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

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Background—The bone marrow contains a variety of blood vessels that have different functions in bone marrow maintenance and hematopoiesis. Arterioles control the flow of blood into bone marrow compartments, and sinusoids serve as a conduit to the bloodstream and as niches for megakaryocyte development. Most studies of bone marrow vasculature, including studies quantifying changes in the marrow vascular by microvascular density, do not differentiate between different types of marrow vessels. Recognizing changes in different types of blood vessels after chemotherapy exposure or during leukemia development has important physiological implications. We hypothesized that the functional heterogeneity of marrow vasculature could be recognized through the use of functional markers such as tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie2) expression or 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate with acetylated low-density lipoprotein (DiI-Ac-LDL) uptake.

Methods and Results—When transgenic mice with green fluorescent protein (GFP) expressed downstream of the Tie2 promoter were injected with Ac-LDL, Ac-LDL was specifically endocytosed by sinusoids, and Tie2 expression was more pronounced in the arteries, arterioles, and transitional capillaries. Combining these 2 functional endothelial markers and using confocal microscopy to obtain 3-dimensional images, we identified transitional zones where arterioles emptied into the sinusoids. Alternatively, coinjection of lectin with DiI-Ac-LDL has a similar result in normal mice, as seen in Tie2/GFP mice, and can be used to differentiate vessel types in nontransgenic mice.

Conclusions—These results demonstrate that bone marrow vasculature is functionally heterogeneous. Methods to study changes in the marrow vasculature using microvascular density or quantifying changes in the vascular niche need to take this heterogeneity into account. (Circulation. 2009;120:1910-1918.)

Key Words: bone marrow ■ endothelium ■ microcirculation ■ vasculature

Bone marrow is the major hematopoietic organ in adult mammals. Bone marrow sinusoids are specialized capillary beds that separate the synthetic bone marrow from the bloodstream. Sinusoids control cellular traffic into and out of the marrow parenchyma. Sinusoidal endothelial cells have no basement membrane, and the individual cells are fenestrated.1,2 Because of the critical role that bone marrow sinusoids are thought to play as hematopoietic niches and conduits to the circulation,3–5 distinguishing sinusoids from other vessels when describing and quantifying changes in the marrow vasculature is critically important.

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Recent studies suggesting that the bone marrow sinusoids serve as niches for hematopoietic stem cells, hematopoietic progenitors, and megakaryocyte precursors have led to studies focused on the effects of various stresses on the bone marrow vasculature. Furthermore, a number of studies have demonstrated changes in bone marrow microvascular density that occur in diseases such as leukemia, multiple myeloma, and myelodysplasia. It is not clear how these stresses induce marrow angiogenesis or what particular types of vessels are increased or decreased in these conditions.

Because of their critical role as a vascular niche, we were interested in developing methods to specifically identify bone marrow sinusoids. We tested a number of previously described immunohistochemical methods to identify the bone marrow sinusoids and found that most of these antigens either were not expressed or were unable to distinguish sinusoids from other
types of marrow vessels. Studies in the 1980s established that reticuloendothelial sinusoids in the bone marrow, liver, and spleen endocytosed acetylated low-density lipoprotein (Ac-LDL) conjugated to the fluorescent dye 11,1’dioctadecyl -3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) and that this in vivo uptake was relatively specific to sinusoidal beds.

Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie2) is a receptor tyrosine kinase for the angiopoietin-1. Tie2 is expressed by vascular endothelial cells. Using transgenic mice expressing reporter genes downstream from the Tie2 promoter to identify blood vessels in the mesentery, researchers discovered that the Tie2 promoter is much more active in the vascular endothelial cells.8,9 Using transgenic mice expressing reporter genes downstream from the Tie2 promoter to identify blood vessels in the mesentery, researchers discovered that the Tie2 promoter is much more active in the arteriolar than the venular side of the vascular system.10

We hypothesized that the functional heterogeneity of the marrow vasculature could be recognized through the use of functional markers such as Tie2 expression or uptake of DiI-Ac-LDL. We have demonstrated that endocytosis of DiI-Ac-LDL and Tie2 promoter activity distinguishes sinusoidal endothelium from presinusoidal arterioles and transitional capillaries. Distinguishing marrow sinusoids from other types of marrow vessels is a prerequisite to mechanistic studies related to marrow angiogenesis and the fate of the vascular niche.

