Diabetes Mellitus Induces Bone Marrow Microangiopathy

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Objective—The impact of diabetes on the bone marrow (BM) microenvironment was not adequately explored. We investigated whether diabetes induces microvascular remodeling with negative consequence for BM homeostasis.

Methods and Results—We found profound structural alterations in BM from mice with type 1 diabetes with depletion of the hematopoietic component and fatty degeneration. Blood flow (fluorescent microspheres) and microvascular density (immunohistochemistry) were remarkably reduced. Flow cytometry verified the depletion of MECA-32+ endothelial cells. Cultured endothelial cells from BM of diabetic mice showed higher levels of oxidative stress, increased activity of the senescence marker β-galactosidase, reduced migratory and network-formation capacities, and increased permeability and adhesiveness to BM mononuclear cells. Flow cytometry analysis of lineage− c-Kit+Sca-1+ cell distribution along an in vivo Hoechst-33342 dye perfusion gradient documented that diabetes depletes lineage− c-Kit+Sca-1+ cells predominantly in the low-perfused part of the marrow. Cell depletion was associated to increased oxidative stress, DNA damage, and activation of apoptosis. Boosting the antioxidative pentose phosphate pathway by benfotiamine supplementation prevented microangiopathy, hyperperfusion, and lineage− c-Kit+Sca-1+ cell depletion.

Conclusion—We provide novel evidence for the presence of microangiopathy impinging on the integrity of diabetic BM. These discoveries offer the framework for mechanistic solutions of BM dysfunction in diabetes. (Arterioscler Thromb Vasc Biol. 2010;30:498-508.)

Key Words: diabetes ■ microangiopathy ■ oxidative stress ■ progenitor cells

Diabetic patients have ischemic complications more frequently than nondiabetic subjects and also show a worse clinical outcome after an ischemic event. This prognostic disadvantage is partly dependent on diabetes-induced impairment of reparative angiogenesis. The contribution of circulating cells in maintenance of vascular integrity and recovery from ischemic complications has been also acknowledged. Tissue injury triggers the bone marrow (BM) to release progenitor cells (PC) and monocytes with proangiogenic capacities into the peripheral circulation.1–3 A default version of this cellular response may account for the weakened healing capacity in diabetes. However, whether diabetes may damage stem cells (SC) inside the BM either directly or by altering their microenvironment remains to be elucidated.

Maintenance of BM homeostasis is dependent on the interaction between SC and cells of the supportive microenvironment, where SC self-renew, differentiate, or die. Regulatory components of the niche include endothelial cells (EC), mesenchymal cells, and adipocytes. The cellular composition and location of the niche are associated with specialized functions. For instance, the vascular niche, composed of lineage-committed PC, mature hematopoietic cells, stromal cells, and cells of the fenestrated sinusoidal endothelium, preside over the trafficking of cells and solutes between the marrow and circulation.4 The osteoblastic niche, located near the endosteal bone and its trabecular projections, is regarded as the main repository of primitive SC of the marrow.5 The low-oxygenated osteoblastic microenvironment is ideal to maintenance of SC quiescence, with SC differentiation occurring along the oxygen ascent toward the vasculature.6,7 However, some endosteal niches are well-perfused, being enmeshed in microvessels that penetrate the bone, and are thereby equally influenced by signals from osteoblasts and EC and by chemical cues from the circulation.8 Furthermore, SC scattered between the 2 main niches may represent transition entities moving back and forward between the endosteum and vasculature.9

In this study we investigated the status of vascular cells, hematopoietic cells, and their niches in BM of diabetic mice. Results show profound marrow remodeling with depletion of the hematopoietic component and presence of a so-far-unreported form of microangiopathy. Importantly, cell deple-
tion more prominently affected the osteoblastic niche because of the generation of a steeper perfusion gradient across the marrow. Inhibition of oxidative stress prevented BM microangiopathy, hypoperfusion, and hematopoietic cell depletion.

Materials and Methods

A detailed, expanded Materials Methods section is available (available online at http://atvb.ahajournals.org).

Animal Procedures

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (the Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office. Type 1 diabetes (T1D) was induced in male CD1 mice (Charles River, Margate, Kent, United Kingdom) by streptozotocin.10 Age-matched male CD1 mice injected with the vehicle of streptozotocin served as controls. Diabetes was assessed by measurement of glycemia at fast and glycosuria.

At 4 weeks from diabetes induction, T1D subgroups were randomly assigned to receive benfotiamine (BFT; 70 mg/kg body weight per day) or vehicle (1 mmol/L HCl) in drinking water for 24 weeks. Nondiabetic age-matched vehicle-treated male mice served as controls.

Measurement of Marrow Blood Flow

Blood flow was assessed by fluorescent microspheres.

Bone Fixation, Decalcification, and Sectioning

Bones were cleaned from muscle and connective tissue, fixed, decalcified, and finally processed for paraffin embedding.

Morphometric Measurements

Total volume of the marrow was computed from longitudinal and cross BM sections on an Olympus BX40 microscope. Giemsa, Trichrome Masson, and Gomori staining were performed to identify the structural composition of BM.

