IHC: Immunohistochemistry

INTRODUCTION

The hematopoietic system supplies the human body with more than 100 billion mature blood cells every day (Boulais et al., 2015). Hematopoietic stem cells (HSCs) are responsible for the constant renewal of blood by forming blood and immune cells. HSCs are located at the top of the hematopoietic hierarchy and give rise to all blood and immune cells throughout life (Figure 1A) (Boulais et al., 2015). They are capable of producing all blood cell lineages, and posses the unique ability to respond to stress and rapidly restore hematopoietic homeostasis by giving rise to needed cell types (Mendelson et al., 2014; Forsberg et al., 2009).

The bone marrow niche

Adult HSCs are primarily found in the bone marrow and it is known that HSC niches within the bone marrow provide an environment that is essential for life-long HSC function (Ugarte et al., 2013). The bone marrow niche is a local tissue microenvironment that integrates the functions of many cell types to maintain and regulate stem cells (Figure 1B) (Mendelson et al., 2014; Morrison et al., 2014; Celso et al., 2011). The integration of both intrinsic and extrinsic factors and signals allows the niche to balance HSC self-renewal, differentiation, proliferation and quiescence (Forsberg et al., 2009; Celso et al., 2011). The inherent difficulty of maintaining and expanding HSCs *in vitro* is likely due to the inability to mimic this complex microenvironment ex vivo. Indeed, the dynamic molecular interactions between HSCs and their endogenous microenvironment have yet to be fully understood

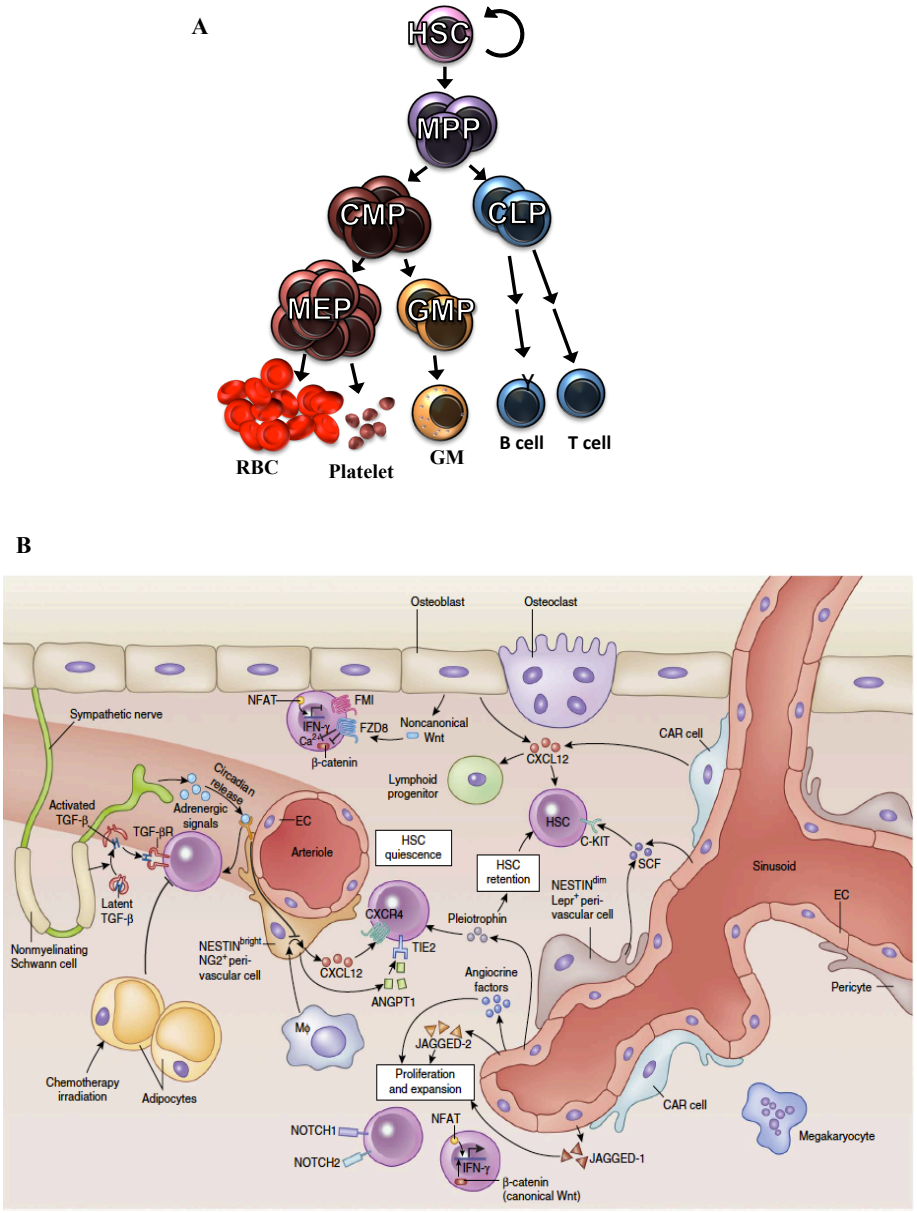

Figure 1. HSC hierarchy and the bone marrow niche
(A) Hematopoietic lineage tree shows that HSCs are multipotent, self-renewing progenitor cells that give rise to blood and immune cells. See page ix for all abbreviations. Figure adapted from Forsberg lab group lineage tree. (B) The bone marrow niche is a complex microenvironment that is composed of many cell types, chemokines, cytokines and signaling pathways that function to regulate and maintain stem cells. There are many pieces of the niche that scientists are working towards understanding and the potential for discovering new cellular interactions and how these interactions influence HSC behavior still exist. Figure published in Mendelson et al., 2014.

(Celso et al., 2011). Although expansion strategies in the presence of cytokines increase the amount of HSCs in culture, a loss of stem cell properties occurs after HSC division (Perdomo-Arciniegas et al., 2011). The majority of proliferating progeny lose regenerative potential within a few days in culture (Weidner et al., 2013). A deeper understanding of the molecular factors and key cellular players is needed to improve the culturing of HSCs for stem cell transplants.

The HSC niche has to be able to accommodate the ability of HSCs to periodically leave the bone marrow, enter the circulation and then re-enter at a later time (Celso et al., 2011). These processes are known as migration and homing. The ability of HSCs to perform these functions is exploited therapeutically during bone marrow transplantations (Celso et al., 2011). HSCs have been successfully used in transplantation therapies for more than 50 years (Smith-Berdan et al., 2011). HSC transplantation can be used to treat both genetic and acquired disorders of the hematopoietic system including cancers such as multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's lymphoma and other blood and immune diseases such as β-thalassemia and sickle cell anemia (Mendelson et al., 2014). However, despite all of the advancements made in regenerative medicine, we still do not have a complete mechanistic understanding of the factors that regulate HSC trafficking and engraftment (Smith-Berdan 2015), which are essential for successful transplants.

The role of the vasculature in HSC trafficking

Recent findings in the Forsberg lab indicate that the vascular endothelium plays important roles in regulating HSC trafficking into and out of bone marrow niches (Smith-Berdan et al., 2015). The bone marrow can be broadly divided into two general compartments: hematopoietic and stromal compartments. The hematopoietic compartment contains HSCs, hematopoietic stem and progenitor cells (HSPCs) and mature blood cells, while the stromal compartment is mostly composed of fibroblasts, mesenchymal stem cells, osteoblasts, and cells of the vascular system such as endothelial cells (Kopp et al., 2005; Winkler et al., 2010). During trafficking, HSCs, leukocytes, and other cells traverse vessel walls and therefore must interact with vascular cells (Ugarte et al., 2013; Smith-Berdan et al., 2015; Itkin et al., 2016). Bone marrow endothelial cells participate in key cellular processes such as forming the vessels necessary for the delivery of oxygen and nutrients to cells within the niche as well as providing signals that regulate HSC development and hematopoiesis (Itkin et al., 2016). These cells also regulate cellular trafficking by forming a mechanical barrier that prevents entry into and out of the bone marrow (Mazo et al., 1998; Kopp et al., 2005; Itkin et al., 2016). Active migration through vascular cells has also been suggested to involve adhesive interactions between blood cells and bone marrow endothelial cells (Mazo et al., 2008; Forsberg et al., 2009). For example, transendothelial trafficking in human umbilical vein endothelial cells (HUVECs) was previously shown to be dependent on the expression of surface receptors and

adhesion molecules, which were inducible by inflammatory cytokines (Shimizu et al. 1992; Zimmerman et al., 1992; Kopp et al., 2005).

The bone marrow vasculature: sinusoids and sinusoidal endothelial cells

The bone marrow vasculature consists of a network of thin-walled, fenestrated sinusoidal endothelial cells (SECs) and smooth-muscle arterioles (Hooper et al., 2009). The vascular surface area in the bone marrow is predominately composed of SECs (Hooper et al., 2009). Sinusoids are specialized venules containing many pores that form a network of discontinuous, fenestrated vessels throughout the bone marrow (Morrison et al., 2014). The structure of the sinusoidal wall is unique in that it is made up of a single layer of SECs and does not contain any supporting cells (Kopp et al., 2005). Therefore, due to their lack of a regular vessel wall, sinusoids have a high level of permeability that allows cells to pass into and out of the bone marrow (Kopp et al., 2005; Morrison et al., 2014).

Previous studies have shown that SECs play a critical role in the regulation of hematopoiesis (Hooper et al., 2009). Numerous studies have also shown that large numbers of HSCs and HSPCs are found near or are associated with sinusoidal vessels within the bone marrow and that HSCs are more likely than other hematopoietic cells to be immediately adjacent to a sinusoid (Wang et al., 2013; Morrison et al., 2014). The close proximity of HSCs to sinusoids seems logical when considering that SECs have a unique expression of adhesion molecules, which are thought to play a role in HSC trafficking and engraftment (Hooper et al., 2009). It has been hypothesized that

sinusoids are best suited for hematopoietic cell migration due to their permeability and high expression of adhesion molecules, such as vascular cell adhesion molecule 1 (Kopp et al., 2005; Kunisaki et al., 2014).

Vascular cell adhesion molecule 1 (VCAM-1)

VCAM-1 is an immunoglobulin-like transmembrane adhesion molecule that is highly conserved in evolution (Ulyanova et al., 2005). It was originally isolated from HUVECs and was shown to be cytokine-inducible (Terry et al., 1997). Since then, its expression has been shown to be inducible in many tissue vascular beds following injury or stress and the upregulation of VCAM-1 on endothelial cells is associated with many diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (Ulyanova et al., 2005; Koni et al., 2001). VCAM-1 is not only expressed on the surface of endothelial cells, but also cells of the osteoblast lineage, HSCs, HSPCs and certain types of hematopoietic cells such as B cells, granulocytes and macrophages (Ulyanova et al., 2005).

VCAM-1 plays many roles throughout the body beginning during development. Deletion of the *VCAM-1* gene is embryonic lethal as it is required in several tissues for development, including cardiac tissue and the placenta (Terry et al., 1997; Ulyanova et al., 2005). Expression on bone marrow stromal cells is thought to play a role in B cell development and the retention of immature lymphocytes to the bone marrow (Koni et al., 2001; Ulyanova et al., 2005). However, the most well characterized function of VCAM-1 involves its role in leukocyte trafficking into

tissues (Koni et al., 2001; Terry et al., 1997). Mature leukocytes must undergo a sequence of distinct adhesion steps to migrate into a tissue from the peripheral blood (Mazo et al., 1998). Leukocyte trafficking involves leukocytes rolling on blood vessel walls where they become activated by molecular cues, firm adherence to vessel cells, and finally transmigration into the tissue (Koni et al., 2001). The primary tethering event is mediated by selectins, which allow cells to slowly roll along the surface of endothelial cells (Mazo et al., 1998). Integrin activation and upregulation occurs when a rolling cell encounters a chemoattractant, which binds to surface receptors on the cell and triggers an intracellular signaling cascade (Mazo et al., 1998). The integrin then binds to receptors on endothelial cells, which allows the leukocyte to stop rolling and adhere to the endothelial cell before migrating through the blood vessel wall into the tissue (Mazo et al., 1998). Previous studies have shown that VCAM-1 mediates the rolling and adherence of leukocytes to endothelial cells (Koni et al., 2001). This involvement suggests that VCAM-1 may also play a similar role in the trafficking of other hematopoietic cells into the bone marrow.