**Methods**

**Mice**

Six- to 8-week-old C57/BL6 and FVB mice, transgenic C57BL/6-Tg(N(ActBEFGFP)1OsB green fluorescent protein (GFP) donor mice,11 and Tg(TIE2GFP) 287Sato/J (Tie2/GFP) mice on the FVB background were purchased from The Jackson Laboratory (Bar Harbor, Me). Both male and female mice were used in these studies. These studies were approved by the University of Florida Institutional Animal Care and Use Committee.

**In Vitro DiI-Ac-LDL Labeling**

Marrow cells were flushed from the marrow cavity with PBS. Erythrocytes were lysed with ammonium chloride (0.15 mol/L of NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L Na2EDTA; pH 7.4). Cells were incubated with 10 μg/mL Dil-Ac-LDL in DMEM at 37°C for 4 hours. The cells were washed twice with PBS and labeled with phycoerythrin-conjugated antibodies for CD11b, GR1, B220, c-Kit, and CD31 (BD Biosciences, San Jose, Calif).

**In Vivo DiI-Ac-LDL Labeling**

Healthy or irradiated C57BL/6, GFP, or Tie2/GFP mice were injected in the retroorbital plexus with 1 μg DiI-Ac-LDL/g body weight (Biomedical Technologies, Stoughton, Mass). Four hours after injection, animals were killed. Marrow cells were flushed and labeled as in the in vitro experiments.

**Tissue Processing for DiI-Ac-LDL and Immunostaining**

Four hours after retroorbital injection with DiI-Ac-LDL, animals were killed. Bones were fixed in freshly prepared 4% paraformaldehyde in PBS for 24 hours. Whole bones were sectioned with the Cryogenic Tape Transfer System (Intruments Inc, Hackensack, NJ). Alternatively, the marrow core was removed by opening the marrow chamber with scissors under the dissecting microscope and carefully lifting the marrow out of the bone. The marrow core was visualized directly under the fluorescent microscope.

**Lectin and Dil-Ac-LDL Coinjection**

DiI-Ac-LDL labeling was performed as described above. Five minutes before the animal was killed, 5 μg/g body weight of FITC-conjugated lectin from Lycopersicon esculentum (tomato) (Vector Laboratories, Burlingame, Calif) or from wheat germ (MP Biomedical, Irvine, Calif) was injected into the retroorbital plexus. Bone marrow was processed the same way as for DiI-Ac-LDL–labeled Tie2/GFP marrow.

**DiI-Ac-LDL and Immunohistochemical Double Staining**

DiI-Ac-LDL–labeled bone marrow sections were incubated with anti-CD31 or anti-von Willebrand factor antibody at 4°C overnight. Donkey anti-rat secondary antibody conjugated to Alexa-Fluor 488 (Molecular Probes, Carlsbad, Calif) was used for fluorescent detection.

**Immunohistochemical Staining for MECA-32 Expression**

Paarfin-embedded sections were treated with DAKO Target Retrieval Solution at 95°C for 20 minutes. Rat anti-mouse MECA-32 antibody 1:10 (Pharriingen, San Diego, Calif) was added to the sample and incubated at 4°C overnight. Staining was detected with an ABC Elite Kit with diaminobenzidine as the chromogen (Vector Laboratories). Slides were counterstained with hematoxylin.

**Fluorescent Microscopy and Confocal Analysis**

Light microscopy and fluorescent images were captured on an Olympus BX 51 microscope equipped with Optronics Magnafire digital camera system. Confocal imaging and lambda scanning were performed on a Leica TCS SP2 AOB5 spectral confocal microscope.

**Results**

**Endocytosis of DiI-Ac-LDL by Bone Marrow Cells Differed Depending on Whether the Exposure Occurred In Vitro or In Vivo**

