Angiogenic Murine Endothelial Progenitor Cells Are Derived From a Myeloid Bone Marrow Fraction and Can Be Identified by Endothelial NO Synthase Expression


Objective—Endothelial progenitor cells (EPCs) contribute to postnatal neovascularization and are therefore of great interest for autologous cell therapies to treat ischemic vascular disease. However, the origin and functional properties of these EPCs are still in debate.

Methods and Results—Here, ex vivo expanded murine EPCs were characterized in terms of phenotype, lineage potential, differentiation from bone marrow (BM) precursors, and their functional properties using endothelial NO synthase (eNOS)—green fluorescent protein transgenic mice. Despite high phenotypic overlap with macrophages and dendritic cells, EPCs displayed unique eNOS expression, endothelial lineage potential in colony assays, and angiogenic characteristics, but also immunologic properties such as interleukin-12p70 production and low levels of T-cell stimulation. The majority of EPCs developed from an immature, CD31+Ly6C+ myeloid progenitor fraction in the BM. Addition of myeloid growth factors such as macrophage–colony-stimulating factor (M-CSF) and granulocyte/macrophage (GM)-CSF stimulated the expansion of spleen-derived EPCs but not BM-derived EPCs.

Conclusion—The close relationship between EPCs and other myeloid lineages may add to the complexity of using them in cell therapy. Our mouse model could be a highly useful tool to characterize EPCs functionally and phenotypically, to explore the origin and optimize the isolation of EPC fractions for therapeutic neovascularization. (Arterioscler Thromb Vasc Biol. 2006;26:1760-1767.)

Key Words: endothelial progenitor cells ■ myeloid cells ■ neovascularization ■ lineage differentiation ■ eNOS

Human peripheral blood (PB) contains bone marrow (BM)–derived progenitor cells with angiogenic properties.1–3 These cells have the potential to differentiate toward endothelial cells (ECs) and are therefore named endothelial progenitor cells (EPCs). Transplantation of EPCs has been shown to be effective in animal models for re-endothelialization4,5 and adult neovascularization6,7 as well as in human patient studies aimed to enhance myocardial regeneration after acute myocardial infarction.7 Although EPCs are used in clinical trials, the exact phenotypic and lineage/differentiation parameters of ex vivo–expanded EPCs are poorly defined, and it is not clear which cell populations will be most effective in repair studies. EPCs can be derived from CD34+ as well as CD34− or CD34low cells and can be isolated and expanded ex vivo using BM aspirates and PB CD14+ mononuclear cell fractions.8–12 In many studies, EPCs are characterized by their adhesive spindle-like morphology, staining with the EC-binding lectin Ulex europaeus agglutinin (Ulex), and the capacity to endocytose acetylated low-density lipoprotein (acLDL).2,11 Although this may generally suffice for EPC studies dealing with EPCs obtained from healthy animal models or humans, the different culture conditions and sources used may lead to a large heterogeneity and functionally suboptimal EPC populations.13,14 It has even been suggested that transplantation of certain cell fractions may contribute to adverse side effects.15 Clinical studies demonstrated that in patients experiencing diabetes and hypertension, the number of circulating EPCs is severely decreased, and the cells are dysfunctional.16–19 This altered phenotype of EPCs could contribute to and might even endow the progression of the pathogenesis of ischemic vascular disease in these patients.

It has been shown that cells from the myeloid lineage (eg, EPCs) show a wide phenotypic overlap20 and, as we demonstrate here, that Ulex and the uptake of acLDL, among other often used endothelial markers, are not specific for EPCs. Therefore, discrimination is difficult between EPCs and other myeloid cells such as dendritic cells (DCs) and macrophages.
(Mps), which are also in close contact with the vascular system. Myeloid progenitor cells exhibit a very high plasticity, and under different circumstances, a precursor cell can be skewed toward alternative differentiation directions. To better characterize the nature of the angiogenic myeloid cell (EPCs) compared with other myeloid cells and mature ECs, we first performed a detailed comparative phenotypic and functional analysis of cells stimulated to differentiate into EPCs, DCs, or Mps starting from the same progenitor cell populations. Second, we used a transgenic mouse model expressing endothelial NO synthase (eNOS) fused to green fluorescent protein (GFP). The expression of the transgene is driven by the native human eNOS promoter and the transgenic mice show an endothelium-specific expression pattern in many different organs. Therefore, this transgenic mouse model is expected to precisely distinguish cells from the EC lineage from other myeloid cells.