The role of VCAM-1 in HSC extravasation

The trafficking of HSCs from the blood to the bone marrow and vice versa appears to involve a cascade of events similar to that of leukocyte extravasation (Laird et al., 2008). In order to migrate into the bone marrow, HSCs must be able to adhere to vessel walls with enough strength to overcome the stress exerted by the flowing blood of the circulation (Laird et al., 2008). In leukocyte trafficking, the

primary tethering event is mediated by selectins (Mazo et al., 1998). However, in the process of hematopoietic cell recruitment from the peripheral blood to the bone marrow, VCAM-1 on bone marrow microvessels has been shown to have an overlapping role with selectins (Koni et al., 2001). Previous studies that looked at selectin-independent rolling of HSPCs in the bone marrow microvessels in the skulls of mice showed that the interaction between VCAM-1 and its main binding partner, integrin α4 (ITGα4), could initiate and sustain cell-cell interactions in bone marrow venules and sinusoids (Mazo et al., 1998; Celso et al., 2011). These studies also showed that ITGα4 /VCAM1 is the major selectin-independent pathway for rolling of HSPCs on bone marrow microvessels (Mazo et al., 1998; Celso et al., 2011). Whether or not the same holds true for HSCs and the exact route of entry remain to be discovered. A schematic of how this process may occur in HSCs can be seen in Figure 2.

A recent discovery in the Forsberg lab suggested that SECs expressing both VCAM-1 and Robo4 promote HSC extravasation (Smith-Berdan et al., 2015). Robo4 is single transmembrane cell-surface receptor that is a member of the Robo family of guidance receptors that respond to secreted proteins (Brose et al., 1999; Long et al., 2004). It is expressed on both endothelial cells and HSCs (Smith-Berdan et al., 2011 and 2015).

Smith-Berdan et al. 2015 showed that wild type HSCs engraft poorly in mice lacking endothelial Robo4, which led to the discovery that a distinct subpopulation of

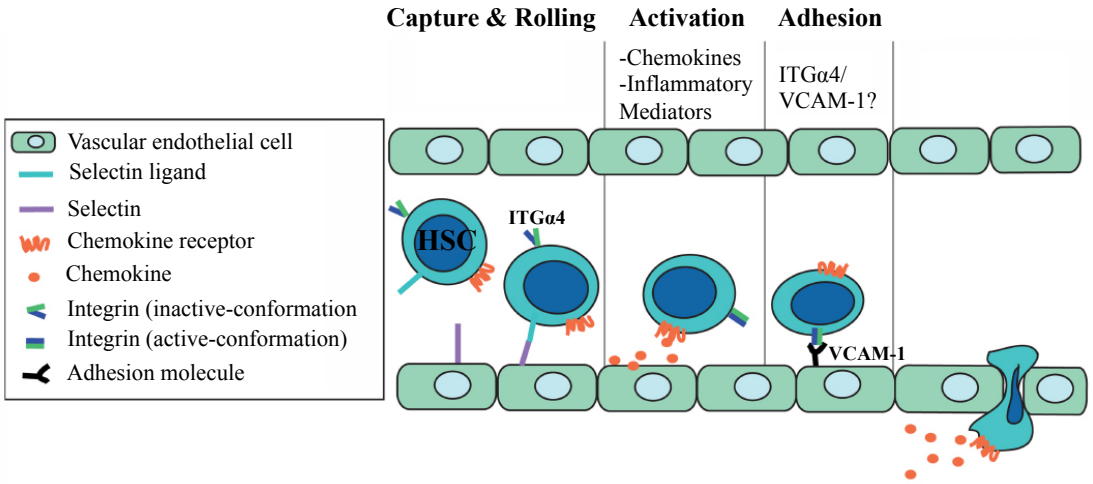

Figure 2. Proposed mechanism for HSC extravasation
The trafficking of HSCs into and out of the bone marrow is believed to involve a similar sequence of events to that of leukocyte extravasation. HSCs may undergo a selectin-dependent pathway in which it is believed that the interaction between ITGα4 and VCAM-1 is crucial for both capture and adhesion. Whether or not the expression of ITGα4 and VCAM-1 on HSCs, vascular endothelial cells, or both play a role in this process remains to be discovered. Figure adapted from Wendt et al., 2015.

bone marrow endothelial cells in wild type mice express high levels of VCAM-1. This population was drastically decreased in *Robo4* knock out (*Robo4*$^{-/-}$) mice (Figure 3A) and further characterization of this VCAM-1^{+} population revealed that these cells are likely SECs (Figure 3B) (Smith-Berdan et al., 2015). To fully understand this finding, the consequences of *Robo4* deletion on the bone marrow vasculature were also investigated. The number of VCAM-1^{+} SECs was significantly reduced and the bone marrow vasculature was also poorly developed in *Robo4*$^{-/-}$ mice (Figures 3A and 3C) (Smith-Berdan et al., 2015). When blocking antibodies to ITGα4, the binding partner of VCAM-1, were applied to endothelial cells in transwell migration assays, HSC transendothelial migration was significantly impaired (Figure 3D) (Smith-Berdan et al., 2015). This finding is consistent with a direct role for VCAM-1 in HSC extravasation (Smith-Berdan et al., 2015). Furthermore, endothelial Robo4 was found to be necessary for HSC translocation across vessel walls and impaired HSC extravasation from the blood to the bone marrow was a cause of poor engraftment in *Robo4*$^{-/-}$ mice (Smith-Berdan et al., 2015). Collectively, these results suggested that SECs play important roles in HSC extravasation and engraftment. For the current study, we hypothesized that SECs, as marked by VCAM-1, mediate the trafficking of hematopoietic stem cells from blood to bone marrow and from bone marrow to blood (Figure 3E).

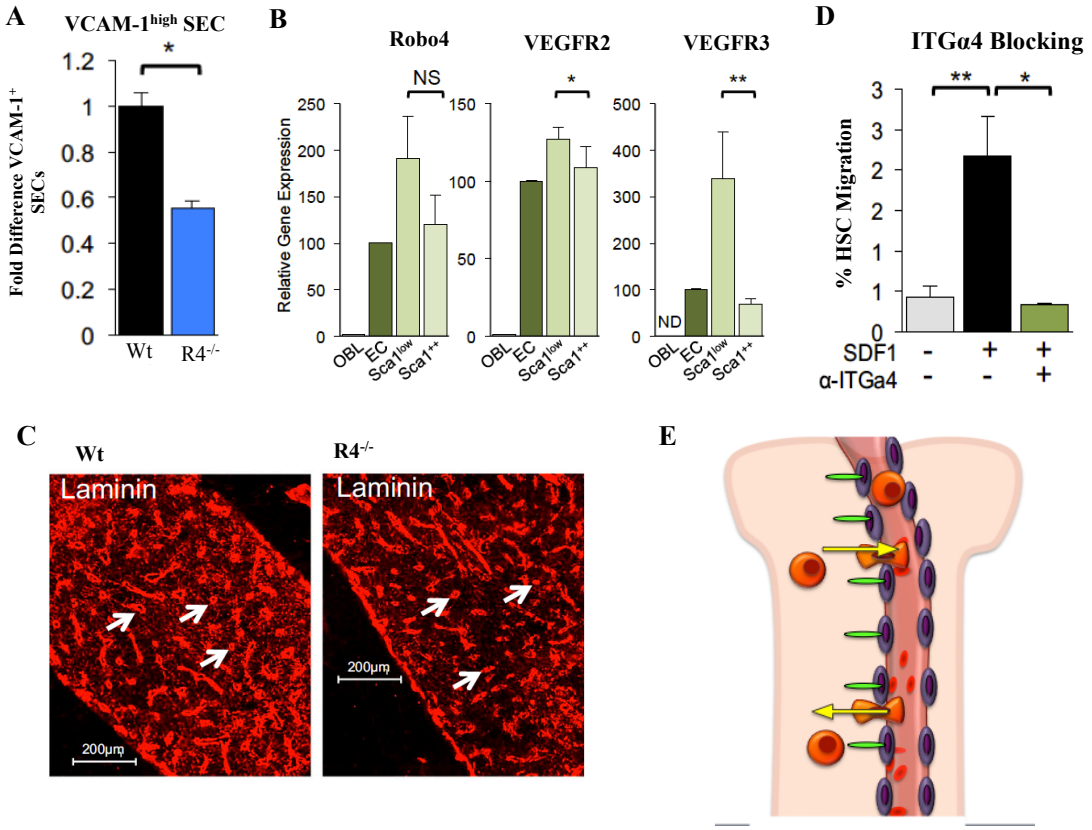

Figure 3. Characterization of bone marrow endothelial cells in wild type and *Robo4*$^{-/-}$ mice

(A) Flow cytometry analysis of bone marrow ECs from wild type (wt) and *Robo4*$^{-/-}$ mice (R4$^{-/-}$) revealed that total numbers of VCAM-1^{+} SECs were decreased in *Robo4*$^{-/-}$ mice. (B) Relative gene expression of Robo4, VEGFR2 and VEGFR3 in sorted bone marrow cell populations by quantitative RT-PCR. SECs (CD45^{-}/Ter119^{-}/CD31^{+}/Tie2^{+}/Sca1^{low}) show robust expression of VEGFR3 compared to all other populations confirming that these Sca1^{low} cells are in fact SECs. Sca1^{++} (or Sca1^{high}) cells were shown to be arteriole cells. (C) Fluorescence microscopy analysis of bone marrow sections shows poorly formed sinusoids (white arrows) in *Robo4*$^{-/-}$ mice compared to wild type. Bone marrow sections were stained with α-laminin. (D) Preincubation of wild type EC monolayers with anti-ITGα4 inhibits HSC transendothelial migration *in vitro*. (E) Schematic of the hypothesis for the current study. We believe that SECs expressing VCAM-1 mediate the trafficking of HSCs from blood to bone marrow and from bone marrow to blood. SECs lining sinusoids within the vasculature are shown in purple, VCAM-1 expressed on SECs is shown in green, and HSCs are shown in orange. Figure designed by Stephanie Smith-Berdan. All experiments in this figure were designed and performed by Stephanie Smith-Berdan and published in Smith-Berdan et al., 2015

RESULTS

Endothelial-specific dyes can be used to label specialized endothelial sub-populations in wild type mice

Since we hypothesize that specific endothelial cell (EC) subsets mediate the trafficking of HSCs from the blood to the bone marrow, we aimed to identify an approach to independently label these distinct populations using cell type-specific dyes, rather than a very complex set of cell surface markers. Our goal was to label specific cell types, such as SECs and arterioles, using single colors for both flow cytometry, which would allow us to quantitate cell numbers under different conditions and sort them for functional assays or for RNA content, and for immunohistochemistry (IHC), in which we could determine how vascular structures are affected under different conditions and identify which cells interact with these structures. In addition, we also wanted to find a way to identify the population of SECs that are VCAM-1^{+}, without using VCAM-1 as the marker. This would allow us to assess the number and organization of SECs in *VCAM-*$1^{-/-}$ mice by both flow cytometry and IHC. Currently, the panel of antibodies needed to identify distinct EC subsets, such as arteriole cells versus SECs, is quite extensive. Between the two subsets, there is differential expression of only two markers, VEGFR3 and Sca1 (Hooper et al., 2009). Although the expression of VEGFR3 is generally considered to be specific to SECs, the expression pattern of Sca1 on ECs is not as well defined. Some groups report that SECs are Sca1^{-} while others report that SECs are actually Sca1^{+} but at lower levels than arterioles (Hooper et al., 2009; Smith-Berdan et al.,

2015). Sca1 is also expressed on many hematopoietic cells so it can be very hard to use as a single marker for differentiating between cell types (Holmes et al. 2007). The VEGFR3 antibody that is currently available does not work well for flow cytometry analysis so we aimed to find a different way of definitively identifying SECs, as well as arterioles, that could be used for both flow cytometry and IHC.