DiI-Ac-LDL has been used as an endothelial marker for >20 years.12 However, DiI-Ac-LDL uptake is not specific when used in vitro because other cells such as macrophages that are equipped with scavenger receptors also endocytose the compound. When flushed bone marrow cells are exposed to DiI-Ac-LDL, a large proportion of the cells endocytosed DiI-Ac-LDL, as demonstrated by flow cytometric analysis (Figure 1A). There were 2 DiI-Ac-LDL–positive populations identified, 1 with a higher Ac-LDL intensity (Ac-LDL high) than the other one (Ac-LDL low). Within the DiI-Ac-LDL–positive population, 40% expressed integrin αM (CD11b or membrane attack complex type 1). The majority of these cells were in the DiI-Ac-LDL high population. Only 12% of the cells within the CD11b–positive population endocytosed DiI-Ac-LDL. Cells expressing glycosyl phosphatidylinositol–linked protein Ly6A (Gr-1), a marker for granulocytes, had a similar staining pattern, with 13% uptaking DiI-Ac-LDL. Interestingly, cells expressing the CD45 isoform and B-cell marker CD45R (B220) also endocytosed DiI-Ac-LDL, but these cells were in the DiI-Ac-LDL low population. There were 2 well-separated B220-positive populations. The B220high population included mature B cells, and the B220low population included pro-B, pre-B, and immature B cells. The B220high population endocytosed DiI-Ac-LDL, but the B220low population did not. Platelet endothelial cell adhesion molecule-1 (CD31) –expressing cells endocytosed Ac-LDL. However, the CD31–positive, DiI-Ac-LDL–positive cells were not endothelial cells. This point is illustrated later. CD31 is also expressed on many leukocyte subsets, including monocytes and erythrocyte progenitors. The stem cell marker c-kit (CD117) is known to be associated with hematopoietic stem and progenitor cells. Some c-kit–positive cells also endocytosed low levels of...
DiI-Ac-LDL. This low level of DiI-Ac-LDL uptake appeared as a well-separated group from the DiI-Ac-LDL high and the DiI-Ac-LDL negative population. The reason behind the low-level DiI-Ac-LDL endocytosis in vitro by various types of hematopoietic cells is under investigation. Nevertheless, these data demonstrate that in vitro exposure to DiI-Ac-LDL leads to a large variety of other cell types besides endothelial cells and macrophages endocytosed DiI-Ac-LDL.

In contrast to in vitro exposure, when mice were injected with DiI-Ac-LDL 4 hours before death, very few bone marrow cells in the prepared single-cell suspension had endocytosed DiI-Ac-LDL (Figure 1B). In particular, very few CD11b- or G4-1-positive granulocytes had endocytosed DiI-Ac-LDL. We interpreted this result as suggesting that DiI-Ac-LDL remained intravascular and that exposure of macrophages and granulocytes was prevented by the endothelial barrier.

The lack of DiI-Ac-LDL uptake in the flow cytometric analysis was not a result of the failure of endothelial cells to endocytose DiI-Ac-LDL, as can be seen in Figure 2. When marrow cells were obtained by flushing the bone marrow cavity, endothelial cells that had endocytosed DiI-Ac-LDL either were destroyed by the shear forces of flushing or were still connected to each other and discarded as cell clumps.

The result was that no endothelial cells labeled by DiI-Ac-LDL were present in the single-cell suspensions. Attempts to disaggregate the bone marrow sinusoidal endothelial cells with enzymatic digestions such as collagenase and dispase led to similar results.

These results demonstrate that DiI-Ac-LDL uptake is not a specific marker of endothelial cells in cultured murine bone marrow cells. Furthermore, it demonstrates that flow cytometry cannot be easily used to analyze bone marrow endothelial cells because the endothelial cells are not present in single-cell suspensions.

**DiI-Ac-LDL Uptake After Retroorbital Injection Specifically Identifies Bone Marrow Sinusoids and Allows 3-Dimensional Analysis With Fluorescent Confocal Microscopy**

The analysis of whole-bone-marrow cores from DiI-Ac-LDL-injected mice was in striking contrast to the single-cell suspension of the flow cytometric results. The marrow core contained abundant DiI-Ac-LDL-labeled sinusoids forming a network of irregular tubules evenly distributed throughout the bone marrow (Figure 2A). When imaged by
confocal microscopy, the detailed 3-dimensional structure of the sinusoidal system was evident (Figure 2B and Figure I of the online-only Data Supplement). The specificity of DiI-Ac-LDL labeling of sinusoidal endothelial cells (red) can be seen in a section of bone marrow from a GFP transgenic mouse with the enhanced GFP cDNA expressed under the control of a chicken β-actin promoter and cytomegalovirus enhancer (Figure 2C). Endocytosed DiI-Ac-LDL was compartmentalized within lysosomes in endothelial cells, producing a granular intracellular signal throughout the sinusoidal wall. Figure 2D shows a bone marrow section from an animal 4 days after receiving 950 cGy radiation. Vastly dilated sinusoids have endocytosed DiI-Ac-LDL. The individual sinusoidal endothelial cells can be identified (white arrows). Because of intramedullary hemorrhage, the dye has been endocytosed by other cells such as macrophages (yellow arrows) (scale bar=40 μm). E, A section from a mouse 9 days after transplantation from a GFP donor showing that sinusoids were the only vessels that have endocytosed DiI-Ac-LDL (white arrows). A straight arteriole (based on the line of elongated nuclei [pink arrows]) was DiI-Ac-LDL negative (scale bar=30 μm). F, Cells lining the central sinus also endocytose DiI-Ac-LDL (scale bar=120 μm).