Immunostaining

To determine capillary and sinusoid density, BM sections were stained with Isolectin IB4 (endothelial marker). Capillaries were recognized as small, regular endothelial structures whose lumen-size does not exceed the diameter of an erythrocyte, whereas sinusoids were identified as irregular vessels, lined by a thin layer of Isolectin IB4-positive EC, and able to contain several erythrocytes (Supplementary Figure I, available online at http://atvb.ahajournals.org). Arterioles were recognized by the vascular smooth muscle cell marker α-smooth muscle actin and Isolectin IB4. The number of capillaries, sinusoids, and arterioles was counted through the entire area of marrow and expressed as average density per mm² of tissue. Additionally, vascular endothelial (VE)-cadherin-2 was used to visualize vascular niches. The endosteal surface lined by osteoblasts was visualized by an anti-neural-cadherin antibody.11 Mouse c-Kit and Sca-1 antigens were used to identify hematopoietic PC, and Ter119 was used to identify erythroblast cells. DNA damage was assessed by staining for p-H2AX.12 A list of used antibodies is

Figure 1. BM remodeling in T1D mice. A, Representative images of hematoxylin and eosin staining of femurs from control and T1D mice (scale bars, 500 μm). High magnifications of epiphysis and metaphysis show decreased cell density and empty spaces corresponding to fat accumulation in the marrow of the T1D mouse (scale bars, 100 μm). Box and whiskers graphs show minimum to maximum values of marrow volume (B), marrow cellular density (C), relative abundance of fat (D), and bone thickness (E). n=7 mice per group. *P<0.05 and **P<0.01 vs controls.
reported in Supplementary Table I (available online at http://atvb.ahajournals.org).

Selection of BM EC
Freshly harvested BM cells were immunomagnetically depleted of CD11b-expressing cells to eliminate myeloid/monocyte fraction and cultured on 0.1% gelatin in DMEM 20% fetal bovine serum supplemented with AcSDKP to avoid SC and fibroblasts contamination. When confluent, cells were analyzed by flow cytometry and immunocytochemistry to assess the expression of endothelium-specific markers. Using the same isolation protocol, confluent BM EC were used in functional studies.

Functional and Western Blot Assays on BM EC
Cell senescence was assessed by measuring β-Gal activity and reactive oxygen species (ROS) using MitoTracker Red CM-H2XROS probe. Migration was assayed using a 24-well transwell set-up and in vitro network formation on matrigel. For static

Figure 2. Microangiopathy in BM of T1D mice. Reduced vascular density and erythrocyte extravasation in T1D BM (A–D). Arrowheads point to vascular structures. Scale bars, 100 μm and 20 μm (I and II). BM EC depletion and increased BM EC apoptosis in diabetes (E, F). n=8 mice per group. *P<0.05 and **P<0.01 vs controls.
adhesion, BM EC were cultured to confluence on 0.1% gelatin-coated glass covers and treated overnight with tumor necrosis factor-α (10 ng/mL). Next, BM mononuclear cells (MNC) from controls mice were prelabeled with calcein-AM, resulting in green fluorescence, and allowed to adhere for 30 minutes on BM EC. Samples were then washed and adherent BM MNC were counted using confocal fluorescent microscopy. To study the influence of flow, confluent BM EC were stimulated as described and mounted onto the microscope stage using a perfusion, open and closed mini chamber system (LaCon) and connected to a perfusion pump. Adhesion was visualized by phase-contrast microscopy and recorded in real time. Transendothelial electric resistance was evaluated by electric cell-substrate impedance sensing. To study transendothelial migration of BM MNC prepared from controls or T1D mice, cells were prelabeled with PKH67 (Sigma) and then left to migrate toward SDF-1 or vehicle through BM EC monolayers on coated transwell filters. Finally, protein expression of phosphorylated VE-cadherin and protein tyrosine kinase 2 in BM EC was measured by Western blot.

Isolation of Marrow Cells From Trabecular Bone
Hematopoietic stem cell isolation kit (Millipore UK) was used for isolation of marrow cells from trabecular bone.

Colonies-Forming Cell Assay
Freshly harvested BM cells from trabecular bone were seeded on methylcellulose (1×10^5 cells/dish) and cultured for 14 days before scoring colonies.

Flow Cytometry Analysis
Freshly harvested BM cells were washed with ice-cold Hank balanced salt solution containing 0.5% bovine serum albumin and 0.02% sodium azide. BM cells were then stained in the same buffer with anti-lineage mixture (Alexa 488), anti-Sca-1 (phycoerythrin), anti-CD34 (Alexa 647), and anti-Kit (Alexa 750 or Alexa 647 when CD34 was omitted). To recognize EC, BM cells were stained with anti-MECA-32 (Biotin), followed by streptavidin-allophycocyanin conjugate. To detect apoptosis, BM cells were stained with annexin V (fluorescein isothiocyanate). ROS-positive cells were identified using CM-H2DCFDA. Distribution of BM cells according to BM perfusion gradient was evaluated using the Hoechst 33342 (Hoe) dye. Briefly, Hoe was injected through the tail vein and the animals were euthanized 10 minutes later to collect the hind limb BM. Cells in microenvironments that are well-perfused by blood are those exposed to the

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**Figure 3.** T1D-induced phenotypic alterations of BM EC. Microphotographs (scale bars, 100 μm) and bar graph illustrating ROS levels (A) and β-Gal activity (B) in BM EC. C, Migration of BM EC toward SDF-1 and vascular endothelial growth factor A. D, Endothelial network formation by BM EC plated on matrigel (scale bars, 500 μm). Adhesion of BM MNC to nondiabetic (C) BM EC or T1D BM EC under static conditions (E) and under the influence of shear flow (F). Western blot analysis of VE-cadherin-pY731 and protein tyrosine kinase 2-pY402 (G). Transendothelial migration of BM MNC toward SDF-1 (100 ng/mL) or vehicle (V) using BM EC isolated from controls (H, left) or T1D mice (H, right) seeded on transwell inserts. For each assay, 3 separate experiments in triplicates were averaged. *P<0.05, **P<0.01, and ***P<0.001 vs controls.
highest concentrations of Hoe, whereas cells in microenvironments that are less perfused are exposed to lower concentrations of Hoe. Flow cytometry identification of cells stained high or low with Hoe (Hoehigh and Hoelow, respectively) allowed for recognition of cell distribution in high-perfused vs low-perfused regions of BM (Supplementary Figure II). Flow cytometry was performed on FACSCanto II and FACSLSRII (BD Biosciences) equipped with FACSDiva software (BD Biosciences). Data were represented using “logical” displays. A list of used antibodies is reported in Supplementary Table II.