The specificity of two fluorescent dyes was tested in wild type mice *in vivo* to test whether the dyes were specifically labeling the presumed cell types. Sinusoidal specific Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) (Kunisaki et al., 2013; Li et al., 2009) and arteriole specific Alexa Fluor 633 hydrazide (Alexa 633) (Kunisaki et al., 2013; Shen et al., 2012) were retro-orbitally injected into wild type mice. I harvested femurs and tibias four hours after injection and prepared them for either flow cytometry analysis or IHC. Using known surface markers for sinusoidal and arteriole cells, the identity of the cells labeled with Dil-Ac-LDL or Alexa 633 was determined. This allowed us to determine whether one or a combination of the two stains could be used to identify subpopulations of bone marrow vascular cells and whether they could be used as a substitute for the VCAM-1 marker on SECs in *VCAM-1*$^{-/-}$ mice.

Flow cytometry analysis revealed that the Dil-Ac-LDL labeled ECs (CD45^{-} /Ter119^{-}/CD31^{+}/Sca1^{+}) that were highly positive for VCAM-1 by cell surface expression (Figure 4A). In this stain CD45 is used to label hematopoietic cells, Ter119 labels erythrocytes and cells in the erythroid lineage, and CD31 labels vascular cells. ECs expressing high levels of VCAM-1 were previously shown to

possess all of the characteristics of SECs, including high VEGFR3 expression (Figure 3B). Thus, Dil-Ac-LDL is accurately labeling the VCAM-1^+ SECs by flow cytometry. However, the dye does not appear to be working when observed by IHC (Figure 4B). Unlike previous published data (Kunisaki et al., 2013) the dye does not seem to be specifically labeling SECs in bone marrow sections, despite using the same protocol on multiple attempts. Rather than only labeling SECs, Dil-Ac-LDL appears to be weakly labeling all cells and no sinusoid like structures can be seen within the bone marrow (Figure 4B). Therefore, we sought to establish another method of labeling SECs by IHC. Previous studies have shown that two other well-characterized markers could be used to label SECs in the bone marrow, laminin and VEGFR3 (Smith-Berdan et al., 2015; Hooper et al., 2009). Laminin labels the basal lamina of all blood vessel types in the bone marrow, so while it does label SECs, it will also label arterioles as well (Nombela-Arrieta et al., 2013; Acar et al., 2015). As VEGFR3 has been shown to be specific for SECs and works well for IHC (Hooper et al., 2009), we tested whether VEGFR3 could be used to specifically label SECs in our hands. Previous attempts to use VEGFR3 by IHC had failed in the lab. However, an in-depth literature review revealed that an antigen retrieval protocol had to be performed on the fixed bone sections in order for the stain to work. After performing an antigen retrieval protocol, the VEGFR3 staining closely matched that of laminin where SECs were labeled (Figure 4C). Therefore, rather than using Dil-Ac-LDL, VEGFR3 can be used to specifically label SECs by IHC, while Dil-Ac-LDL can be used to label SECs

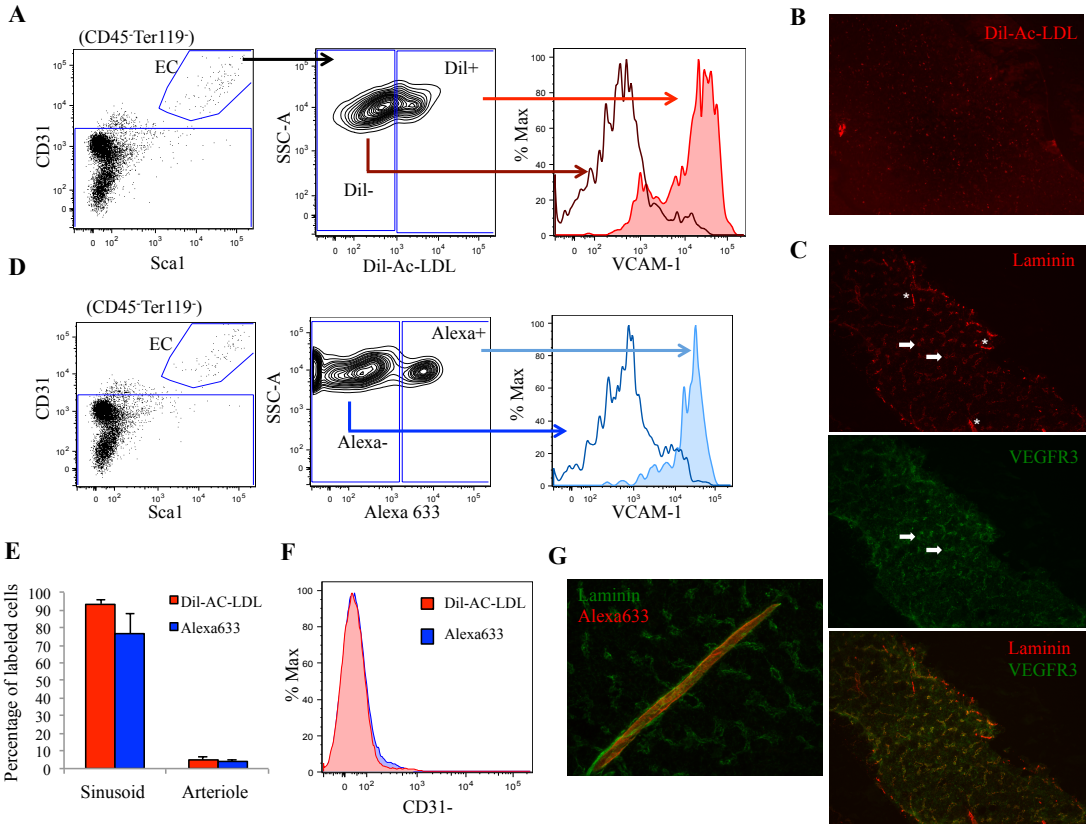

Figure 4. Identifying specialized endothelial subpopulations using endothelial-specific dyes

(A) Flow cytometry analysis of endothelial sub-populations being stained by sinusoid specific Dil-Ac-LDL revealed that Vcam1^{high} SECs are being stained by this method. (B) Dil-Ac-LDL appears to be labeling all cells within the bone marrow and no sinusoid structures can be seen by fluorescence microscopy. (C) VEGFR3 co-stains with laminin on SECs by fluorescence microscopy. Wild type bone marrow sections were stained with α-laminin and α-VEGFR3 antibodies. Individual channels and the overlay are shown. Asterisks denote arteriole cells and arrows point towards sinusoids. (D) Flow cytometry analysis of endothelial sub-populations being stained by arteriole "specific" Alexa 633 revealed that Vcam1^{high} SECs are being stained by Alexa 633 rather than Vcam^{low} arterioles (E) Quantification of sub-populations being stained by each dye by flow cytometry analysis. (F) Both Dil-AC-LDL and Alexa 633 show specificity for endothelial cells and are not staining CD31^{-} stromal cells. (G) IHC staining of Alexa 633 shows specificity for arteriole cells.

Error bars represent SEM.

Experiments designed and performed by Susan Calhoun

for flow cytometry analysis. By using two independent approaches to measure SECs, flow cytometry and IHC, we will be able determine whether *VCAM-1*$^{-/-}$ mice contain fewer sinusoidal cells, and whether the structural integrity of vessels made up of these cells is altered.

We next tested the specificity of Alexa 633 to determine if it was specifically labeling arteriole cells (CD45$^-$/Ter119$^-$/CD31$^+$/Sca1$^+$/VCAM-1^{low}) *in vivo*. Flow cytometry analysis revealed that Alexa 633 stained cells in the SEC compartment, rather than staining VCAM-1^{low} arteriole cells (Figure 4D and 4E). To determine if Alexa 633 was staining other types of stromal cells, such as osteoblasts (CD45$^-$/Ter119$^-$/CD31$^-$/Sca1$^-$/CD51$^+$) or mesenchymal stem cells (CD45$^-$/Ter119$^-$/CD31$^-$/Sca1$^+$/CD51$^+$) (Winkler et al., 2010) the CD31$^-$ compartment was examined. Both Dil-Ac-LDL and Alexa 633 showed specificity for vascular ECs (CD31$^+$) in the stromal compartment (Figure 4F). However, by IHC staining, Alexa 633 did appear to be exclusively staining arteriolar vessels in the bone marrow (Figure 4G). It is important to note that Alexa 633 has only previously been used for IHC and has not been used for flow cytometry analysis (Kunisaki et al., 2013; Shen et al., 2012). Since Alexa 633 binds to elastin fibers on arterioles (Shen et al., 2012), it is possible that the processing of the bones during cell preparation for flow cytometry analysis, which involves physically crushing the bones and digestion with collagenase, disrupts this bond. Therefore, a different method for labeling arterioles using a single color by flow cytometry analysis still needs to be determined. Taken together, the results from our dye labeling experiments indicate that Dil-Ac-LDL will be used for labeling

SECs by flow cytometry, while VEGFR3 and Alexa 633 will be used by IHC to label SECs and arterioles, respectively. These labeling methods will be very useful for quantifying and visualizing the vasculature of the bone marrow in *VCAM-1*$^{-/-}$ mice using both flow cytometry and microscopy in future studies.

Generating inducible mouse models with total deletion of VCAM-1 and endothelial cell specific deletion

To understand the effects that deleting VCAM-1 has on HSC extravasation, we decided to generate inducible VCAM-1 knockout mouse models. We recently discovered that there were far fewer VCAM1^{+} SECs in *Robo4*$^{-/-}$ mice and that these mice had impaired HSC extravasation from the blood to the bone marrow (Smith-Berdan et al., 2015). VCAM-1 is expressed on the surface of endothelial cells (Celso et al., 2011) and since HSCs must interact with the vascular endothelium during extravasation (Smith-Berdan et al., 2015), we hypothesized that VCAM-1 is important for this. We also found that blocking integrin α4 (ITGα4), the binding partner of VCAM-1, impaired HSC transendothelial migration in transwell assays (Smith-Berdan et al., 2015) (Figure 3D). This suggests that VCAM-1 has a direct role in HSC extravasation. However, since both HSCs and ECs express VCAM-1 and ITGα4, we do not know whether VCAM-1 on HSCs or ECs, or both, is necessary for efficient HSC extravasation.

In order to address this issue, we generated inducible VCAM-1 knockout mouse models using two different CreERT2 transgenic mouse lines. Inactivation of

genes in a tissue-specific manner is dependent on the ability of recombinases to induce the specific deletion of a gene that has been flanked by recombination sites, also known as loxP sites in this system (Terry et al., 1997). Cre recombinase is a 38kDa enzyme derived from the P1 bacteriophage that catalyzes recombination between two 34-base pair DNA recognition sites (loxP sites) (Feil et al., 2009; Terry et al., 1997). It is highly efficient at removing the intervening DNA, which generates a null allele in all cells where Cre is active and all future descendants of such cells (Feil et al., 2009). CreERT2 recombinases are ligand-dependent chimeric Cre recombinases that have been developed to allow for inducible gene deletion by treating the mouse with tamoxifen (Feil et al., 2009). CreERT2 contains a mutated version of the human estrogen receptor ligand-binding domain, which is highly sensitive to tamoxifen, but not to natural estrogens or other physiological steroids (Feil et al., 2009; Monvoisin et al., 2006). An advantage of this system is decreased background activity (recombination) when tamoxifen is not present (Feil et al., 2009). Figure 5A depicts a schematic of the CreERT2/loxP system. When no tamoxifen is present, CreERT2 is retained in the cytoplasm by heat shock protein 90 (hsp90) (Monvoisin et al., 2006). The binding of tamoxifen to ERT2 disrupts the interaction with hsp90 and allows Cre to be transported into the nucleus where it can recombine the loxP flanked DNA and thus excise the targeted gene (Figure 5A) (Monvoisin et al., 2006; Feil et al., 2009).