**Figure 2.** Injected DiI-Ac-LDL specifically marked the venular side of the murine marrow vasculature, including the narrow sinusoids (n=5 to 10). A, Flushed bone marrow from the tibia of a C57BL/6 mouse that was injected with Dil-Ac-LDL demonstrates the sinusoidal network throughout the marrow (scale bar=600 μm). B, Confocal image of the network of sinusoids from mouse bone marrow injected with Dil-Ac-LDL (scale bar=150 μm). C, A section of the bone marrow of a GFP transgenic mouse that was injected with Dil-Ac-LDL shows the specificity of the Dil-Ac-LDL marker for the sinusoids (scale bar=50 μm). D, A section of bone marrow 4 days after receiving 950 cGy radiation. Vastly dilated sinusoids have endocytosed Dil-Ac-LDL. The individual sinusoidal endothelial cells can be identified (white arrows). Because of intramedullary hemorrhage, the dye has been endocytosed by other cells such as macrophages (yellow arrows) (scale bar=40 μm). E, A section from a mouse 9 days after transplantation from a GFP donor showing that sinusoids were the only vessels that have endocytosed Dil-Ac-LDL (white arrows). A straight arteriole (based on the line of elongated nuclei [pink arrows]) was Dil-Ac-LDL negative (scale bar=30 μm). F, Cells lining the central sinus also endocytose Dil-Ac-LDL (scale bar=120 μm).

**Tie2/GFP Transgenic Mice Show Increased Activity of the Tie2 Promoter in Arteriolar Relative to Sinusoidal Endothelium**

Tie2 is a receptor tyrosine kinase that is expressed by vascular endothelial cells, hematopoietic stem cells, and vasculogenic monocytes. The Tie2 promoter is more active in arteriolar than venular endothelium in the mesenteric and diaphragmatic vasculature. In healthy Tie2/GFP mice, the structures expressing GFP most actively were arteries and arterioles. They formed branched structures in the bone marrow to deliver blood from the nutrient arteries to the smaller arterioles and capillaries (Figure 3A). The expression of GFP was particularly strong in arteries (Figure 3B) recognized by the very large luminal diameter and by multiple layers of smooth muscle cells surrounding them (pink arrows). The presence of smooth muscle could be inferred by the pattern of regularly spaced nuclei of the smooth muscle cells surrounding the arterioles, as can be recognized in hematoxylin and eosin staining and immunostaining for smooth muscle actin (Figure II of the online-only Data Supplement). GFP was also intensely expressed by arterioles (Figure 3C, red arrows) with nuclei of the smooth muscle cell forming an evenly spaced pattern along the vessel (pink arrows). Another type of vessels that were 10 to 15 μm in diameter and had no surrounding muscular sheath also expressed Tie2/GFP (orange arrow in Figure 3C and green vessel in Figure 3F). The blue arrows in Figure 3C indicate some strong GFP-expressing capillaries with 4- to 6-μm diameters.
The sinusoids had a much larger diameter and avidly endocytosed DiI-Ac-LDL. Sinusoidal endothelial cells expressed GFP in the perinuclear region but had much less cytoplasmic GFP expression than arterioles or capillaries (yellow arrows in Figure 3D and 3E). Low cytoplasmic GFP may be due to the thinner walls of the sinusoids compared with arterioles. However, their cytoplasm was intensely labeled by DiI-Ac-LDL relative to the arterioles, which had negligible DiI-Ac-LDL uptake. GFP signals on sinusoidal endothelial cells from frozen sectioned marrow core were lost during sample processing (yellow arrows in Figure 3B and 3F). By using a confocal lambda scanning technique, we demonstrated that the weak green signals from the sinusoidal endothelial cells were true GFP and not due to autofluorescence (Figure III of the online-only Data Supplement).

DiI-Ac-LDL Uptake and Tie2 Promoter Activity Together Define Functional Differences Between Endothelial Beds and Transitional Zones From Arterioles to Sinusoids