Statistics
Differences between multiple groups were compared by analysis of variance, followed by a Holm-Sidak multiple comparison test. Two-group analysis was performed by t test (paired or unpaired as appropriate). *P<0.05 was considered significant.

Results
Diabetes Reduces BM Volume and Cellularity
First, we compared the BM structure of T1D mice at 27 to 30 weeks from the onset of diabetes to age-matched nondiabetic controls. Diabetes remarkably reduced the hematopoietic fraction and caused fat accumulation and osteopenia (Figure 1). No structural alteration was observed at 10 days after diabetes induction (data not shown), discounting an acute toxic effect of streptozotocin on the BM.

Microangiopathy in Diabetic BM
Cumulative vascular density was reduced by 2.9-fold in BM of T1D mice (*P<0.001 vs controls). Analysis of perfused vessels, identified by binding of intracardially injected isolecitin IB4, revealed a consistent reduction of sinusoids, capillaries, and arterioles. Furthermore, the microvasculature appeared fragmented with bleeding into the surrounding marrow (Figure 2A–D).

Flow cytometry analysis of BM single-cell suspensions, using an antibody specific for the EC marker MECA-32, confirmed BM EC depletion and increased BM EC apoptosis in diabetes (Figure 2E, F).

Functional Alterations of Diabetic BM EC
BM EC were isolated from T1D and control mice and their purity was confirmed by flow cytometry and immunocytochemistry (Supplementary Figure III). We found that T1D BM EC express higher levels of mitochondrial ROS (Figure 3A) and cell senescence marker β-galactosidase (Figure 3B), are unresponsive to chemoattractant stimuli, like SDF-1 and vascular endothelial growth factor A (Figure 3C), and fail to form network structures on matrigel (Figure 3D). Furthermore, we observed an increased adhesion of BM MNC to T1D BM EC under static conditions and after introduction of shear flow (Figure 3E, F).

Another hallmark of diabetic microvasculature is its augmented permeability. Confluent T1D BM EC showed a 14%±2% reduction in transendothelial resistance compared to controls BM EC (*P<0.05), which was abrogated by the ROS scavenger N-Acetyl-cysteine, pinpointing oxidative stress as a determinant of altered cell–cell interaction. ROS facilitates transendothelial migration of BM-derived PC through phosphorylation of VE-cadherin by the redox-sensitive protein tyrosine kinase 2,15,16 We found that T1D BM EC have higher phosphorylation levels of VE-cadherin (at tyrosine 731, the β1-catenin binding site) and protein tyrosine kinase 2 (at tyrosine 402, which is the autophosphorylation site for protein tyrosine kinase 2) compared with control BM EC (Figure 3G). Furthermore, T1D BM MNC transmigrate as efficiently as control BM MNC in the presence of nondiabetic endothelium (Figure 3H, left). In contrast, nonspecific migration of BM MNC was enhanced and SDF-1–stimulated migration was abol-
ished in the presence of diabetic endothelium, thus suggesting endothelial barrier dysfunction in T1D (Figure 3H, right).

Diabetes Causes Depletion of BM Sca-1posc-Kitpos Cells

Immunohistochemical analysis documented the reduction of Sca-1posc-Kitpos (SK) cells in BM of T1D, especially at the level of the osteoblastic niche, identified by staining osteoblast lining with neural-cadherin (Figure 4, Supplementary Figure IV). Furthermore, considering longitudinal and coronal sections of BM, we verified that the distance of SK cell clusters of the osteoblastic niche to sinusoids is longer in marrow of T1D mice (9.0±0.4 cell diameters) compared to that in controls (5.5±0.4 cell diameters; *P<0.001).

Flow cytometry analysis confirmed the effect of diabetes on reducing the relative frequency of Lineage− SK (LSK) cells in marrow of the femoral cavity or trabecular bone, a porous plexus enriched with SC and osteoblasts (Figure 5A).5 We also found that the subfraction of primitive CD34pos LSK cells is remarkably reduced in T1D marrow (3.6±0.7 per 100 000 BM cells) compared to controls (27.0±3.0 per 100 000 BM cells; *P<0.01). Concordantly, colony-forming-unit assays showed a reduced formation of multipotent PC colonies (colony forming unit granulocyte-erythroid-makrophage-megakaryocyte [c.f.u. GEMM]) by trabecular BM cells of T1D mice (Figure 5B). However, the colony-forming activity of lineage-committed PC was similar in diabetic and control mice, suggesting compensation downstream to multipotent PC.

Diabetes Reduces BM Perfusion

T1D mice showed a remarkably reduced BM perfusion at the level of femur (0.17±0.01 vs 0.27±0.02 mL/min per gram in controls; *P<0.01) and tibia (0.11±0.01 vs 0.18±0.03 mL/min per gram in controls; *P<0.01).

Figure 5. T1D depletes BM LSK cells. A, Flow cytometry analysis of PI− lineage− c-Kit+ Sca-1+ cells. n=7 mice per group. B, Colony forming unit (cfu) assay of marrow cells harvested from trabecular bone. n=5 mice per group. *P<0.05 and **P<0.01 vs controls.
Predominant LSK Cell Depletion in the Hypoperfused Part of the Marrow

We then determined the relative position of LSK cells with respect to in vivo Hoe dye perfusion gradient.7 Hoe was injected intravenously and then the degree of uptake of the dye by BM cells from different locations was evaluated by flow cytometry. We found that 53% of total LSK cells are located in the Hoelow perfusion region of controls BM, but this fraction decreased to 21% in T1D BM (Figure 6A, central panel). Reversing the gating procedure, we analyzed the...
abundance of LSK cells in total cells and lymphomonocyte fraction of each Hoe perfusion area (Figure 6B). Results confirmed the selective depletion of LSK cells of the low-perfused zone of T1D BM, whereas the high-perfused zone, which corresponds to the predominant localization of MECA-32 pos BM EC (eg, the vascular niche), was relatively preserved. MECA32/H11001 EC were reduced overall in T1D BM (Figure 6C) and, considering their relative distribution, also shifted from the low to the high Hoe perfusion area (Figure 6A, right).