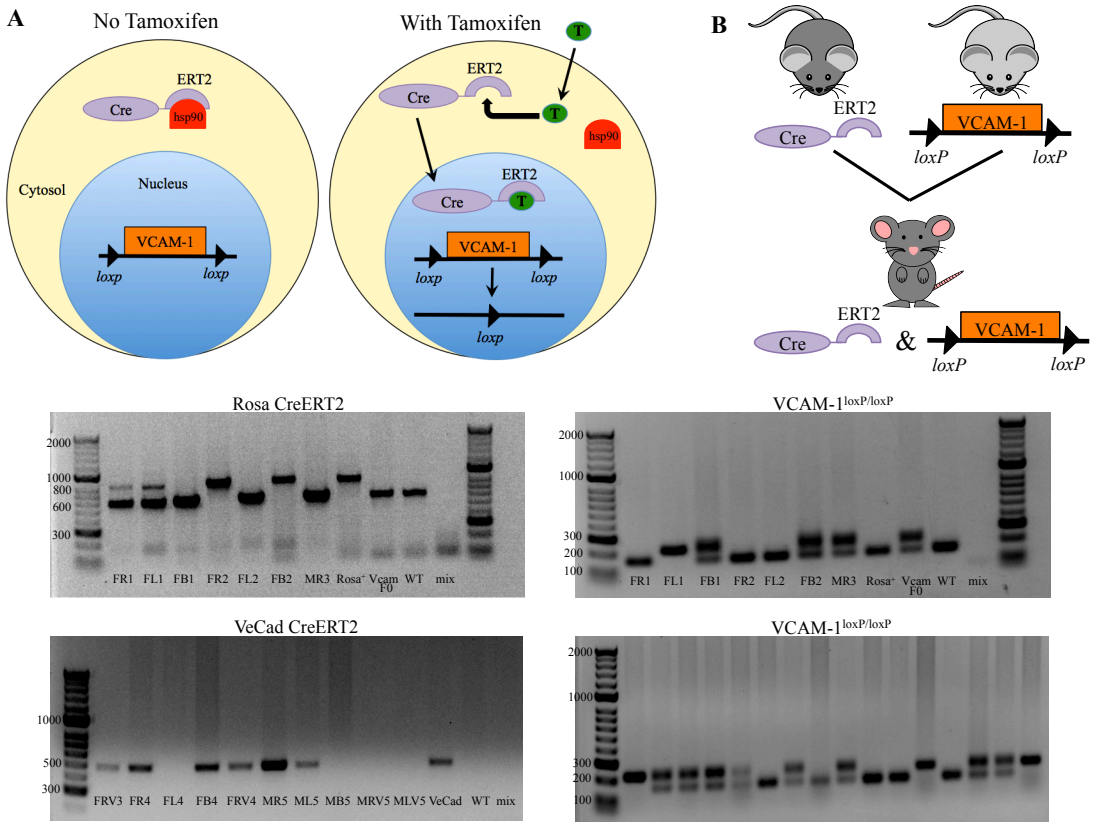

Figure 5. Generating inducible VCAM-1 knockout mouse models using CreERT2 mouse lines

(A) Schematic of how tamoxifen inducible CreERT2 recombinase works. Green circle represents tamoxifen. (B) Schematic of how different inducible CreERT2 mouse lines can be crossed to our *VCAM-1*$^{loxP/loxP}$ mouse to generate cell specific *VCAM-1* knockout mice. (C) Mice that were heterozygous for both Rosa-CreERT2 and *VCAM-1*$^{loxP/loxP}$ were crossed to each other and the resulting PCR analysis of genomic DNA isolated from the ears of 4-week-old pups is shown. Mice homozygote for Rosa-CreERT2 should have a band at 825 base pairs (bp) while heterozygotes will have bands at 825 bp and 603 bp. Mice homozygous for the floxed *VCAM-1* allele should have a band at 226 bp while heterozygotes will have bands at 226 bp and 162 bp. (D) Mice that were positive for VeCad-CreERT2 and heterozygous for *VCAM-1*$^{loxP/loxP}$ were crossed to each other and the resulting PCR analysis of genomic DNA isolated from the ears of 4-week-old pups is shown. Mice containing the VeCad-CreERT2 should have a band at 548 bp. Mice homozygote for the floxed *VCAM-1* allele should have a band at 226 bp while heterozygotes will have bands at 226 bp and 162 bp.

Genotyping performed by Susan Calhoun

In this study, two different CreERT2 mouse lines were crossed to *VCAM-1*$^{loxP/loxP}$ mice. The *VCAM-1*$^{loxP/loxP}$ mice contain loxP sites on either side of the promoter region and exon 1 of the *VCAM-1* gene and therefore yields deletion of the *VCAM-1* gene promoter and the first exon when crossed to a Cre recombinase mouse (Koni et al., 2001). Previous attempts to further investigate the roles of VCAM-1 using non-inducible genetic knock out mice were met with limited success because *VCAM-1* deficiency during fetal development causes embryonic lethality (Gurtner et al., 1995; Terry et al., 1997; Koni et al., 2001). Development of a conditional *VCAM-1* knockout mouse was therefore needed so that the effects of adult and cell type-specific VCAM-1 deletion could be studied. Figure 5B shows a schematic of the creation of the double transgenic. These mice will be useful for future studies in which the deletion of VCAM-1 from specific cell types is being investigated.

To create a mouse with an EC-specific deletion of VCAM-1, we crossed a VE-cadherin-CreERT2 (VeCad-CreERT2) mouse to a *VCAM-1*$^{loxP/loxP}$ mouse. The VeCad-CreERT2 mouse is under the regulation of the vascular endothelial cadherin (VeCad) promoter and has been previously shown to be very useful for knockout studies targeting the endothelium (Monvoisin et al., 2006; Wang et al; 2010). These mice were given to us by Eugene Butcher, MD from Stanford University. Unfortunately, when these mice arrived they were not on the same genetic background as the *VCAM-1*$^{loxP/loxP}$ mice. In order to get these mice on the same genetic background, which would be essential for all comparative studies as well as

future transplantation studies, they had to be backcrossed to wild type mice (Black 6) for seven generations. During this time, the specificity of the Cre was investigated to determine if expression of Cre was specific to ECs and if there was any background activity when tamoxifen was not present. To determine the specificity of the Cre, I sorted ECs and B cells from VeCad-CreERT2 mice and performed Quantitative RT-PCR to determine Cre mRNA levels in each cell type. Our analysis revealed that only ECs expressed robust levels of Cre (Figure 6A). To further confirm the specificity of the Cre expression in ECs and also determine whether the Cre was being activated in the absence of tamoxifen, VeCad-CreERT2 mice were crossed to mT/mG dual-color reporter mice. The mT/mG mice contain a dual-color cassette inserted into the Rosa26 locus, which allows for ubiquitous reporter expression (Figure 6B) (Muzumdar et al., 2007; Boyer et al., 2012;). Upon Cre activation, all cells in which the Cre is expressed, and their descendants, will switch from expression of tomato fluorescent protein (TOM) to green fluorescent protein (GFP) (Figure 6B). Flow cytometry analysis of bone marrow ECs showed that between 50-70% of VCAM-1^{+} ECs expressed GFP when Cre was activated by the administration of tamoxifen compared to less than 5% of ECs expressing GFP from control when no tamoxifen was administered (Figure 6C). To check for off target Cre activation, I also assayed hematopoietic stem and progenitor cells, also known as KLS ($cKit^{+}/Lin^{-}/Sca1^{+}$), B cells, T cells, and granulocytes/macrophages (GM). Analysis revealed that none of these cell types showed any significant GFP expression when treated with tamoxifen (Figures 6D and 6E). Controls were transgenic mice that were not treated with

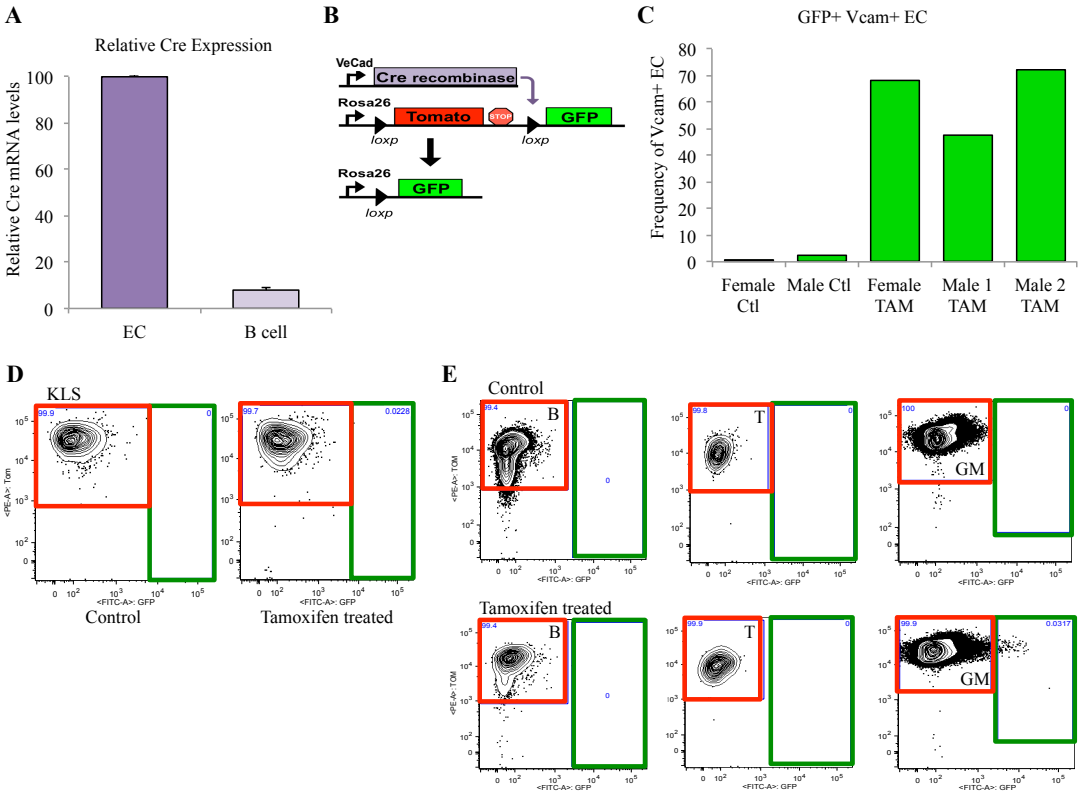

Figure 6. VeCad-CreERT2 mice can be used to generate a mouse model with endothelial specific deletion of VCAM-1

(A) ECs isolated from VeCad-CreERT2 mice express robust mRNA levels of Cre while B cells show minimal expression. (B) VeCad-CreERT2 mice were crossed to mT/mG dual color reporter mice to determine cell specific expression of Cre. Figure adapted from Boyer et al., 2012. (C) Flow cytometry analysis of bone marrow EC populations, KLS populations (D) and mature blood cells: B cells, T cells, and GMs (E) from VeCad-CreERT2 crossed to mT/mG mice reveal that there is no off target expression of Cre when tamoxifen (TAM) is administered. Controls were transgenic mice that were not treated with tamoxifen.

Experiments designed and performed by Susan Calhoun

tamoxifen. Taken together, these results suggest that our VeCad-CreERT2 mice can be used to generate a mouse model with endothelial-specific deletion of VCAM-1.

Since both HSCs and ECs express VCAM-1, we also wanted to create a mouse model in which we could delete VCAM-1 from HSCs. In order to accomplish this, we crossed a Rosa-CreERT2 mouse to a *VCAM-1*$^{loxP/loxP}$ mouse. Although this cross will result in a total deletion of VCAM-1 from all cell types, we reasoned that we could start by using this mouse model to isolate HSCs that do not express VCAM-1. These cells will be useful in future migration and transplantation assays where we would like to observe the effects of deleting VCAM-1 on HSCs. Ultimately, the goal is to cross the *VCAM-1*$^{loxP/loxP}$ mouse to a Cre strain that is hematopoietic-specific such as a Vav-CreERT2 mouse. Currently, both crosses of mice, VeCad-CreERT2 crossed to *VCAM-1*$^{loxP/loxP}$ and Rosa-CreERT2 crossed to *VCAM-1*$^{loxP/loxP}$, are breeding to build up the colonies. In order for the mice to be useable, they need to contain both transgenes so that they are homozygous for the floxed *VCAM-1* allele and also contain the respective Cre alleles. Genotyping data for all crosses can be seen in Figures 5C and 5D. Once these mice are on a homozygous background, they will be used for future migration and transplantation assays in which the affects of VCAM-1 deletion on either ECs or HSCs are investigated in both *in vitro* and *in vivo* assays.