**Materials and Methods**

For detailed methods, please see the online Materials and Methods, available at http://atvb.ahajournals.org.

**Animals**

C57BL/6J and FVB wild-type mice 6 to 22 weeks of age were used. eNOS-GFP transgenic mice with C57BL/6J as well as FVB background were generated and bred as described previously.

**Isolation and Differentiation of Murine EPCs, DCs, and Mps**

Single-cell BM suspensions were prepared by flushing femora and tibiae. BM isolates were used to culture EPCs, Mps, and DCs for 7 days using optimal culture conditions to differentiate the cells. For activation, EPCs, Mps, and DCs were incubated overnight with lipopolysaccharide (LPS) at day 6, and overnight culture supernatants were collected and frozen for cytokine measurements.

**Antibodies and Conjugates for Cell Sorting, Flow Cytometric, and Immunohistochemical Analysis**

Antibodies against ER-MP12 (CD31), ER-MP20 (Ly-6C), F4/80, mouse endothelial cell antigen (MECA)-20 MECA-32, CD11c, major histocompatibility complex (MHC) class II, CD14, kinase insert domain receptor (KDR), Sca-1, c-kit, CD34, vascular endothelial–cadherin, and Flt-1 receptors were used to phenotype and characterize cells. Flowcytometric analyses were done with proper isotype controls for the antibodies. For lectin staining, cells were stained with rhodamine-labeled Bandeiraea simplicifolia lectin and Ulex europaeus agglutinin-I. The uptake of Dil-labeled acLDL cells was measured by flowcytometry.

**Cell Sorting**

Before sorting of cells, labeled cell suspensions were filtered more than a 30-µm pore size sieve to avoid clogging of the nozzle. The purity of the sorted cell suspensions exceeded 95%.

**Cytokine Detection**

Interleukin-10 (IL-10) and IL-6 ELISA kits (Biosource) and IL-12p40 and IL-12p70 ELISA kits (R & D Systems) were used according to manufacturer protocol.

**Mixed Leukocyte Reaction Assay**

Allogeneic mixed leukocyte reactions (MLRs) were performed to evaluate the ability of the various cells to stimulate a T-cell response.

**In Vitro Angiogenesis Assay**

Conditioned media (16 hours; serum-free medium) were obtained from 6-day EPC cultures and applied on an in vitro angiogenesis assay using human umbilical vein ECs (P3) as tube forming cells. After 14 hours, tube formation was measured and quantified.

The ability of labeled EPCs, DCs, and Mps to incorporate or participate in the formation of vessel-like structures was tested using the same in vitro angiogenesis assay kit.

**Endocytosis Assay**

Uptake of dextran–fluorescein isothiocyanate was done at 37°C for 30 minutes, and cells were washed and then measured by fluorescence-activated cell sorter.

**Real-Time Quantitative Polymerase Chain Reaction**

Quantitative analyses of mRNA levels of eNOS were measured using iCycler polymerase chain reaction technology. GAPDH and actin were used as normalization genes.

**EPC Colony Formation**

An established colony forming unit–endothelial cell assay was used to assess the property of EPCs to proliferate and to differentiate to ECs.

**Statistical Analysis**

Results are expressed as mean±SD. P values of P<0.05 were considered statistically significant (Student t test).

**Results**

For supplemental Figures I through IV, please see the online supplement, available at http://atvb.ahajournals.org.