Using the combination of DiI-Ac-LDL uptake, Tie2 promoter expression, and 3-dimensional imaging, we observed transitional zones between arterioles and sinusoids. An example of a transitional zone is shown in Figure 4 (also see Figure IV of the online-only Data Supplement). The cytoplasm of endothelium from capillaries that were <5 \( \mu \)m in diameter (small white arrows) expressed GFP strongly. These small-diameter capillaries emptied into tubes with larger diameters, perinuclear GFP expression, and weaker cytoplasmic GFP expression (larger white arrows in the higher-magnification panels). These vessels in turn emptied into sinusoids that were characterized by intense DiI-Ac-LDL uptake and perinuclear GFP expression (pink arrows). The dramatic shift from the very narrow capillaries that strongly expressed GFP to the wider-diameter sinusoids that weakly expressed GFP and showed avid DiI-Ac-LDL uptake occurred through short transitional vessels. These transitional vessels had some features of the vessels they connected: the relatively strong Tie2/GFP expression and lack of uptake of Ac-LDL of the straight capillaries, as well as the perinuclear GFP expression and larger tube diameter of the sinusoids. These features of transitional vessels have not been previously described. The different characteristics of the 3 different types of endothelium in this transitional zone demonstrate functional differences between the endothelial cells that line these different types of vessels.

In the diaphysis, the arteriole-to-sinusoid transition points tended to cluster within small areas of the bone marrow, in contrast to the sinusoids, which were distributed throughout. The low-power view of a core with Tie2 and DiI-Ac-LDL staining in Figure V of the online-only Data Supplement gives a better understanding of the distribution of the arterioles, the transition zones, and the sinusoidal area in the diaphysis. In this area, the major transition zones were not located on the endosteal surface. The majority of vessels located close to or on top of the endosteum surface were thin straight capillaries and sinusoids.
In the metaphysis, there were fewer arterioles. Some sinusoids were longitudinally oriented toward the ends of the bone (Figure 5A). There were some transitional vessels in this area (Figure 5B, white arrow), but it lacked a large transition zone as seen in the diaphysis. More examples of the vessel distribution in diaphysis and metaphysis in sectioned bone marrow are shown in Figure VI of the online-only Data Supplement.

DiI-Ac-LDL and Lectin Coinjection Differentiates Vessel Types in Nontransgenic Mice

The methods to differentially identify arterioles, capillaries, and sinusoids described above require Tie2/GFP transgenic mice. A method to differentiate vessels in nontransgenic mice would be more generally applicable. In pursuing this goal, we first tried to use Tie2 antibodies to detect the expression of Tie2 protein on bone marrow blood vessels, but the staining was weak and the background was high. Because DiI-Ac-LDL specifically labels the sinusoids, a second method was necessary to label other vessel types. We coinjected FITC-conjugated lectin with DiI-Ac-LDL. As Figure 6A shows, arteries and arterioles fluoresced green and did not uptake Ac-LDL. In contrast, the sinusoids were stained by lectin and endocytosed DiI-Ac-LDL, leading to both green and red fluorescence (yellow). In Figure 6B, we can see a thin capillary (green) connected to wider-caliber transition vessel (yellow vessel) that resembles the transition vessel in Figure 4. Coinjection of lectin with DiI-Ac-LDL serves as a second method that can be used to differentiate vessels in all types...
of mice. However, because of the smaller size of the lectin molecule, some lectin leaked out of the vessels and stained surrounding cells (pink arrows in Figure 6A and 6B). Therefore, staining with lectin resulted in a “dirtier” image than using Tie2/GFP mice. Lectin also stained vessels in other organs such as heart and liver at levels rivaling the GFP expression in Tie2/GFP mice. Figure VII of the online-only Data Supplement shows examples of the vascular staining patterns in other organs labeled by coinjection of FITC-conjugated wheat germ lectin with DiI-Ac-LDL.

**Discussion**

Recent interest in the role of the bone marrow sinusoids as niches for hematopoietic progenitors and stem cells has led to efforts to understand the cellular and molecular mechanisms by which the sinusoids develop and are maintained. An important prerequisite to this is the ability to distinguish the sinusoids from other types of vascular structures in the bone marrow.

Transgenic mice expressing GFP downstream of the Tie2 promoter provide a useful tool to visualize vessels in the murine bone marrow. However, Tie2 promoter activity is not equivalent in all blood vessel types. Specifically, Tie2 reporter activity is more pronounced in arteries and arterioles. Anghelina et al reported a “dead-end” distribution of the Tie2/LacZ vessels in the mouse diaphragm. Similarly, we observed pronounced Tie2 reporter activity in the arteries, arterioles, and capillaries, with faint perinuclear expression of the GFP reporter in the venular sinusoids. The perinuclear location of GFP expression in the sinusoidal endothelial cells has not been previously reported. However, lambda scanning confirmed that this signal was due to Tie2 promoter activity, not autofluorescence.