Our previous studies showed that transendothelial migration of HSCs across wild type EC layers *in vitro* was blocked by pre-incubation of ECs with an antibody to the VCAM-1 binding protein ITGα4 (Figure 3D) (Smith-Berdan et al., 2015). This

suggests that VCAM-1 plays a direct role in HSC extravasation. We know that VCAM-1 and ITGα4 are expressed on both ECs and HSCs, but it is not known if expression on one cell type matters more than the other. In an attempt to answer this, we decided to use *in vitro* migration assays using blocking antibodies to directly test whether VCAM-1 on HSCs or ECs is important for HSC extravasation. We also wanted to expand on our previous work and see if blocking ITGα4 on HSCs had the same effect on HSC migration as when it is blocked on ECs.

Blocking VCAM-1 on HSCs has no effect on active migration *in vitro* when EC layers are not present

In order to directly test whether VCAM-1 promotes active extravasation *in vitro* and whether ITGα4 matters on HSCs, we blocked VCAM-1 and ITGα4 on hematopoietic cells using blocking antibodies and analyzed their ability to migrate through transwell membranes. Wild type lineage-depleted bone marrow cells were pre-incubated with anti-VCAM-1 or anti-ITGα4 before being placed in the upper compartment of the transwell. The ability of lineage-depleted cells to migrate through the porous membrane towards the chemoattractant SDF1 was assessed by flow cytometry analysis of the cells that migrated to the bottom chamber (Figure 7A). One of the strengths of this assay is the ability to quantify different cell types from the same well to see if cells other than HSCs are affected, such as B cells or multipotent progenitors (MPPs) (Figure 9C). In these experiments we also aimed to determine if

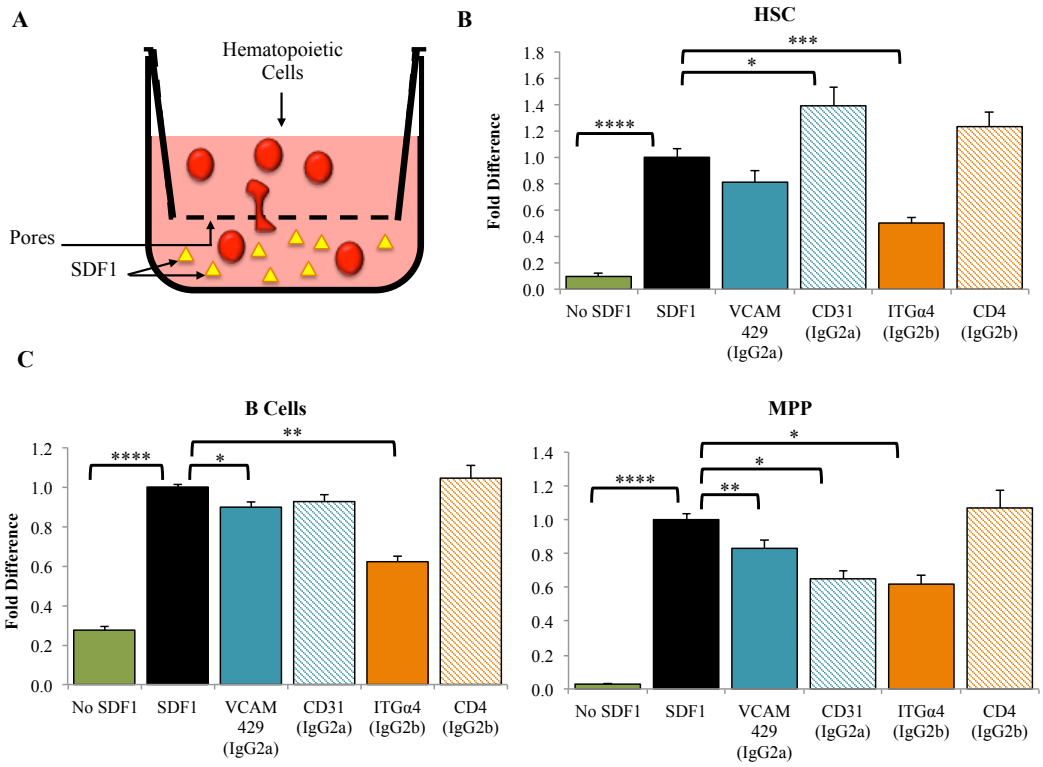

Figure 7. Blocking VCAM-1 on HSCs has no effect on active migration *in vitro* when EC layers are not present

(A) Schematic of experimental set up to measure active cell migration across transwell inserts lacking an EC layer (created by Stephanie Smith-Berdan) (B) VCAM-1 blocking antibody applied to hematopoietic cells has no significant effect on active migration of HSCs towards SDF1 in vitro but blocking ITGα4 significantly inhibits the migration of HSCs (C) The migration of B cells and MPPs were also affected when these cells were pretreated with VCAM-1 and ITGα4 antibodies. All cell types were quantified from the same well. n= 5 independent experiments with each condition performed in triplicate. Error bars represent SEM. P values were determined using a paired t test *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005 All experiments performed by Susan Calhoun

we could identify a different control for the VCAM-1 429 clone since the IgG2 isotype control used in previous experiments was having an effect on migration. We decided to test CD31 as a control for VCAM-1 clone 429 because it is expressed on the surface of hematopoietic cells (Baumann et al., 2004; Smith-Berdan et al., 2011) and was previously shown to have no effect on HSC transendothelial migration in transwell assays when ECs were pre-treated with anti-CD31 antibody (Smith-Berdan et al., 2015). It is also the same IgG class as the VCAM-1 429 clone, so we reasoned that it could serve as a useful control in these assays. CD4 served as the control for ITGA4 as it is not expressed on HSCs or ECs and is the same IgG class as the ITGα4 antibody. Blocking VCAM-1 had no significant effect on the migration of HSCs, while significantly impairing the ability of B cells and MPPs to migrate. (Figures 7B and 7C). Blocking ITGα4 on hematopoietic cells significantly impaired the migration of HSCs, B cells and MPPs in transwell assays (Figures 7B and 7C). These results support our previous finding that ITGα4 plays a key role in the migration of hematopoietic cells and its expression appears to be important on both ECs and HSCs.

Since HSCs express higher levels of VCAM-1 than B cells and MPPs (Lai et al., 2005; Ulyanova et al., 2005), we reasoned that the concentration of anti-VCAM-1 was not sufficient to have a measureable effect on HSC migration. As B cells and MPPs express lower levels of VCAM-1, it is possible that the effect was more pronounced in these cell types, and that an effect on HSCs was not observed because saturating conditions were not achieved. Increasing the concentration of anti-VCAM-

1 by three fold had no effect on the migration of HSCs, B Cells or MPPs when compared to the original concentration of 10 ug/ml (Figures 8A and 8B). Therefore, the results that we observed in our previous experiments were not due to a concentration dependent effect. Although we no longer saw a significant effect on the migration of B cells and MPPs, this could be due to the amount of replicates that were done. For the first set of experiments in which we saw a significant decrease in migration for B cells and MPPs, those results are representative of five individual experiments, while the results from increasing the concentration of anti-VCAM-1 are from two individual experiments. The results are trending toward B cells and MPPs having decreased migration but there are not enough replicates for it to be significant. Together, these results suggest that blocking VCAM-1 on HSCs does not significantly affect HSCs that are not undergoing transendothelial migration. However, a better understanding of what may be going on would be obtained through transendothelial migration assays using cultured ECs since this scenario closely mimics what is seen *in vivo*.

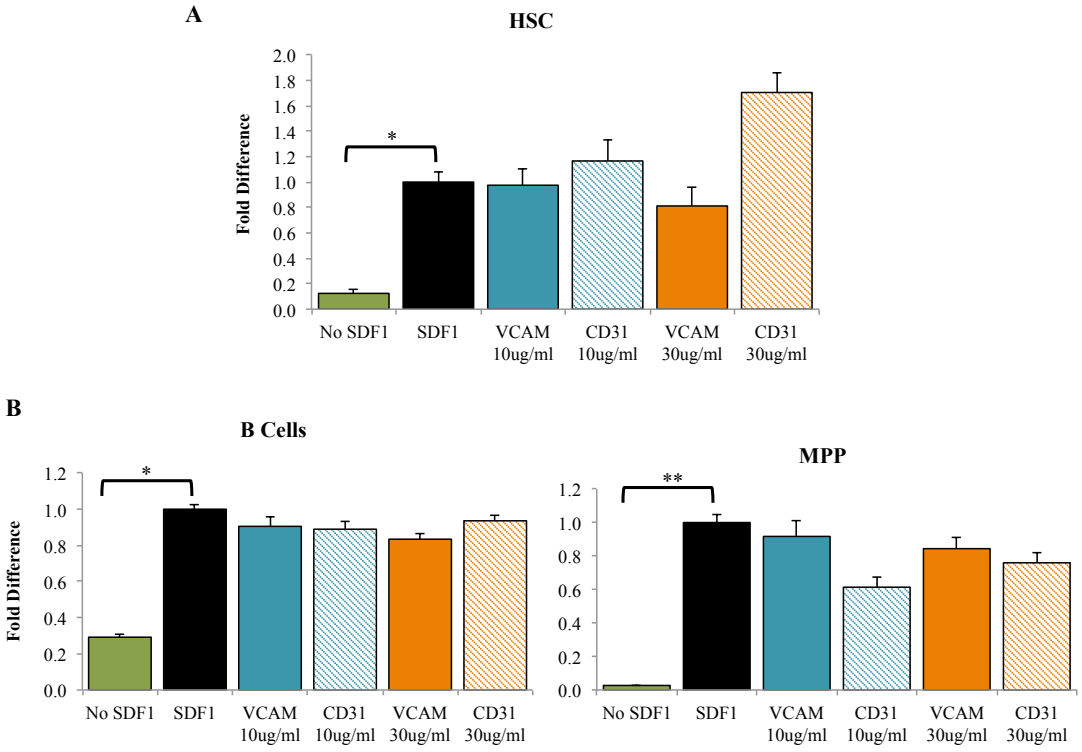

Figure 8. Increasing the concentration of VCAM-1 antibody has no significant effect on active migration

(A) VCAM-1 blocking antibody applied to hematopoietic cells at concentrations of 10 ug/ml and 30 ug/ml have no significant effect on active migration of HSCs towards SDF1. (B) The migration of B cells and MPPs are not affected by increasing the concentration of VCAM-1. All cell types were quantified from the same well. n= 2 independent experiments with each condition performed in triplicate. Error bars represent SEM. P values were determined using a paired t test *$p<0.05$, **$p<0.005$ All experiments performed by Susan Calhoun.