**Morphological and Phenotypic Comparison of EPCs, DCs, and Mps Derived From BM**

Because of the high phenotypic overlap of EPCs with other cells of the myeloid lineage, it is important to define the criteria that characterize EPCs in more detail. To that end, we first investigated morphological and functional differences between EPCs, DCs, and Mph cultures obtained from mouse BM. In Figure 1A (top), the distinct morphology of the different cells at day 7 is shown. EPCs showed typical spindle-shaped morphology, DCs displayed long-extended dendrites or veils, and Mps were more rounded up and attaching. EPCs were capable of binding Ulex and taking up acLDL to the same extent as Mps. DCs stained for Ulex but hardly took up acLDL particles. Therefore, Ulex staining combined with the uptake of acLDL are not appropriate markers restricted to EPCs.

Next, we determined the expression of surface markers to further characterize EPCs (CD31, MECA-20, MECA-32, BS-1 lectin, Flt-1, c-kit, Sca-1, KDR, VE-cadherin, and CD14), DCs (CD11c and MHCII), and Mps (F4/80, CD11b; Figure 1B; supplemental Figure 1). EPCs displayed a higher expression of MECA-20, CD14 and CD31, in comparison to Mps and DCs (Figure 1B). EPCs and Mps showed a lower expression level of CD11c and MHCII when compared with DCs. The Flt-1 receptor is highly upregulated in total population of the EPCs but also on a small population of DCs (supplemental Figure 1). MECA-32 antibody showed expression on a very small subset of the EPCs and no expression on the EPCs.
fraction when compared with DCs and Mphs. Thus, a unique marker specifically defining EPCs was lacking. At best, EPCs could be characterized and distinguished from DCs and Mphs as spindle-shaped cells that were CD31hi, MECA-20 hi, Flt hi, and F4/80lo.

Functional Comparison of EPCs, DCs, and Mphs Derived From BM
Conditioned medium (CM) of EPCs, Mphs, and DCs was tested for supporting formation of tube-like structures in an in vitro angiogenesis assay. Although CM of DCs and Mphs hardly showed any induction of tube-like structures, EPC CM significantly augmented the formation of tube-like structures (supplemental Figure IIA). Second, using confocal microscopy, we compared the 3 different cell types for their ability to incorporate into or to participate in the formation of tube-like structures. Although DCs, Mphs, and EPCs all attached to the protrusions of the EC, only EPCs were able to specifically adhere to and line up in tube-like structures (supplemental Figure IIB, arrows). Thus, only EPCs and not DCs or Mphs display genuine proangiogenic properties by both factor production and participation in tube formation.

Next, we addressed functional properties specific for Mphs and DCs. Mphs endocytose to clear the body of pathogens, whereas DCs mainly use their endocytic properties to present antigens to T lymphocytes, for instance, in an MLR. Because expected, mature/activated DCs were able to trigger T-cell proliferation. EPCs activated by LPS could do this as well but to a lesser extent (supplemental Figure IID). Unstimulated EPCs hardly induced T-cell proliferation (data not shown).

To evaluate the cytokine profile of EPCs compared with that of DCs and Mphs, we measured IL-6, IL-10, IL-12p70, and IL-12p40 in CM of nonstimulated and LPS-stimulated cells. Although DCs and Mphs were capable of producing all 4 cytokines, EPCs secreted detectable levels of IL-12p70 and IL-12p40 only. IL-12p70 was produced by the EPCs to a similar level as Mphs and DCs, and LPS stimulation of the EPCs strongly enhanced this IL-12p70 production (supplemental Figure IIE). IL-12p40 was produced by EPCs, although to a lower extent than by DCs and LPS-stimulated Mphs. IL-12p70 has been shown to be an active subunit of IL-12, which can regulate T-cell-mediated immune responses by promoting Th1 development. It is striking that IL-12p70 is the predominant IL-12 subtype produced by EPCs.

Concluding, only EPCs have the capacity to induce in vitro angiogenesis, yet they share with DCs and Mphs the capability to endocytose and are also able to act, to some extent, as activated protein C with IL-12–producing capacity.