Increased Oxidative Stress in Diabetic BM

Next, we measured levels of oxidative stress in BM cells using CM-H$_2$DCFDA, a cell-permeable intracellular ROS indicator. Flow cytometry analysis showed that ROS high SK cells are greatly increased in T1D BM (Figure 7A). We also verified the presence of higher mitochondrial ROS levels in BM MNC from T1D BM (Figure 6C) and, considering their relative distribution, also shifted from the low to the high Hoe perfusion area (Figure 6A, right).

Excessive oxidative stress reportedly causes DNA damage and reduces the lifespan of BM SC. Levels of p-H2AX (Ser139), a marker of double DNA strand breaks, were 2.5-fold higher in T1D BM cells compared to controls (Figure 7C). Because H2AX is phosphorylated by ataxia telangiectasia mutated, we analyzed ataxia telangiectasia mutated expression by quantitative polymerase chain reaction and found it 2.6-fold higher in T1D BM cells compared to controls. Furthermore, flow cytometry analysis of Annexin V-positive cells unraveled the increased apoptosis of SK cells from BM of T1D mice (Figure 7D).

Stimulation of Antioxidative Mechanism Prevents Microangiopathy and LSK Cell Depletion

We found that diabetes reduces the activity of transketolase and G6PDH, the rate-limiting enzymes of the pentose phosphate pathway, which represents a fundamental source of antioxidant equivalents and substrates for DNA synthesis and repair (Figure 8A, B).

We then asked whether activation of this antioxidative mechanism may protect BM from diabetes-induced damage. Boosting the thiamine-dependent enzyme transketolase by BFT supplementation (Figure 8A) restored G6PDH activity (Figure 8B) and prevented microangiopathy (Figure 8C) and hypoperfusion of diabetic BM (Figure 8D). Furthermore,
BFT prevented oxidative stress (Figure 8E) and p-H2AX elevation (Figure 8F) in T1D BM cells. Importantly, these effects of BFT were associated to prevention of LSK cell depletion, both in terms of absolute number (Figure 8G) and relative proportion to total BM cells (Figure 8H), and inhibition of apoptosis (Figure 8I). Analysis of cell distribution across the Hoe perfusion gradient confirmed the protective action of BFT against diabetes-induced LSK cell depletion (Figure 8J, K).

Discussion

Here we show for the first time to our knowledge the presence of diabetic microangiopathy altering the marrow milieu. Microvascular rarefaction was associated with endothelial dysfunction, encompassing reduced migratory capacity, impaired angiogenic activity, increased adhesiveness, and endothelial barrier disruption. Importantly, these defects were observed after culturing diabetic BM EC in normal glucose, in line with the recent demonstration of epigenetic changes caused by transient hyperglycemia.18

Previous studies have documented the important role of the BM endothelium in maintenance of marrow homeostasis through paracrine and physical interaction with other cells of the marrow.19,20 Another important function of BM vasculature is to deliver nutrients and oxygen to marrow cells. The
peculiar distribution of microvasculature creates differentially perfused environments across the marrow. The most primitive stem cells are believed to reside in the osteoblastic niche at the lowest end of the physiological perfusion gradient, protected from oxidative stress.6,7 However, recent studies demonstrated that a large fraction of endosteal stem cells is enmeshed in vessel networks.21 In diabetic BM, the ongoing microvascular rarefaction inevitably alters the pathlength for oxygen and nutrient diffusion, and, as a consequence, an increasing fraction of marrow becomes critically hypoperfused and secluded from the influence of the vascular niche. Our results indicate that LSK cells of the osteoblastic niche can barely survive in such a harsh environment. However, the BM vasculature can offer an ultimate shelter, as documented by the relative conservation of LSK cells in the perivascular space. To the best of our knowledge, the only precedent for marrow cell depletion in the hypoxic microenvironment, often identified with the osteoblastic niche, is represented by the hematopoietic decline described in aging rodents.8 The model of accelerated senescence fits well with diabetic BM remodeling, because in both conditions fat accumulation occurs along with osteopenia. The mechanism that underpins aging-induced and diabetes-induced increases in adipocyte abundance remains unknown. Fat accumulation could serve not only to fill the empty marrow, pushing marrow cells toward the vasculature, but also to participate in the ongoing diabetic remodeling by secreting paracrine factors and proinflammatory cytokines.22 Of note, a similar remodeling was observed in obese leptin-receptor mutant mice, a model of insulin-resistant type 2 diabetes (P. Madedd, unpublished observations, 2009).

The physiological gradient of ROS acts as a signaling mechanism governing functional compartmentalization of stem cells. Those precious cells, necessary for regeneration of almost all the rest of the whole organism, reside in the “low-risk zone,” ideal for maintenance of quiescence. The function of the ROS high zone adjacent to the marrow vasculature instead is to facilitate stem cell maturation.6 Under pathological conditions, however, excessive production of ROS might endanger the viability of stem cells. Genetically modified mice, lacking essential components of the regulatory system that maintain ROS within the physiological range, show accelerated stem cell senescence and progressive bone marrow failure,23–25 replicating the situation observed in mice exposed to the oxidant buthionine sulfoximine.17 Our data show that an elevation in adipocyte abundance remains unknown. Fat accumulation could serve not only to fill the empty marrow, pushing marrow cells toward the vasculature, but also to participate in the ongoing diabetic remodeling by secreting paracrine factors and proinflammatory cytokines.22 Of note, a similar remodeling was observed in obese leptin-receptor mutant mice, a model of insulin-resistant type 2 diabetes (P. Madedd, unpublished observations, 2009).