Tie2 expression by hematopoietic stem cells and monocytes has been reported in several articles. However, the Tie2/GFP mice used in our experiments showed little GFP expression in these cell types. Using the Tie2 antibody used by Yano et al. to identify Tie2-positive hematopoietic stem cells and monocytes, we found very few Tie2-positive stem cells and monocytes. The majority of these Tie2-positive cells identified by antibody staining were GFP negative in our Tie2/GFP mice (see Figure VIII of the online-only Data Supplement for flow cytometric comparison of Tie2/GFP expression with Tie2 antibody in our Tie2/GFP mice).

Injection of DiI-Ac-LDL led to the fluorescence of the entire venular side of the marrow vasculature, including the sinusoids, venules, and the central sinus, but not the arteriolar side of vessels. The failure of Ac-LDL to label arterial vessels and capillaries in other tissue has also been noticed. In culture, endothelial cells isolated from the arterial vessels were able to endocytose Ac-LDL. It was found that the effect of the shearing force of blood flow decreased the efficiency of Ac-LDL endocytosis by aortic endothelial cells. Shear flow inhibited scavenger receptor-mediated endocytosis and resulted in decreased uptake of Ac-LDL. This lack of efficient uptake of Ac-LDL on the arterial side of vessels provided us with a tool to distinguish between histologically different types of blood vessels.

By combining levels of Tie2 promoter activity and uptake of Ac-LDL, the transition between arteriolar to venular vessels became apparent. The differences in morphology, level of Tie2 promoter activity, and efficiency of uptake Ac-LDL allowed these transition vessels to be neatly separated from the arterioles and sinusoids that are connected by these vessels. Brookes and Revell described transition points between the arterioles and sinusoids called the arteriovenous junctions. A funnel-shaped junction was found where arterioles and venules met. However, in other studies using India ink injected into the femoral artery or vein to visualize the marrow vasculature, the junctions were found to be more gradual, widening from the arteriole to sinusoids occurring over 2 or 3 microscopic fields. With the functional labeling method reported here, the 2 types of vessels can be differentiated clearly and the transitional vessels linking arterioles to the sinusoids can be identified unambiguously.

The transition zones contain a high concentration of capillaries. Compared with the evenly distributed sinusoids, the uneven distribution of the capillaries could have an effect on the calculation of vascular density, particularly when the staining methods do not differentiate between these 2 vessel types. When the transitional zone is sectioned, more vessel cross sections would be present compared with the sections outside the zone. In addition, because of the small diameter of the capillaries and their presence in high concentrations in specific areas, the presence of these vessels could be falsely identified as newly “sprouted” vessels or “neovessels.”

The significance of these different vascular beds in the bone marrow relative to human disease is currently poorly understood. For instance, could autoimmune vasculitis or other vascular abnormalities within the bone marrow cause bone marrow failure? The effect of chemotherapy and radiation on the marrow sinusoids leads to intramedullary hemorrhage, but to what degree does damage to these structures cause bone marrow failure in heavily pretreated cancer patients? Furthermore, what types of vessels are increased leukemic and myelodysplastic states where there is increased microvascular density? We are developing staining methods that can be used in human samples to differentiate various types of marrow vasculature that might lead to answers to these questions.

Bone marrow sinusoids are highly specialized capillaries and do not always express the antigens that are expressed in other capillary beds. Furthermore, the mouse bone marrow sinusoids differ from human sinusoids in that not all antigens expressed in human marrow vasculature such as CD31, CD34, and von Willebrand factor are expressed by blood vessels in the murine bone marrow. This is true despite the fact that some of these proteins are expressed abundantly in vessels of other murine organs such as the liver. Examples of staining patterns of other widely used agents in the murine bone marrow are given in Figure IX of the online-only Data Supplement. One important drawback to our methods is that DiI-Ac-LDL
staining and lectin staining require blood flow through a vessel for exposure to the agent and could be misleading in states that cause impaired marrow blood flow. A summary of the characteristics and the distributions of different vessels illustrated here is shown in Figure 7. This work demonstrates that studies of bone marrow vasculature require careful choice of markers, depending on the vessels of interest.

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Disclosures
None.