Tracking EPC Differentiation by Using the Endothelial-Specific Marker eNOS Coupled to GFP
Because there was a considerable phenotypic and also some functional overlap between the EPCs, DCs, and Mphs, we
aimed to specifically track BM-derived cells differentiating toward the endothelial lineage (EPCs). Therefore, a transgenic mouse model was used in which the mice show an endothelium-specific GFP expression pattern. When BM of eNOS-GFP transgenic C57BL/6J mice was harvested (day 0), a small population of cells (~0.05% of total cells) expressed GFP in the transgenic mice, which is not present in control BM isolates (day 0) of wild-type mice (Figure 2A). At day 7 of culture under EPC culture conditions, ~15% (n=6; representative experiment shown) of the attached cells were GFP+ in the transgenic EPCs. There is a high autofluorescent background of cells in the EPC cultures at day 7 in both FL1 and FL2 channels, which is seen in transgenic BM cultures as well as wild-type BM cultures.

When EPC cultures were flow-sorted at day 7 and the GFP− and GFP+ populations were replated separately at the same concentrations, only the GFP+ fraction (by definition, expressing eNOS; supplemental Figure IV) cells displayed the typical EPC morphology of spindle-shaped cells. The GFP− population hardly reattached, indicating that these did not represent EPCs.

To ensure that the GFP reporter specifically tracks ECs and EPCs, BM cells of the transgenic mice were cultured with either granulocyte/macrophage (GM)-CSF to differentiate them to DCs or with M-CSF for Mph differentiation (Figure 2B). In the Mph culture, no GFP+ cells were present, and >90% of the culture was F4/80+ in the DC culture, only a very small percentage (3%) of cells was found to express GFP at a low level. However, these GFP+ cells did not express CD11c, suggesting that these few GFP+ cells were not DCs.

To assess the property of BM-derived EPCs to differentiate and proliferate in an in vitro colony assay and to exclude the possibility of a minute fraction of mature ECs growing out in our cultures, we performed an established CFU-EC assay. BM of transgenic mice was plated on fibronectin-coated dishes for 48 hours, and nonattaching cells were then replated and assessed for colony outgrowth (GFP+ colonies). There were hardly any cells attached to the plates after 2 days, and these few cells did not survive or proliferate in the next 3 days (data not shown). However, the nonattaching fraction did form GFP+ colonies as shown in Figure 2C. This observation was extended when we sorted out the very small GFP+ population, presumably corresponding to a minute fraction of mature ECs in BM. When cultured under EPC culture conditions, the GFP+ population did not survive and did not expand (data not shown), whereas the GFP− population proliferated significantly and differentiated into eNOS+ GFP+ cells.

We conclude that using this mouse model EPC differentiation can be tracked, allowing identification and separation of true EPCs from cells not committed to the endothelial lineage.

**EPC Differentiation Varies Between Different Mouse Strains**

To further explore commitment of BM-derived cells toward the endothelial lineage, GFP expression was followed in time up to 7 days. Because there could be differences between mouse strains, we studied the kinetics of EPC differentiation in 2 different genetic backgrounds: C57BL/6J and FVB eNOS-GFP transgenic mice. At day 0, there was no significant difference in the already very low number of GFP+ cells. At day 1, the attached cells were GFP− (Figure 3A); however, at day 4, eNOS-GFP+ cells appeared in both strains that expanded further in time. At day 4, a trend of higher numbers of GFP+ cells was observed in the FVB background mice, but this was not statistically significant. At day 7, >4-fold more GFP+ cells were observed for FVB over the C57BL/6J strain (FVB mice 65% ± 11 GFP+ cells [n=6] versus C57BL/6J 15 ± 7.5 [n=6]; *P<0.01; Figure 3B). These data
indicate that eNOS-expressing EPCs can be derived from BM of both strains tested but more readily from FVB mice.

**Ex Vivo–Expanded EPCs From BM Are Mainly Derived From a Specific Myeloid Precursor Fraction**

Next, we investigated which subfraction of the BM contains progenitors for EPCs. Based on a 2-color flow cytometry analysis with ER-MP12 (anti-CD31) and ER-MP20 (anti-Ly-6C), total BM cells can be separated in 6 phenotypically and functionally distinct subsets. We previously showed that 3 of these subsets contain myeloid progenitor cells that can give rise to Mphs and DCs. Here, based on CD31/Ly-6C profiles, all 6 subsets were flow-sorted from total BM of both eNOS-GFP transgenic mice and cultured under EPC conditions (Figure 4).