The extensive remodeling of bone marrow observed in diabetic mice may not inspire therapeutic optimism. However, previous studies showed that glucose-lowering therapies can restore progenitor cell function to some extent.28 Similarly, in genetically modified animals unable to modulate ROS production, antioxidant administration restored the reconstitutive capacity of hematopoietic stem cells, thereby preventing bone marrow failure.23,24 Our study newly shows that benfotiamine stimulates antioxidative defense through activation of transketolase and protects vascular and LSK cells from oxidative stress and apoptosis.

Conclusion
In conclusion, our results demonstrate the deleterious effect of diabetes on bone marrow homeostasis. Our characterization of the molecular and cellular signature of diabetic pathology in bone marrow along with successful results of BFT treatment may lead to beneficial therapies for human disease. Whether thiamine derivatives may clinically reverse BM failure in diabetes represents the objective of future investigation.

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Disclosure
None.

References


SUPPLEMENTARY MATERIAL

DETAILED METHODS

Animal procedures - The experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (the Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office. Type 1 diabetes (T1D) was induced in male CD1 mice (Charles River) by streptozotocin (STZ). Non diabetic controls consisted in age-matched CD1 male mice injected with the vehicle of STZ. Measurements of glycemia at fast and glycosuria were performed during follow-up to confirm the persistence of diabetes.

Benfotiamine (BFT) supplementation - At 4 wk from diabetes induction, T1D mice were randomly assigned to receive BFT (70mg/kg body weight daily) or vehicle (1mmol/L HCl) in drinking water for 24 wk. Non-diabetic age-matched vehicle-treated mice were studied as a control reference. BFT concentration in drinking water was modified according to consumption. The effect of diabetes and BFT treatment on the activity of the thiamine-dependent enzyme transketolase and glucose-6-phosphate dehydrogenase (G6PDH) was measured in enriched bone marrow (BM) mononuclear cells (MNCs), as described.  

Measurement of marrow blood flow (BF) - BF to the femur and tibia BM was assessed by fluorescent microspheres as reported.

Bone fixation, decalcification and sectioning - Femoral bones were cleaned from muscle and connective tissue and fixed with 4% buffered formalin for 24 h at room temperature (RT). Bones were decalcified in 10% formic acid for 24 h at RT and then kept in PBS and processed for paraffin-embedding. Paraffin sections of marrow were cut at 3 μm thickness for histological analyses.

Morphometric measurements - Total volume of the marrow was computed from longitudinal and cross BM sections on an Olympus BX40 microscope using an ocular objective provided with a 42 points grid (Wild Heerbrugg Instruments Inc.), which defines an area of 0.2 mm². The following parameters were obtained using Giemsa, Trichrome Masson and Gomori staining: -Fractional Volume (%) of bone, hematopoietic component, fatty tissue and collagen -Bone Thickness (μm)

Immunofluorescence on marrow and analysis of vascular profiles - Paraffin embedded BM sections (3μm thick) were kept at 60°C for 10 min and deparaffinized in xylene and rehydrated through passages in alcohol (100% to 70%) and distilled water. Heat-induced antigen retrieval was carried out in 10 mM sodium citrate buffer (pH 6) for 15 min in a microwave oven. Sections were cooled down to RT, washed in distilled water and then rinsed in PBS. In order to block unspecific binding of antibodies, samples were incubated for 30 min with 1% bovine serum albumin (BSA) or 10% goat serum in PBS. For isolectin staining of endothelial cells (ECs), sections of BM obtained from mice, which received an intracardiac (i.c.) injection of biotin-conjugated isolectin IB₄, 20 min before sacrifice, were incubated with streptavidin-AlexaFluor-488 or -AlexaFluor-568 for 1 h at RT. A double staining for Isolectin IB₄ and α-smooth muscle actin (α-SMA) was performed to recognize arterioles, by adding to the above an additional 2h-incubation step with a monoclonal α-SMA-Cy3 antibody at RT. To recognize erythrocytes, BM sections were incubated with a rat anti-mouse monoclonal antibody against TER119, followed by goat anti-rat secondary antibody Alexa-488-conjugated. The antibody identifies also erythroblasts, but the two cell types can be distinguished because erythroblasts are nucleated whereas erythrocytes are non-nucleated. Perfused vessels were visualized with Isolectin IB₄ as above. In all staining procedures, nuclei were visualized by DAPI (4’,6-diamidino-2-phenylindole) staining. Slides were finally mounted with Fluoromount-G mounting medium.
Analysis of BM cells by immunofluorescence - In order to reduce non-specific antibody binding, BM sections were exposed to 10%-20% serum of the species in which secondary antibodies were developed. Quenching of autofluorescence was achieved by immersion of sections in an alcoholic solution of Sudan Black. In addition, each primary antibody solution contained 10% BSA and 10% specific serum. Sections were incubated with isotype-matched controls or specific primary antibodies to detect marrow hematopoietic cells (CD45) and Sca-1pos c-Ki67pos (SK) cells. The reactions were visualized by Fluorescein isothiocyanate-IgG (FITC), tetramethyl rhodamine isothiocyanate -IgG (TRITC) and Cy5-IgG conjugated secondary antibodies to allow the simultaneous detection of multiple antigens. Nuclei were stained by DAPI. The analysis of putative SC clusters was conducted on the entire femur. Moreover, LK cells were found as individual elements or nested in clusters of two or more (range 2 to 5; average 2.3 cell/cluster) closely adjacent cells, predominantly located in the epiphysis and metaphysis. This cellular configuration, strongly suggestive of a niche, was confirmed by the documentation of the engagement of LK cells with the osteoblastic and vascular aspects of the BM through the adherens junctions, N-cadherin and VE-cadherin, respectively. A list of Abs and reagents used for immunohistochemistry can be found in the Supplementary Table I.