Preliminary data suggests that blocking VCAM-1 on ECs has no effect on the ability of HSCs to migrate towards SDF1 *in vitro*

Since VCAM-1 is also expressed on ECs, we wanted to see if blocking VCAM-1 on ECs would have any effect on HSC transendothelial migration *in vitro*. ECs were isolated from wild type mouse lungs by previously established methods (Lim et al., 2006) and cultured until confluent. We used lung, rather than bone bone marrow, as source of endothelial cells because ECs are about 40 times more numerous in lungs and previous experiments in the lab revealed that the tissue origin of the ECs did not have a significant effect on the ability of HSCs to migrate towards SDF1 (Smith-Berdan et al., 2015). When culturing ECs, it is important to pay close attention to their morphology as they begin to reach confluency. Cultured ECs have a cobblestone appearance that is distinct from other cell types (Figure 9A). Once the cultured ECs reached confluency, they were plated onto transwell membranes. After cell expansion and barrier formation occurred, ECs were pre-incubated with anti-VCAM-1 antibodies before wild type lineage-depleted bone marrow cells were placed in the upper compartment of the transwell. The ability of lineage-depleted cells to migrate through the EC layer towards SDF1 was assessed by flow cytometry analysis of the cells that migrated to the bottom chamber (Figure 9B). Blocking VCAM-1 on ECs had no effect on the migration of HSCs, B cells or MPPs towards SDF1 (Figures 10A and 10B). However, no statistics were calculated on these data because they only represent one independent experiment. As these experiments were

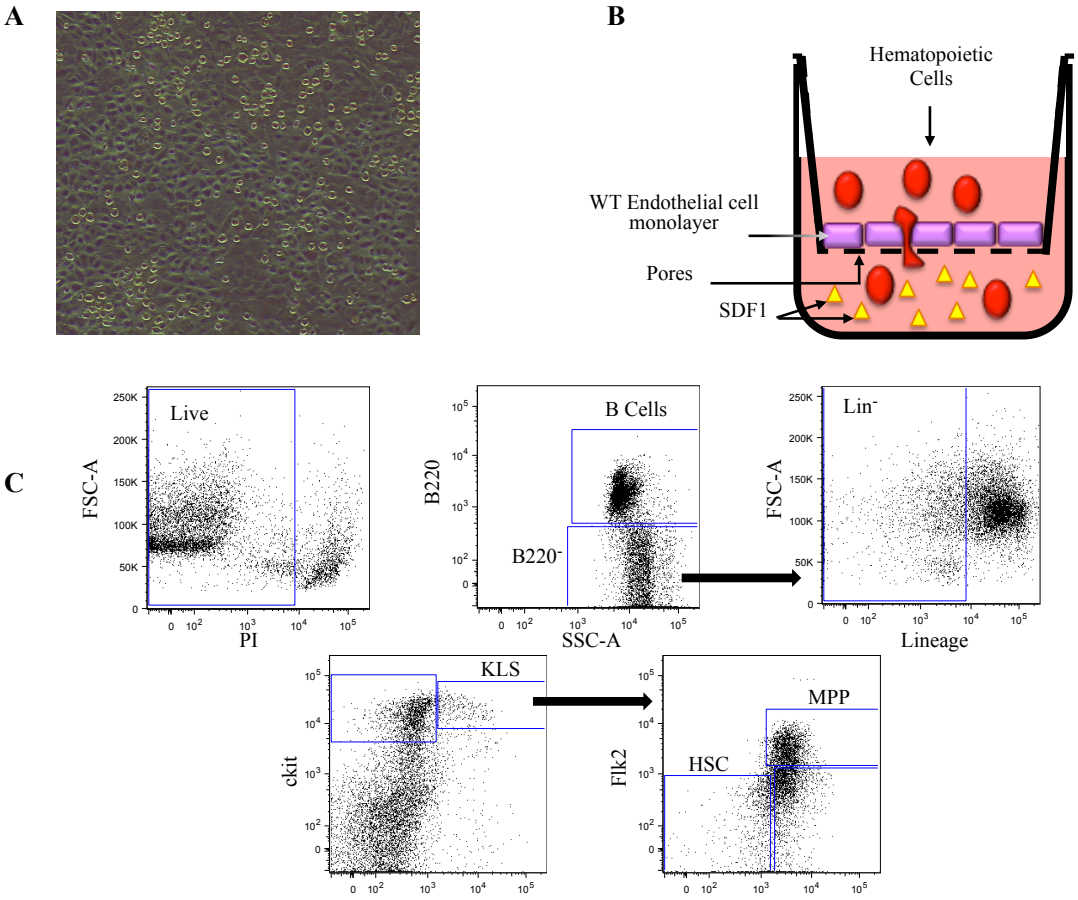

Figure 9. *In Vitro* migration assay to test whether VCAM-1 promotes active extravasation

(A) Confluent monolayers of P0 endothelial cells isolated from wild type mice show cobblestone appearance typical of cultured endothelial cells. (B) Schematic of experimental set up to measure transendothelial migration across WT endothelial cell layers (created by Stephanie Smith-Berdan) (C) Gating strategy used it isolate cell types of interest. All cell types were quantified from the same well.

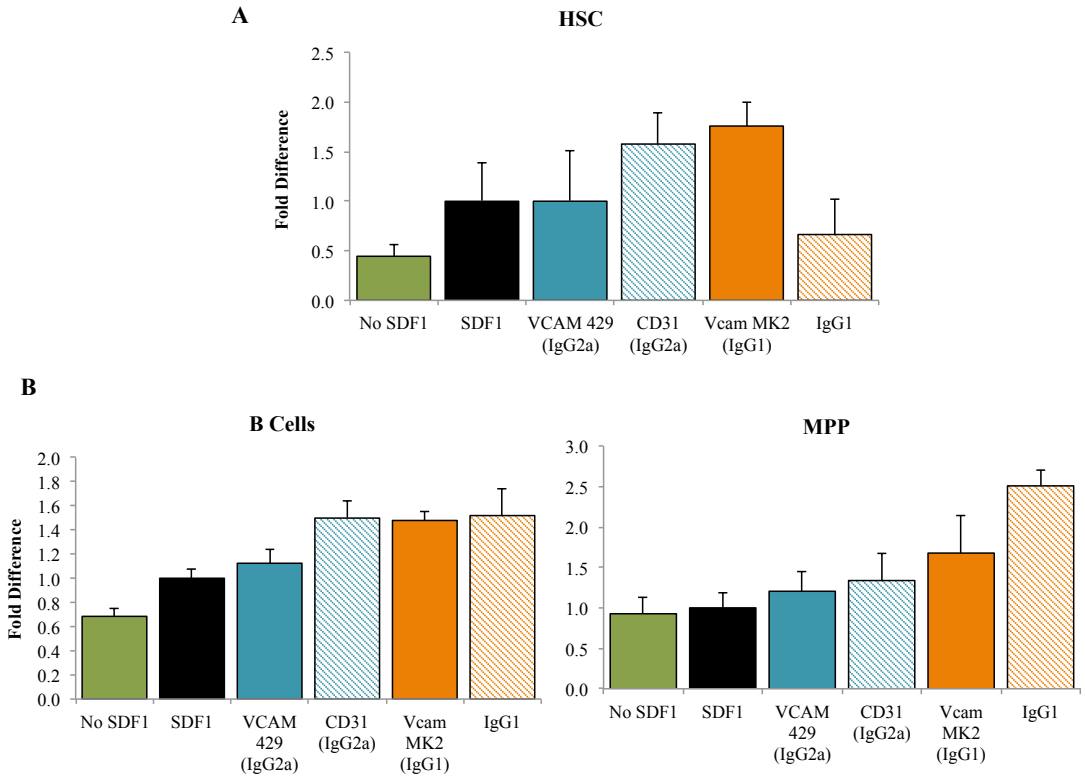

Figure 10. Blocking VCAM-1 on ECs has no effect on HSC transendothelial migration towards SDF1 *in vitro*

(A) VCAM-1 blocking antibodies (clones 429 and MK2) applied to wild type EC layers have no effect on transendothelial migration of HSCs towards SDF1 in vitro (B) The migration of B cells and MPPs is not affected. All cell types were quantified from the same well. n= 1 independent experiment with each condition performed in triplicate. Experiment performed by Susan Calhoun

underway, a critical component of the EC culture media became unavailable for purchase for an extended period of time. Endothelial cell growth supplement (ECGS) contains growth-promoting factors for vascular endothelial cells and is required for the growth of ECs in culture (Lim et al., 2006). A replacement product was offered by the distributing company but was determined to be non-compatible for our experiments (data not shown). At this point it is too early to draw any conclusions from these data, and these blocking assays will need to be repeated before it can be determined whether VCAM-1 on ECs is necessary for transendothelial migration.

Blocking VCAM-1 on hematopoietic cells has a significant effect on the ability of HSCs to migrate through EC layers towards SDF1 *in vitro*

Although we did not see significant effects on HSC migration when VCAM-1 was blocked on HSCs in experiments in which no EC layers were present, we aimed to test the hypothesis that expression of VCAM-1 on HSCs may be necessary to interact with ECs. As in the previous section, wild type ECs were isolated and plated onto transwell membranes. After expansion and barrier formation, wild type lineage-depleted bone marrow cells were pre-incubated with anti-VCAM-1 antibodies before being placed in the upper compartment of the transwell. The ability of lineage-depleted cells to migrate through the EC layer towards SDF1 was assessed by flow cytometry analysis (Figures 9B and 9C). Blocking VCAM-1 on hematopoietic cells had a significant inhibitory effect on the ability of HSCs to migrate through EC layers towards SDF1 (Figure 11A). This effect was seen with two different clones of

VCAM-1 blocking antibodies used, clone 429 and clone MK2. Both clones significantly reduced the migration of HSCs, while no effects were seen for B cells or MPPs (Figure 11B). These results suggest that VCAM-1 on HSCs is important for active extravasation across wild type EC layers *in vitro*.

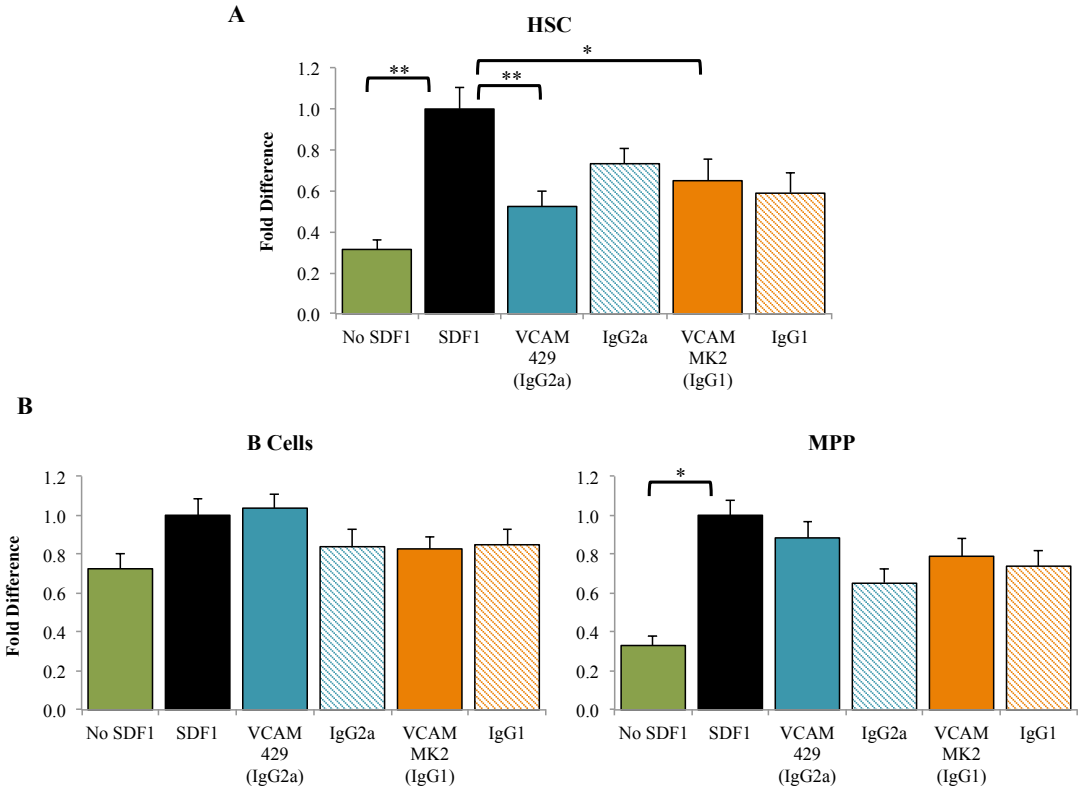

Figure 11. Blocking VCAM-1 on HSCs significantly inhibits transendothelial migration towards SDF1 *in vitro*

(A) VCAM-1 blocking antibodies (clones 429 and MK2) applied to hematopoietic cells has a significant effect on transendothelial migration of HSCs towards SDF1 in vitro (B) The migration of B cells and MPPs are not affected. n= 4 independent experiments with each condition performed in triplicate. Error bars represent SEM. P values were determined using a paired t test *$p<0.05$, **$p<0.005$

All experiments performed by Susan Calhoun

DISCUSSION AND FUTURE DIRECTIONS

The results presented here suggest that VCAM-1 on HSCs is important for HSC extravasation when ECs are present. By using *in vitro* migration assays, we were able to show that when no ECs were present, blocking VCAM-1 on HSCs, using an anti-VCAM-1 antibody, had no significant effect on the migration of HSCs towards SDF1. This result suggested that VCAM-1 on HSCs is not necessary for migration when HSCs do not encounter a barrier. However, when ECs are present, blocking VCAM-1 on HSCs does significantly impair their ability to migrate through EC layers, while having no effect on the migration of B cells or MPPs. Taken together, these results provide insight into what may be happening *in vivo* when HSCs must interact with ECs to migrate into and out of the bone marrow, and the specific adhesion molecule involved in this interaction.