In 4 of 4 sort experiments, GFP+/H11001 EPCs appeared in the cultures derived from the CD31+/Ly-6C+ (P4) subset. As expected, significantly fewer GFP+ cells appeared in the culture of eNOS-GFP C57BL/6J background compared with the eNOS-GFP FVB. We demonstrated previously that nearly 80% of this CD31+/Ly-6C+ (P4) cell fraction is comprised of myeloid progenitor cells, indicating that the majority of EPCs are derived from these cells. In 2 of 4 experiments, we observed a few GFP+ cells in the CD31+/Ly-6C- (P6) subset but only in the FVB background. In 1 of 4 sorting experiments, a very small fraction of GFP+ cells was also seen in the CD31dim/Ly-6Clo (P1/2) subfraction. Because this fraction contains lymphoid progenitor cells and hematopoietic stem cells, it could be that it takes longer to induce EPC differentiation from this fraction or that the necessary factors are missing in the in vitro culture system used here. In conclusion, the main source of EPCs from BM is the CD31+/Ly-6C+ (P4) subset, whereas DCs and Mphs can also be differentiated from the P1/2 and P6 fraction.

Additional phenotyping of the CD31+/Ly-6C+ (P4) subpopulation compared with cultured EPCs and mature ECs (bEnd3 cells) showed that c-kit was markedly present in the BM P4 fraction but very minor in the EPC cultures and absent in the mature EC cultures (supplemental Figure IIIB). A subpopulation of 11% of this P4 fraction showed Sca-1 expression, whereas Sca-1 expression seemed to be highly present on EPCs as well as mature ECs. The observation that Sca-1 is expressed on ECs and even a possible function of expression of Sca-1 on ECs has been proposed previously by Luna et al. Vascular endothelial growth factor-1 (Flt-1) is highly expressed on eNOS+ cells, whereas KDR is not yet detectable. VE-cadherin is positive on a small subset of cells and has been confirmed by immunohistochemical staining (data not shown). CD31 is upregulated in EPC fraction and showed an even higher expression on mature ECs (supplemental Figure IIIIC). Myeloid markers such as CD11b were downregulated on GFP+ EPCs and even further on mature ECs, especially when compared with Mphs and DCs. BS-1 lectin staining of the total population of both EPCs and
mature ECs was confirmed by flowcytometric as well as immunohistochemical analyses. We conclude that with the notable exception of Sca-1, EPCs express higher levels of progenitor/stem cell markers than mature ECs and begin to express EC-specific markers while downregulating classical myeloid markers, consistent with a further narrowing of differentiation potential toward the EC lineage.

### Spleen-Derived EPCs Can Be Expanded Using Myeloid-Specific Growth Factors

The therapeutic potential of EPCs has elicited a number of studies that demonstrated that myeloid growth factors can stimulate recruitment, differentiation, or outgrowth of EPCs and may have favorable effects on their function. Therefore, the effects of GM-CSF and M-CSF on EPC differentiation from BM were determined. Addition of these myeloid growth factors to the cultures lowered the numbers of EPCs (GFP^+^ cells) derived from the BM precursors (Figure 5A). Other sources than BM have been used to derive human EPCs and murine EPCs. Human EPCs can be cultured from CD14^-^ mononuclear cell fractions isolated from PB mononuclear cells (PBMCs) or from CD34^-^ progenitor cells isolated from G-CSF-mobilized PB stem cells, umbilical cord blood, or BM. Murine EPCs have been cultured from BM and spleen. The mononuclear cell fraction of the spleen is often used as a homologue of PBMCs from mice because it is described as a reservoir of PB stem/progenitor cells. Spleen-derived murine EPCs have similar functional (angiogenic) and phenotypic characteristics as BM-derived EPCs (data not shown), but they show a lower proliferation capacity. Using the same culture conditions as described above for the generation of BM-derived EPCs, spleen-derived cultures yielded 10- to 50-fold lower numbers of GFP^+^ EPCs (Figure 5B). Addition of myeloid growth factors to spleen-derived cultures showed an increase in the number of EPCs. Thus, addition of myeloid growth factors as GM-CSF and M-CSF could be useful for expanding PB- or spleen-derived EPCs ex vivo but not for BM derived EPCs.