Detection of DNA double-strand breaks - DNA damage was assessed by immunohistochemistry exposing BM sections to anti-phospho histone 2AX (p-H2AX) antibody followed by biotin/strepatavidin reaction and visualized by DAB precipitation (ABC system). Nuclei were counterstained with hematoxylin.

Culture and characterization of BMECs used for functional studies - Freshly harvested BM cells were immunomagnetically depleted of CD11b-expressing cells to eliminate myeloid/monocyte fraction and cultured on 0.1% gelatin in DMEM 20% FBS, supplemented with tetrapeptide Ac-Ser-Asp-Lys-Pro (AcSDKP) in order to avoid SCs and fibroblasts contamination. Cells were then analyzed by flow cytometry and immunocytochemistry to assess the percentage of cells expressing endothelium-specific markers (vWF, VCAM-1 or NOS3, isoelectin IB4, DiI-acLDL).

FACS Sorting of BMECs- BM cells were stained with anti MECA-32 (Biotin) followed by Streptavidin-PE and anti CD45 (APC). Sorting was performed on FACS vantage cell sorter (BD Biosciences). MECA-32posCD45neg BMECs were separated on a FACS vantage cell sorter using the following gating procedure. Total BM cells were first gated for propidium iodide (P1, PIneg) and then PIneg BM cells were divided in MECA-32pos CD45neg (P2) and MECA-32pos CD45pos (P3). Only MECA-32posCD45neg BMECs were used.

Immunofluorescence on BMECs - BMECs were seeded on 8-well chamber slides coated with 0.1% gelatin at a density of 4x10^4 cells/well and incubated overnight in a humidified incubator at 37°C in an atmosphere containing 5% CO₂ to allow adhesion. Cells were then fixed in 2% paraformaldehyde in PBS for 15 min and incubated in 5% normal serum of the same species as secondary antibody diluted in 0.1% TritonX-100/PBS for 30 min at RT. Cells were then incubated overnight with primary antibodies against vWF, VCAM-1 or NOS3, followed by AlexaFluor 488-conjugated goat anti-rabbit secondary antibodies for 1 h at RT, in the dark. Matched isotype controls were included in each staining to check for unspecific binding of primary antibodies. After staining for vWF, cells were incubated in 1% BSA/PBS for 20 min at RT and then exposed to biotin-conjugated isoelectin IB4 for 1 h at 37°C, followed by Alexa Fluor 568-conjugated streptavidin for 1 h at RT. Slides were mounted with fluorescence mounting medium containing DAPI and analyzed using an Olympus BX40 fluorescence microscope.

Adherent cells growing in 8-well slides were also assessed for the uptake of 1,1-diocadecyl-3,3',3'-tetra-methyl-indo-carbo-cyanine labelled acetylated low density lipoproteins (DiI-acLDL) and binding of FITC-labelled isoelectin IB4 and then mounted as described above.

Detection of senescent BMECs by staining for β-Galactosidase (β-Gal) activity - BMECs were seeded on 8-well chamber slides as described above and assessed for β-Gal activity at pH 6 using Senescence Detection Kit (Calbiochem) according to the manufacturer’s instructions. Briefly, cells were fixed in
fixative solution and then incubated with a staining solution containing 1 mg/mL X-gal in N-N-dimethylformamide at 37°C overnight. Cells were then observed under a day light microscope for development of blue colour. Five microphotographs/well were taken at x200 magnification and the number of positive cells per microphotograph (tot area: 0.2 mm²) was quantified and expressed as percentage of the total number of cells in the same microphotograph. Three separate experiments in triplicates were analyzed and averaged.

MitoTracker assay - BMECs were seeded on 8-well chamber slides as described above and then assessed for reactive oxygen species (ROS) production using MitoTracker® Red CM-H₂XROS, a mitochondria-selective ROS scavenger and fluorescent probe (Molecular Probes), according to manufacturer’s instruction. MitoTracker® Red CM-H₂XROS is a reduced, non-fluorescent compound that fluoresces when oxidized by ROS after uptake into the mitochondrial matrix of respiring cells. Fluorescent images were captured using an Olympus BX40 fluorescence microscope applying identical exposure settings to all conditions. Fluorescence intensity was evaluated by Image Pro® Plus software. Three separate experiments in triplicates were analyzed and averaged.

Migration assay - Migration was assayed using a 24-well transwell setup (Costar) using polycarbonate filters (membrane pore size: 8 μm). Three separate experiments in triplicates were analyzed and averaged.

Matrigel assay - Cultured BMECs (3x10⁴ cells in a total volume of 100μL EBM-2) were added on top of 100μL gelled, growth-factors-enriched matrigel. After 16 h at 37°C, gels were washed gently with PBS and fixed with 2% paraformaldehyde, followed by H&E staining (to visualize cells) and then mounting with glycerol. Five random view fields were photographed for each sample (40X magnification) in phase contrast (Olympus). A number of n=3 samples per group was analyzed in triplicate.

Static adhesion of BMMNCs to BMECs - Non diabetic (C) or T1D murine BMECs were cultured to confluence on 0.1% gelatin-coated glass covers and treated with TNF-α (10 ng/mL) overnight. Next, C or T1D BMMNCs, pre-labelled with Calcein-AM, resulting in green-fluorescence, were allowed to adhere for 30 min on BMECs. Samples were then washed 5 times carefully with PBS supplemented with 1mM CaCl₂ and 1mM MgCl₂. Samples were fixed, permeabilized and stained for F-actin using fluorescent phalloidin. Adherent BMMNCs were counted in 5 random view-fields using confocal fluorescent microscopy.

Adhesion of BMMNCs to BMECs-under flow - C or T1D BMECs were cultured to confluence and stimulated with TNF-α, as described above. Next, cells were mounted onto the microscope stage using a POC-mini chamber system (LaCon) and connected to a perfusion pump. Using low physiological flow conditions, 1x10⁶ C BMMNCs per mL were perfused over the BMECs. Next, fluid-flow was increased every 5 min. Adhesion was visualized by phase-contrast microscopy and recorded in real-time. From each experiment, 5 view-fields were analyzed.