References
Changes in the bone marrow microvasculature have been demonstrated to result from the presence of certain forms of leukemia in the bone marrow. Increases in the number of vascular cross sections within a given section of the bone marrow have been the primary method of demonstrating increases in marrow vascular density. Furthermore, antiangiogenic treatments have been used to treat hematologic malignancies such as multiple myeloma. However, understanding the biology of this extravascular tissue is imperative to developing better targeted therapy. Here, a mouse model is used to demonstrate that the arteriolar and venous vasculatures are physiologically different. Expression of the receptor Tie2 (tyrosine kinase with immunoglobulin and EGF homology domains) was seen predominantly in the arteriolar vessels. Furthermore, endocytosis of the functional endothelial marker acetylated low-density lipoprotein was more pronounced in the marrow sinusoids and veins but not the arterioles. These results suggest that the bone marrow vasculature is functionally heterogeneous. Recognizing this heterogeneity, studies should be undertaken to determine what types of vessels increase in disease states such as multiple myeloma. Understanding this heterogeneity and how disease affects specific types of blood vessels could allow the development of better targeted therapy to treat marrow disorders.
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SUPPLEMENTAL MATERIAL

Supplementary Figure 1. A 3 dimensional step-wise view of the DiI-Ac-LDL labeled sinusoidal system
Supplementary Figure 2. Presence of smooth muscle cells and size of endothelial cell nuclei helped to differentiate different vessel types (n=3-5). A. Immunostaining by anti-alpha smooth muscle actin (SMA) antibody (brown). The SMA positive muscle cells were located along the artery wall (red arrows). The green arrows show elongated arterial endothelial cell nuclei. (Scale bar = 30um). B. H&E staining. The muscle cells surrounding the artery show the same morphology and location (red arrows). The endothelial cells have similar size and shape as in A (green arrows). The sinusoidal endothelial cells lining the sinusoidal wall are smaller and less elongated (yellow arrow) (Scale bar = 30um). C. This panel has been shown in Figure 3 B. The muscle cell nuclei, stained blue by DAPI (red arrows) show the same morphology and location as cells in A and B (red arrows). (Scale bar = 25um). D. H&E staining of an arteriole, which show no obvious smooth muscle cells. The green arrows show the very elongated endothelial cells, similar to the one forming the arteries. The yellow arrow shows a less elongated and smaller sinusoidal endothelial cell. (Scale bar = 25um). E. SMA staining. The arteriole on the lower right corner is SMA positive (brown). The green arrows demonstrate elongated nuclei. The other vessel on the upper left contains endothelial cells with similar size and appearance as the SMA positive arteriole, indicating that they are not sinusoidal endothelial cells, but are SMA negative (green arrows on the left). This vessel is an arteriole/capillary that does not have smooth muscle cells surrounding it. (green arrow). (Scale bar =25 um).
Supplementary Figure 3. Lambda scanning by confocal microscopy demonstrates that green fluorescent emission from the vessels of Tie2/GFP mice is from GFP and not autofluorescence. A. A merged bone marrow confocal image from a Tie2/GFP mouse with Dil-Ac-LDL. (Scale bar = 47 um). B. An enlarged single layer image from the area marked with a white square at panel A (Scale bar = 23um). V1 and V2 were arterioles; S1, S2 were sinusoids; A1, A2 and A3 were other green cells; B1 was the background area showing no green fluorescence. Panel C shows the individual sampling area that was scanned by the confocal microscope using lambda scanning. Emission wavelengths from 500-600 um were scanned in 3 um intervals. D. The graphic view of the plotted spectrum from each scanned sample, showing well separated emission curves from GFP (V1, V2, S1, S2) compared with areas of green autofluorescence (A1, A2, A3, B1). Therefore, the different levels of green signal from the arteries, arterioles, straight capillaries and sinusoidal endothelium were due to localization and quantity of GFP, and not due to autofluorescence.
Supplementary Figure 4. View of transition zone and the neighboring sinusoids dominated area in diaphysis and step-wise view through a transition zone. A and B. Examples of the transition zones and the neighboring sinusoids in the diaphysis. (Scale bar = 150 um). C. Step-wise view through the flattened image in A.
Supplementary Figure 5. Low power view of marrow in the diaphysis from a Tie2/GFP mouse injected with DiI-Ac-LDL. In this area, the major transition zones were not located on the endosteal surface, but some could be seen in the vicinity of the endosteal surface. The majority type of vessels located close to or on abutting the endosteum were thin straight capillaries and sinusoids. (Scale bar = 200um)