Surprisingly, blocking VCAM-1 significantly inhibited the migration of B cells and MPPs when no ECs were present. Previous studies have shown that when *VCAM-1* deficient mice were crossed to Tie2-Cre mice, which allows deletion in hematopoietic cells and endothelium, the offspring had higher proportions of circulating hematopoietic progenitors and B cells in the peripheral blood, and a reduced capacity to recruit B and T cells to the bone marrow in short-term migration assays (Ulyanova et al., 2005; Koni et al., 2001). This could explain the results we saw in the assays in which no EC layers were present. These experiments showed that blocking VCAM-1 on hematopoietic cells significantly inhibited B cell and MPP migration. In assays where ECs were present, there was no significant effect on B cell

or MPP migration, which could be due to fact that there was still VCAM-1 on ECs for these cells to interact with.

To gain further insight into what may really be happening *in vivo,* we have created two lines of inducible transgenic mice that will be extremely useful for determining the effects of VCAM-1 deletion on both ECs and HSCs. Our VeCad-CreERT2 x *VCAM-1*$^{loxP/loxP}$ mice will be used for studying the effects of VCAM-1 deletion on ECs, while our Rosa-CreERT2 x *VCAM-1*$^{loxP/loxP}$ mice can be used for isolating HSCs that do not express VCAM-1. These mice will be used for a variety of future experiments that will be conducted by another member of the Forsberg lab. The mice will be used to repeat the migration assays to determine if we see similar results using knock out cells versus blocking antibodies. To directly test whether VCAM-1 promotes active extravasation, *in vitro* transendothelial migration assays using wild type EC layers and *VCAM-1*$^{-/-}$ HSCs isolated from Rosa-CreERT2 x *VCAM-1*$^{loxP/loxP}$ mice will be performed. Likewise, we also want to test whether VCAM-1 on ECs is important for this process and will do another set of experiments using *VCAM-1*$^{-/-}$ ECs isolated from VeCad-CreERT2 x *VCAM-1*$^{loxP/loxP}$ mice with wild type HSCs. These experiments will be a more direct way of determining if VCAM-1 on ECs, HSCs or both affects active extravasation.

Another question we would like to address is whether the vasculature of the bone marrow is different in mice that do not express VCAM-1 on ECs. Since we believe that SECs expressing VCAM-1 serve as important conduits for HSC extravasation, we will quantify the number, organization and size of sinusoids in bone

marrow sections of mice that do not express VCAM-1 on ECs. Using the results presented here, we now have a way to independently label SECs that are VCAM-1^{+} and arteriole cells without the use of VCAM-1 antibody and other complex antibody stains. For flow cytometry analysis, Dil-Ac-LDL will be used to label SECs, which will allow us quantify the number of SECs present. For IHC of bone marrow sections, VEGFR3 will be used to label sinusoids and Alexa 633 will be used to label arterioles. If VCAM-1 is playing a role in mediating HSC trafficking through SECs, then deleting it may cause a reduction in the number and structural integrity of sinusoids present in the bone marrow.

In order to fully understand the effect of VCAM-1 deletion on the vasculature, we would also like to determine whether VCAM-1 deletion leads to altered vascular permeability *in vivo* using our VeCad-CreERT2 x *VCAM-1*$^{loxP/loxP}$ mice. Our previous findings showed that *Robo4*$^{-/-}$ mice have increased permeability (Smith-Berdan et al., 2015), but whether or not this is due to decreased numbers of VCAM-1^{+} SECs remains to be determined. To test the vascular permeability of the bone marrow vasculature, along with other tissues such as lungs and small intestine, Evans Blue dye will be intravenously injected into wild type and *VCAM-1*$^{-/-}$ mice. Organs with high vascular permeability turn blue. The level of vascular permeability is often readily visualized by eye, but it will also be objectively measured by spectroscopic quantification of the amount of dye incorporated per milligram of tissue (Radu et al., 2013; Smith-Berdan 2015). If endothelial VCAM-1 is important for vascular integrity, we expect to see increased vascular permeability in *VCAM-1*$^{-/-}$ mice

compared to wild type mice. Determining whether VCAM-1 deletion leads to altered vascular permeability will provide valuable insights into the important functional interactions between HSCs and the vasculature. These experiments will also elucidate whether vascular permeability can be targeted and altered to improve HSC translocation across endothelial layers in transplantation therapies.

HSC transplantation therapy involves the movement of HSCs both into and out of the bone marrow (Smith-Berdan et al., 2011; Mendelson et al., 2014). HSCs are commonly mobilized from the bone marrow to the peripheral blood through the use of mobilizing drugs such as AMD3100 (Broxmeyer et al., 2005; Smith-Berdan et al., 2011). Induced mobilization by AMD3100 is rapid and HSCs can be collected from the peripheral blood rather than directly from the bone marrow (Broxmeyer et al., 2005; Smith-Berdan et al., 2011). We would like to determine whether endothelial VCAM-1 regulates the efficiency of HSC mobilization. HSCs in wild type mice and in mice lacking endothelial VCAM-1 will be mobilized from the bone marrow to the blood with AMD3100. One hour later, the peripheral blood will be collected and analyzed by flow cytometry to quantify the number of HSCs that have been mobilized to the blood. If endothelial VCAM-1 regulates HSC mobilization, we expect to see fewer HSCs in the peripheral blood of mice lacking endothelial VCAM-1 compared to wild type. However, another possibility is that we could see more HSCs in the peripheral blood if VCAM-1 deletion leads to altered vascular permeability, causing HSCs to mobilize better in *VCAM-1*$^{-/-}$ mice compared to wild type mice.

Proper HSC function upon transplantation is dependent on the ability of HSCs to travel through the blood system and engraft in the bone marrow (Forsberg et al., 2009). To test whether VCAM-1 on ECs is required for engraftment, GFP-labeled HSCs from wild type mice will be transplanted into irradiated mice lacking endothelial VCAM-1, as well as a wild type controls. To assess long-term donor chimerism, donor contribution to GFP+ donor-derived mature cells in the peripheral blood will be monitored using flow cytometry over a period of 16 weeks and HSC chimerism in the bone marrow will be analyzed at 16 weeks. If endothelial VCAM-1 is important for HSC engraftment, we expect to see decreased levels of donor chimerism and little or no long-term engraftment in mice lacking endothelial VCAM-1.

The ability of HSCs to travel into and out of the bone marrow has great importance for their use in regenerative medicine. In HSC transplantation therapies, HSCs must be able to mobilize from the donor bone marrow and travel to the peripheral blood so that they can be collected. Once transplanted into the recipient, HSCs must home from the blood to the bone marrow. One of the interesting aspects of this process is how directionality is achieved. With the results presented here and the future experiments we have planned, this study we will work towards understanding the molecular factors that regulate the directionality of HSC trafficking. We may find that VCAM- 1 is important for going into the bone marrow but not out, or alternatively that it is important for migration in both directions. Regardless of the outcome, we can use our findings to explicate how directionality is

achieved and regulated. We know from recent studies that inhibiting the binding of VCAM-1 to its binding partner ITGα4, impairs HSC transendothelial migration (Smith-Berdan et al., 2015). This finding suggests that VCAM-1 has a direct role in regulating HSC extravasation. We also now know that blocking VCAM-1 on HSCs significantly impairs their ability to undergo transendothelial migration *in vitro*. These findings can be used to better understand the mechanisms regulating the directionality of HSC trafficking and molecular targets that can be manipulated to improve HSC mobilization and engraftment in clinical therapy.

EXPERIMENTAL PROCEDURES

Mice

Mice were maintained by the University of California Santa Cruz animal facility. All experiments were performed using adult mice (8-12 weeks old). VCAM-1$^{loxP/loxP}$, mT/mG and Rosa CreERT2 transgenic mice, obtained from JAX, were all previously described (Koni et al., 2001; Boyer et al., 2012; Muzumdar et al., 2007; Ventura et al., 2007). VeCad CreERT2 mice, obtained from Eugene Butcher, MD at Stanford University, were previously described (Wang et al., 2010).

Immunohistochemistry

Dil-AC-LDL (20u g per mouse) or Alexa 633 (50 ug per mouse) was retro-orbitally injected into wild type mice. Four hours after injection, mice were perfused with PBS

to remove any unbound dye. Long bones were collected for FACS or immunofluorescence imaging.

Femurs and tibias were post fixed for 30 minutes in freshly prepared 4% paraformaldehyde and incubated in 15% sucrose overnight at 4°C. Bones were moved to 30% sucrose in the morning and allowed to incubate for 6-8 hours at 4°C. Bones were embedded in optimal cutting temperature compound (OCT) in an ethanol/dry ice slurry and stored at -80°C. Bone marrow sections were cut with a tungsten blade to 10 or 20 μm thickness using a Leica Cryo-Jane tape transfer system. Slides were fixed with acetone at -20°C for 10 minutes before staining. Tissue sections were blocked with 10% goat serum at room temperature for 1 hour prior to overnight primary antibody staining at 4°C. Sections were incubated with fluorescently conjugated secondary antibodies for 2 hours at room temperature. Images were acquired using a Keyence Biorevo BZ-9000 digital widefield microscope at the UCSC Life Sciences Microscopy Center.

Antigen Retrieval

For detection of VEGFR3, tissue sections were subjected to antigen retrieval using heat and a sodium citrate buffer (protocol adapted from Jiao et al., 1999). Sections were rinsed three times for 5 minutes each in 0.1 M phosphate buffer (pH 7.4). Sections were then transferred to a koplin jar containing10 mM sodium citrate buffer (pH 8.5) preheated to 80°C in a water bath. Sections were maintained in this solution and temperature for 30 minutes. The koplin jar was then removed from the water bath

and the sections were maintained in the solution while cooling to room temperature. Sections were then rinsed three times for 5 minutes each in 0.1 M phosphate buffer (pH 7.4) before proceeding to blocking step.

Transgenic Mice Genotyping

Offspring were genotyped for either VeCad CreERT2, Rosa CreERT2 or *VCAM-1*$^{loxP/loxP}$ alleles using PCR. DNA was isolated from the ears of 4 week-old offspring by methods previously described (Truett et al., 2000). For VeCad CreERT2, DNA was amplified for 35 cycles: denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and elongation at 72°C for 40 sec with a final elongation step at 72°C for 7 min. The forward primer is directed to VeCad (5' to 3' sequence: TCC TGA TGG TGC CTA TCC TC) while the reverse is directed to the CreERT2 (5' to 3' sequence: CCT GTT TTG CAC GTT CAC CG). The expected product size of the Cre is 548 base pairs (bp). For Rosa CreERT2, DNA was amplified for 40 cycles: denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 1 min with a final elongation step at 72°C for 5 min. Three primers were used for this reaction. The common primer (5' to 3' sequence: AAA GTC GCT CTG AGT TGT TAT), the wild type reverse (5' to 3' sequence: GGA GCG GGA GAA ATG GAT ATG) and the mutant reverse (5' to 3' sequence: CCT GAT CCT GGC AAT TTC G). The expected product sizes are a homozygote band at 825 bp, heterozygote bands at 825 and 603 bp, or a wild type band at 603 bp. For *VCAM-1*$^{loxP/loxP}$, DNA was amplified for 35 cycles: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and

elongation at 72°C for 30 sec with a final elongation step at 72°C for 2 min. Forward primer (5' to 3' sequence: GGG ACG GAT TTT CTT TCC AC) and reverse primer (5' to 3' sequence: GAC TTT GAA GCC CAT TGC AC). The expected product sizes are a homozygote band at 226 bp, heterozygote bands at 226 and 162 bp, or a wild type band at 162 bp.