Electric Cell-substrate Impedance Sensing (ECIS) - C or T1D BMECs were added at 1x10⁴ cells per well (0.8 cm²) to a 0.1% gelatin-coated electrode-array, containing 10 gold-electrodes per array, and grown to confluence. After the electrode-check of the array and the basal transendothelial electrical resistance (TER) of the endothelial monolayer, N-Acetyl cysteine (N-Ac) or DMSO (vehicle) were added and TER was measured on line at 37°C at 5% CO₂ with the ECIS-Model-100 Controller from Applied BioPhysics, Inc. (Troy, NY, USA). After 8 h, data were collected and changes in resistance of endothelial monolayer were analyzed as described previously.

Western blotting of BMECs - BMECs were grown to confluence on 0.1% gelatin-coated dishes (50 cm²), washed twice gently with ice-cold Ca²⁺- and Mg²⁺-containing PBS, and lysed in lysis buffer (25 mM Tris, 150 mM NaCl, 10 mM MgCl₂, 2mM EDTA, 0.02% (w/v) SDS, 0.2% (w/v) deoxycholate, 1% Nonidet P-40, 0.5 mM orthovanadate with the addition of fresh protease-inhibitor-mixture tablets (Roche Applied
Science). After 30 min on ice, cell lysates were collected and the supernatant was separated from the insoluble fraction by centrifugation (14,000 g, 10 min at 4°C). Supernatant was diluted with boiling 2x SDS-sample buffer containing 4% 2-mercaptoethanol (Bio-Rad). The samples were analyzed by SDS-PAGE. Proteins were transferred to 0.45-μm nitrocellulose and the blots were blocked with 5% BSA in Tris-buffered saline with Tween-20 (TBS-T) for 1 h and subsequently incubated at RT with phospho-specific antibodies against VE-cadherin-Y731 and Pyk2-Y402 and with a monoclonal antibody against Tubulin, followed by incubation with goat-α-rabbit-IgG-horseradish peroxidase for 1 h at RT. Between the various incubation steps, the blots were washed 5 times with TBS-T and finally developed with an enhanced chemiluminescence detection system (Amersham Biosciences).

Trans-endothelial migration (TEM) - BMMNC TEM was performed using transwell cell culture inserts equipped with 3μm pore size filters (BD Biosciences).\textsuperscript{11} Inserts were pre-coated with 0.5 μg/mL fibronectin in 0.1% gelatin. BMECs were seeded on coated transwell filters (8x10^4/well) and cultured for 24 h before the experiment was performed in a humidified atmosphere (37°C, 5% CO\textsubscript{2}). SDF-1 (100ng/mL) or vehicle alone was added to the lower compartment of the transwell system in DMEM containing 0.1% BSA. Freshly isolated BMMNCs from C and T1D mice were labeled with PKH67 (Green Fluorescent Cell marker) following the manufacturer’s instruction, re-suspended at 10^5 cell/mL and then added to the top compartment (3x10^5 cells per well in 300 μL). After 24 h incubation at 37°C, non-migrated cells on the upper side of the membrane were removed by scraping, all inserts were fixed for 10 min in methanol, and mounted on slides using Vectashield with DAPI. The number of fluorescent BMMNCs transmigrated to the lower side of the membrane was quantified in duplicates. Five random viewfields at x200 magnification were captured and the number of PKH67-positive BMMNCs was determined. In addition, green fluorescent PKH67-stained BMMNCs migrated to the lower chamber were counted using flow cytometry. Two separate experiments in triplicates were analyzed and averaged.

Isolation of marrow cells from trabecular bone - Hematopoietic Stem Cell Isolation Kit (Millipore UK) was used for isolation of marrow cells from trabecular bone of femurs and tibia. Briefly, the marrow-flushed bones were ground thoroughly with pestle in PBS with 2% FBS so that the bones were opened and broken into small fragments. The bone fragments were washed twice and filtered through a 40μm nylon cell strainer. Bone fragments were incubated in 2 mL of enzyme solution (3 mg/mL Collagenase I & 4 mg/mL Dispase II) for 5 min at 37°C in an orbital shaker at 750 rpm. The bone fragments were then washed with PBS containing 2% FBS and filtered through a 40 μm nylon cell strainer. All collected cells were washed by centrifuging at 400g for 5 min at 4°C, resuspended in PBS containing 2% FBS, filtered through a 40 μm nylon cell strainer and pooled.

Mouse Colony-Forming Cell Assays - BM cells harvested from trabecular bone were washed with PBS containing 2% FBS and plated in 35mm tissue culture dishes in 1.1mL Methylcellulose-Based Medium (StemCell Technologies) according to manufacturer’s instructions. Cells were seeded at 1x10^4 cells/dish and cultured for 14d before scoring colonies. Colonies were distinguished as colony forming unit-erythroid (CFU-E), burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte (CFU-G), colony forming unit-macrophage (CFU-M), colony forming unit-granulocyte-macrophage (CFC-GM), or colony forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFC-GEMM) based on their morphological appearance.

Flow cytometry analysis of freshly isolated total BM cells - Total BM cells were washed with ice-cold Hank’s balanced salt solution containing 0.5% bovine serum albumin and 0.02% sodium azide. BM cells were then stained in the same buffer with anti Lineage Mixture (Alexa 488), anti Sca-1 (PE) and anti c-Kit (Alexa 647). To recognize ECs, BM cells were stained with anti-MECA-32 (Biotin) followed by Streptavidin-APC. After washing, stained cells were examined by flow cytometry on FACS Canto II (BD Biosciences) equipped with FACSDiva software (BD Biosciences). Data were represented using “Logical” displays, as previously reported.\textsuperscript{12}
Annexin V staining: To detect apoptosis, BM cells were stained with Annexin V (FITC) together with PI. Total BM cells (1x10^6) were resuspended in Annexin V binding buffer (BD Biosciences) prior to adding 2.5µg Annexin V and were incubated for 30 min at 37°C in the dark.