Supplementary Figure 6. The comparison of vessel distribution in diaphyseal and metaphyseal areas in sectioned bones (n=3). A. Images from the diaphyseal area. (Scale bars = 400 um, 200 um and 100 um from top to bottom respectively). B. Images from the metaphyseal area. (Scale bars = 400 um, 200 um and 100 um from top to bottom respectively) When the bones were sectioned, the distribution of DiI-Ac-LDL up taking sinusoids and the Tie2/GFP positive arteriole/capillary vessels were easily be distinguished. However, because the sectioning cut off the continuity view of the vessels, the presence of the transitional zone could not be easily seen. The area with a lot of small diameter green vessels could be the transitional zone area, but they can not be readily identified. In these sectioned bone marrow images, we can see some capillaries (white arrows) and some sinusoids (yellow arrows) run long the bone surface. They are directly in contact with the endoseal niches area of the stem cells therefore may have special function in regulating the stem cell functions.
Supplementary Figure 7. FITC conjugated wheat germ lectin and DiI-Ac-LDL double staining in different organs of C57B6 mice (n=2). A. Heart. Lectin stained the same vessels as DiI-Ac-LDL but the intensity was more intense. B, Liver. Lectin positive vessels were everywhere while the DiI-Ac-LDL stained vessels were more prominent in the portal areas. C, Brain. Lectin stained vessels were well defined while DiI-Ac-LDL did not stain specific vessels. D, Intestine. The lectin positive vessels and the DiI-Ac-LDL positive vessels were clearly separated. The DiI-Ac-LDL positive vessels were located at the base of the villae and the lectin positive vessels were present within the villae with very little DiI-Ac-LDL staining. E, Spleen. The majority of vessels were located in the red pulp and lectin and DiI-Ac-LDL co-stained most vessels. Occasionally, FITC-lectin positive vessels were seen within the white pulp. F, Kidney. Lectin labeled epithelial cells on the outer and inner sides of the tubules and DiI-Ac-LDL are cumulated in between. G, Lung. Blood vessels costained with lectin and DiI-Ac-LDL with autofluorescence apparent from the bronchial epithelium. H. Skeletal muscle. Similar to the heart, lectin stained the vessels more prominently than DiI-Ac-LDL.
Supplementary Figure 8. Flow cytometry using phycoerythrin conjugated-Tie2 antibody (CD202 clone TEK4) vs. Tie2/GFP positive cells in Tie2/GFP mice (n=3). A. Flow cytometry of Tie2 positive monocytes. The cells in the left panel were from Lin positive cells from the bone marrow of a Tie2/GFP mice. The Sca-1 and CD11B double positive cells were gated into the middle panel. The Sca-1 and Tie2 double positive cells were gated into the left panel. Only half of the Tie2 positive cells based on the PE-Tie2 antibody binding were GFP positive. B. Flow cytometry of the Tie2 positive cells within the hematopoietic stem cell pool. The cells in the left panel were from gated Lin negative cells from the Tie2/GFP mice bone marrow. The Sca-1 and c-kit double positive cells were gated into middle panel. The Sca-1 and Tie2 double positive cells were gated into the left panel. The majority of the Tie2 positive cells based on the PE-Tie2 antibody binding were GFP negative. C. CD11B positive or negative cells from total living cells were gated to show the ratio of Tie2 antibody positive vs. GFP positive cells. Only a small fraction of the Tie2 antibody positive cells are GFP positive in our Tie2/GFP mice.
Supplementary Figure 9. Other staining methods to identify mouse bone marrow sinusoids. A-B. H&E staining of diaphyseal marrow from a normal C57/B6 mouse (S marks the sinusoids). A. The sinusoids (yellow arrows) were abundant and could be distinguished from capillaries (blue arrow) by shape. (Scale bar = 100um). B. Comparison of the size of endothelial nuclei in an arteriole and a sinusoid. The endothelial cells that lined the arterioles had more elongated nuclei (blue arrows) than the sinusoidal endothelial cells (yellow arrow) (Scale bar = 30um). C-D. vWF was expressed in the mouse hepatic sinusoids (C) (Scale bar = 20um) but not in the bone marrow sinusoidal endothelial cells (D) (Scale bar = 40um). E-F. Fluorescent labeling mouse bone marrow with Dil-Ac-LDL (red) and vWF or CD31 (green). Sinusoidal endothelial cells endocytosed Dil-Ac-LDL (red) but did not express vWF (green) (E) nor CD31 (green) (F) (Scale bar = 15um). G. MECA-32 labeled both arteriole and sinusoids in mouse bone marrow. Megakaryocytes also expressed MECA-32 (Scale bar = 20um). H. Laminin (red) was also expressed by the sinusoidal endothelial cells (S) in mouse bone marrow (Scale bar = 50um). I. Using differential staining method to study the behavior of different vessels in different physiological conditions. Bone marrow core from a Tie-2/GFP mouse 4 days after received 950 cGy radiations and was injected with Dil-Ac-LDL. The sinusoids were dilated (Figure 2D). However, the diameters of the capillaries remained unchanged even thought they are also lack a sheath of smooth muscle and yet these vessels maintained tight junctions and did not seem to be effected by irradiation. However, transitional vessels were distorted by lethal irradiation. (Scale bar = 60um).