Administration of Tamoxifen

Tamoxifen was administered to adult mice via intraperitoneal injection once every 24 hours for a total of 5 consecutive days. A 20 mg/ml solution of tamoxifen was prepared in sterile corn oil and injected at a final concentration of 75 mg tamoxifen/kg body weight. Cross-contamination was a concern so control mice were kept in separate cages. Control mice genotyped positive for CreERT2 and were injected with oil. Mice were sacrificed 7 days after the last injection for further analysis.

EC Isolation and Culture

ECs were isolated from wild type mice as previously described (Lim et al., 2006; Sobczak et al., 2010). Freshly isolated cells were used for flow cytometry analysis. ECs for transwell assays were enriched using CD31-coupled magnetic beads (Miltenyi) and plated onto 10 cm 0.25% gelatin-treated plates. ECs were cultured for up to three passages using media that promotes EC growth: DMEM with high

glucose, 20% fetal bovine serum (FBS), non-essential amino acids (1X), sodium pyruvate (1X), heparin (100 ug/ml), ECGS (60 ug/ml), and primocin (1X).

Transendothelial Migration Assays

Cultured ECs were plated onto 0.25% gelatin-treated transwell inserts and grown to confluency. EC media was changed to a 5% FBS in RPMI the day before the migration was to occur. Bone marrow cells were harvested from wild type mice and lineage depleted by magnetic selection (Dynabeads sheep anti-rat IgG). Cells were pre-treated with antibodies, anti-VCAM-1 (clone 429) (Biolegend), IgG2 (Biolegend), anti-VCAM-1 (clone MK2) (Cedarlane), IgG1 (Biolegend), anti-CD31 (Biolegend), anti-ITGα4 (Santa Cruz Biotech), or anti-CD4 (Biolegend) at a final concentration of 10 ug/ml when appropriate at 37°C for 1 hour. Heat activated cells were placed in the upper chamber of a transwell insert with a 5.0 μm pore size. The bottom wells contained SDF1 (100 ng/ml) for all samples except the No SDF group in each experiment. The migration occurred for 2 hours at 37°C. Cells were collected from the bottom well and analyzed by flow cytometry as previously described (Smith-Berdan et al., 2015).

REFERENCES

Acar, M., Kocherlakota, K., Murphy, M., Peyer, J., Oguro, H., Inra, C., Jaiyeola, C., Zhao, Z., Luby-Phelps, K., and Morrison, S. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature 2015; 526(7571), 126-130

Baumann, C., Bailey, A., Li, W., Ferkowicz, M., Yoder, and M., Fleming, W. PECAM-1 is expressed on hematopoietic stem cells through out ontogeny and identifies a population of erythroid progenitors. Blood 2004; 104, 1010-1016

Boulais, P. and Frenette, P. Making sense of hematopoietic stem cell niches. Blood 2015; 125(17), 2621-2629

Boyer, S., Beaudin, A., and Forsberg, C. Mapping differentiation pathways from hematopoietic stem cells using Flk2/Flt3 lineage tracing. Cell Cycle 2012; 11:17, 3180-3188

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M., and Kidd, T. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 1999; 96, 795-806

Broxmeyer, H.E, Orschell, C.M., Clapp, D.W., Hangoc, G., Cooper, S., Plett, P.A., Liles, W.C., Li, X., Graham-Evans, B., Campbell, T.B., Calandra, G., Bridger, G., Dale, D.C., and Srour, E.F. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J. Exp. Med. 2005; 201, 1307-1318

Celso, C. and Scadden, D. The haematopoietic stem cell niche at a glance. Cell Science 2011; 124, 3529-3535

Feil, S., Valtcheva, N., and Feil, R. Inducible Cre Mice, Methods Mol Biol. 2009; 530, 343-363

Forsberg, C. and Smith-Berdan, S. Parsing the niche code: the molecular mechanisms governing hematopoietic stem cell adhesion and differentiation. Haematologica 2009; 94(11), 1477-1481

Gurtner, G., Davis, V., Li, H., McCoy, M., Sharpe, A., and Cybulsky, M. Targeted disruption of the murine VCAM-1 gene: essential role of VCAM-1 in chorioallantoic fusion and placentation. Genes Dev. 1995; 9, 1-14

Holmes, C. and Stanford, W. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells 2007; 25, 1339-1347

Hooper, A., Butler, J., Nolan, D., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K., James, D., Witte, L., Zhu, Z., Yan, W., Pytowski, B., Rosenwaks, Z., Mittal, V., Sato, T., and Rafii, S. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2 mediated regeneration of sinusoidal endothelial cells. Cell Stem Cell 2009; 4(3): 263–274

Itkin, T., Gur-Cohen, S., Spencer, J., Schajnovitz, A., Ramasamy, S., Kusumbe, A., Ledergor, G., Jung, Y., Milo, I., Poulos, M., Kalinkovich, A., Ludin, A., Kollet, O., Shakhar, G., Butler, J., Rafii, S., Adams R.H., Scadden, D.T., Lin, C., and Lapidot, T. Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature 2016; 532, 323-328

Jiao, Y., Sun, Z., Lee, T., Fusco, F., Kimble, T., Meade, C., Cuthbertson, S., and Reiner, A. A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. Journal of Neuroscience Methods 1999; 93, 149-162

Koni, P., Joshi, S., Temann, U.A., Olson, D., Burkly, L., Flavell, R.A. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. J. Exp. Med. 2001; 193(6); 741-753

Kopp, H.G., Avecilla, S., Hooper, A., and Raffi, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology 2005; 20, 349-356

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei1, Q., Lucas, D., Ito, D., Mar, J., Bergman, A., and Frenette, P.S. Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 2013; 502, 637-643

Kunisaki, Y. and Frenette, P.S. Influences of vascular niches on hematopoietic stem cell fate. Int J Hematol 2014; 99, 669-705

Lai, A., Lin, S., and Kondo, M. Heterogeneity of Flt3-expressing multipotent progenitors in mouse bone marrow. J Immunol 2005; 175, 5016-5023

Laird, D., von Andrian, U., and Wager, A. Stem cell trafficking in tissue development, growth, and disease. Cell 2008; 132, 612-630

Li, X.M., Hu, Z., Jorgenson, M., and Slayton, W. High levels of acetylated low-density lipoprotein uptake and low tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie2) promoter activity distinguish sinusoids from other vessel types in murine bone marrow. Circulation 2009; 120, 1910-1918

Lim, Y.C. and Luscinskas, F.W. Isolation and culture of murine heart and lung endothelial cells for in vitro model systems. Cell-Cell Interactions 2006; 341, 141-154

Long, H., Sabatier, C., Ma, L., Plump, A., Yuan, W., Ornitz, D.M., Tamada, A., Murakami, F., Goodman, C.S., and Tessier-Lavigne, M. Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron 2004; 42, 213-223

Mazo, I., Guiterrez-Ramos, J.C., Frenette, P.S., Hynes, R., Wagner, D., von Andrian, U. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. J Exp Med 1998; 188(3), 465-474

Mendelson, A. and Frenette, P.S. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nature Medicine 2014; 20(8), 833-846

Monvoisin, A., Alva, J., Hofmann, J., Zovein, A., Lane, T., and Iruela-Arispe, M.L. VE-cadherin-CreER^{T2} transgenic mouse: A model for inducible recombination in the endothelium. Developmental Dynamics 2006; 253, 3413-3422

Morrison, S. and Scadden, D. The bone marrow niche for haematopoietic stem cells. Nature 2014; 505, 327-334

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. A global double-fluorescent Cre reporter mouse. Genesis 2007; 45, 593-605

Nombela-Arrieta, C., Pivarnik, G., Winkel, B., Canty, K., Harley, B., Mahoney, J., Park, S.Y., Lu, J., Protopopov, A., and Silberstein, L.E. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. Nature Cell Biology 2013; 15(5), 533-543

Papadimitriou, M., Menter, D., Konstantopoulos, K., Nicolson, G., and McIntire, L.V. Integrin $\alpha_4\beta_1$/VCAM-1 pathway mediates primary adhesion of RAW117 lymphoma cells to hepatic sinusoidal endothelial cells under flow. Clinical & Experimental Metastasis 1999; 17, 669-676

Perdomo-Arciniegas, A.M. and Vernot, J.P. Co-culture of hematopoietic stem cells with mesenchymal stem cells increases VCAM-1-dependent migration of primitive hematopoietic stem cells. Int J Hematol 2011; 94, 525-532

Radu, M. and Chernoff, J. An in vitro assay to test blood vessel permeability. J Vis Exp 2013; (73), e50062

Schechter, R., Miller, O., Yovel, G., Rosenzweig, N., London, A., Ruckh, J., Kim, K.W., Kalchenko, V., Bendel, P., Lira, S.A., Jung, S., and Schwartz, M. Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus. Immunity 2013; 38, 555-569

Shen, Z., Lu, Z., Chhatbar, P., O'Herron, P., and Kara, P. An artery-specific fluorescent dye for studying neurovascular coupling. Nature Methods 2012; 9(3), 272-280

Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. Lymphocyte interactions with endothelial cells. Immunol Today 1992; 13, 106-112

Smith-Berdan, S., Nguyun, A., Hassanein, D., Zimmer, M., Ugarte, F., Ciriza, J., Li, D., Garcia-Ojeda, M.E., Hinck, L., and Forsberg, C. Robo4 Cooperates with CXCR4 to specify hematopoietic stem cell localization to bone marrow niches. Cell Stem Cell 2011; 8,1-12

Smith-Berdan, S., Nguyen, A., Hong, M., and Forsberg, C. ROBO4-mediated vascular integrity regulates directionality of hematopoietic stem cell trafficking. Stem Cell Reports 2015; 4, 255-268

Terry, R., Kwee, L., Baldwin, H.S., and Labow, M. Cre-mediated generation of a VCAM-1 null allele in transgenic mice. Transgenic Research 1997; 6, 349-356

Truett, G.E, Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A., and Warman, M.L. Preparation of PCR quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). BioTechniques 2000; 29, 52-54.

Ugarte, F. and Forsberg, C. Haematopoietic stem cell niches: new insights inspire new questions. The EMBO Journal 2013; 32, 2535–2547

Ulyanova, T., Scott, L., Priestley, G., Jiang, T., Nakamoto, B., Koni, P., and Papayannopoulou, T. VCAM-1 expression in adult hematopoietic and nonhematopoietic cells is controlled by tissue-inductive signals and reflects their developmental origin. Blood 2005; 106, 86-94

Ventura, A., Kirsch, D., McLaughlin, M., Tuveson, D., Grimm, J., Lintault, L., Newman, J., Reczek, E., Weissleder, R., and Jacks, T. Restoration of p53 function leads to tumour regression *in vivo*. Nature 2007; 445, 661-665

Wang , Y., Nakayama, M., Pitulescu, M., Schmidt, T., Bochenek, M., Sakakibara, A., Adams, S., Davy, A., Deutsch, U., Luthi, U., Barberis, A., Benjamin, L., Makinen, T., Nobes, C., and Adams, R.H. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 2010; 465, 483-489

Wang, L., Benedito, R., Bixel1, M.G., Zeuschner, D., Stehling, M., Savendahl, L., Haigh, J., Snippert, H., Clevers, H., Breier, G., Kiefer, F., and Adams, R.H. Identification of a clonally expanding haematopoietic compartment in bone marrow. The EMBO Journal 2013; 32, 219-230

Weidner, C.I., Walenda, T., Lin, Q., Wolfler, M.M., Denecke, B., Costa, I.G., Zenke, M., and Wagner, W. Hematopoietic stem and progenitor cells acquire distinct DNA-hypermethylation during in vitro culture. Scientific Reports 2013; 3:3372

Wendt, E and Keshav, S. CCR9 antagonism: potential in the treatment of inflammatory bowel disease. Clinical and Experimental Gastroenterology 2015; 8, 119-130

Winkler, I., Barbier, V., Wadley, R., Zannettino, A., Williams, S., Levesque, J.P. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood 2010; 116, 375-385

Zimmerman, G.A., Prescott, S.M., and McIntyre, T.M. Endothelial cell interactions with granulocytes: tethering and signaling molecules. Immunol Today 1992; 13, 93-100