ROS staining: Total BM cells were incubated with 100 nM CM-H2DCFDA for 30 min at 37°C in the dark. Cells were washed and resuspended in PBS, containing 1mM CaCl^2, 0.5mM MgCl^2, 0.1% (Wt/vol) D-glucose, and 50µM L-arginine.

Flow cytometry analysis of BMEC cultures - Adherent cells were detached by trypsinization and washed in PBS. The staining method described above was carried out. A list of the primary and secondary Abs can be found in the Supplementary Table II.

Measurement of in vivo BM cell perfusion using Hoechst gradient - The Hoe dye perfusion gradient in murine BM was evaluated as described previously. Briefly, mice were injected with Hoechst 33342 dye (Hoe, Sigma-Aldrich, 0.8mg/mouse, via the tail vein) and then sacrificed exactly 10 min after Hoe injection, a time sufficient to avoid that cells extrude the dye. Marrow cells were immediately flushed out from bones, filtered and suspended in cold Hank’s balanced salt solution (HBSS) containing 2 % FBS and 10 mM L-arginine. The dual emission wavelengths were assessed on a logarithmic scale by FACSLSR (BD Biosciences).

Real-time quantitative RT-PCR (qPCR) - Total RNA was isolated from murine BMMNCs (RNaseasy, Qiagen) and RNA quality confirmed using the RNA Nano LabChip in a bioanalyzer (Agilent). RNA was reverse transcribed (Sensiscript reverse transcriptase, Qiagen) and quantitative PCR was performed in a LightCycler (Roche). Primers for PCR amplification for ATM gene are: forward - GATCTGCTCATTTGCTGCCG; reverse - GTGTGGTGCTGATACTTTTGT. 18SrRNA was used as housekeeping gene. The ΔΔCt obtained was used to find the gene relative expression according to the formula: relative expression=2^-ΔΔΔCt, where ΔΔCt=ΔCt of those genes in experimental groups—ΔCt of the same genes in control group. The analyses were performed on at least 4 samples per time and repeated three times.

Supplemental references

5. Li XM, Hu Z, Jorgenson ML, Wingard JR, Slayton WB. Bone marrow sinusoidal endothelial cells undergo nonapoptotic cell death and are replaced by proliferating sinusoidal cells in situ to maintain the vascular niche following lethal irradiation. Exp Hematol. 2008;36:1143-1156.


Supplemental Tables and Figures with Legends

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Supplementary Figure I. Immunofluorescence identification of marrow microvasculature. (a) Microphotographs showing vascular sinusoids (S, panel i and ii) and capillaries (C, panel i) stained red with Isolectin IB₄. (b) Microphotographs showing marrow arterioles, whose endothelial cell layer is stained green with Isolectin IB₄ and smooth muscle cell layer is stained red with α-SMA.
Supplementary Figure II. Distribution of BM cells across the Hoe gradient. Hoe was injected through the tail vein and the animals sacrificed 10min later to collect the hindlimb BM. Cells in microenvironments that are well perfused by blood are those exposed to the highest concentrations of Hoe, whereas cells in microenvironments that are less perfused are exposed to much lower concentrations of Hoe. Flow cytometry identification of LSK cells staining with high (Hoe$^{\text{high}}$) or low levels of Hoe (Hoe$^{\text{low}}$) allowed for recognition of hematopoietic cell abundance in high-perfused vs. low-perfused regions of BM. Sinusoid (S), hematopoietic stem cell (HSC), osteoblast (OB), osteoclast (OC).
Supplementary Figure III. Isolation and characterization of ECs from murine BM. Freshly collected BM cells were either FACS-sorted to isolate MECA-$^{+}$ pos CD45$^{−}$ neg cells (a) or depleted of CD11b cells by immunomagnetic columns to eliminate the myeloid/monocyte fraction and then cultured in DMEM 10% FBS in the presence of AcSDKP to avoid SC and fibroblast contamination. The purity of culture-isolated cells was analysed by flow cytometry (b). Isolated BMECs were also characterized using immunofluorescence microscopy analysis of endothelial markers (c). Scale bars: 50μm.
Supplementary Figure IV. Diabetes alters the abundance of SK cells. (a) Immunofluorescence staining of c-Kit\textsuperscript{pos} (green) and CD45\textsuperscript{pos} (red) cells in BM: arrowheads point to c-Kit\textsuperscript{pos} cells and arrows point to c-Kit/CD45 double positive cells. c-Kit\textsuperscript{pos} are randomly distributed in the marrow (i) or clustered in groups (ii). (iii) Localization of c-Kit\textsuperscript{pos} cell clusters (green) in the osteoblastic (upper panel) and vascular niche (lower panel). High-magnification inserts show clusters of c-Kit\textsuperscript{pos} cells in contact with the paratrabecular bone or a vWF-labeled sinusoid (S, red). Megakaryocytes are also stained in red by vWF. (iv) Immunofluorescence staining of Sca-1\textsuperscript{pos} (green, arrowhead) and vWF\textsuperscript{pos} (red). (v,vii) Arrows indicate Sca-1 (green) c-Kit (red) double positive cells (SK, yellow fluorescence); arrowheads indicate Sca-1\textsuperscript{neg} c-Kit\textsuperscript{pos} cells. About 80% of c-Kit\textsuperscript{pos} cells coexpress Sca-1, whereas 60% of Sca-1\textsuperscript{pos} cells coexpress c-Kit. Scale bars: i,iii: 50 μm; ii,iv,v,vi: 20 μm. (b) Bar graphs show the distribution of SK cells at the level of epiphysis, metaphysis and diaphysis Values are mean±s.e.m.; n=7 per group. *P<0.05 and ***P<0.001 vs. C.