### Discussion

In this study, we characterized the ex vivo commitment of BM precursors toward ECs in terms of phenotype, lineage potential, differentiation from BM precursors, and angiogenic properties. To address these issues in detail and to have an endothelium-specific marker, we made use of eNOS-GFP transgenic mice. This well-characterized system allows a careful appreciation of the relationship between myeloid and endothelial lineages. Our report emphasizes the high phenotypic overlap and close relationship of EPCs, DCs, and Mφs. Consequently, frequently used markers for EPCs, such as the uptake of acLDL and binding of Ulex, are relatively unspecific because these are also markers for Mφs. Despite this high phenotypic overlap of EPCs, DCs, and Mφs, the capacity of EPCs to support angiogenesis is a unique feature of EPCs when compared with DCs and Mφs. Although we could demonstrate a potent angiogenic capacity in the CM of EPCs, we observed that only a small fraction of the EPCs did incorporate (as do mature ECs) in tubes. The majority of the EPCs appear to function as pericytes and localize around the tubes and under the junctions but do not form an integral part of it. Other investigators also found that attaching cells derived from BM or PBMCs under culture conditions with vascular endothelial growth factor did not differentiate into ECs but stimulated angiogenesis in other ways. Therefore, the term EPCs might not be an adequate definition of the total cell culture because not all cells might become true ECs under the conditions used. Although we generally refer to these attaching cells with angiogenic capacity as EPCs, after the consensus in the field, the term angiogenic myeloid cells may be more appropriate. Nevertheless, the cells referred to as EPCs are different from mature ECs, as demonstrated in the CFU-EC assays and by phenotypic analysis. EPCs also express higher levels of stem cell markers but lower levels of eNOS, although they are clearly positive for this marker.

We showed that there is a strain difference between FVB and C57BL/6J mice in their capacity to generate EPCs from BM precursors. FVB mice are less susceptible for atherosclerosis, and this might possibly indicate a role for the plasticity of BM precursors to differentiate toward ECs. As a corollary, we conclude that C57BL/6J mice might not be the best strain to choose for studying short-term cultured murine EPCs.
A number of studies indicated that myeloid growth factors such as GM-CSF can be used to augment neovascularization in animal models and in patients. In this study, only for spleen-derived EPCs, the number of eNOS-GFP+ EPCs increased. In BM, addition of M-CSF and GM-CSF to the culture resulted in a decreased number of EPCs, probably because of extensive expansion of myeloid progenitors that are driven into another differentiation lineage than ECs, such as DCs and Mφs. It is becoming increasingly apparent that cells of the myeloid lineage display a high plasticity, and that some of these seemingly “lineage-committed” myeloid cells can, under specific growth conditions, differentiate into cells of another lineage with distinct functional properties. For instance, in the presence of inflammatory cytokines, the normal differentiation of monocytes into Mφs can be skewed to yield DCs. Another example is the differentiation of myeloid cells into cells of the mesenchymal lineages. Likewise, several reports have described the myeloid character of ECs. Cultures of adhered mononuclear cells or DCs grown under stringent angiogenic differentiation conditions have been shown to differentiate into endothelial like cells. We argue that this large degree of plasticity among cells of the myeloid lineage and the close phenotypic overlap between many of these different myeloid lineages (including cells that stimulate angiogenesis) caution the use of these cells in clinical cell transplantation protocols aimed to augment neovascularization in peripheral or cardiac ischemia. In particular, when early outgrowth EPCs are derived from patients subject to chronic systemic inflammation, transplanted cells might have suboptimal angiogenic properties or even induce an unwanted immunologic response. In the present study, we observed that LPS-stimulated EPCs cultures have the capacity, although to a low extent, to induce T-cell proliferation in an MLR. Using short cultured cell sorting experiments, we here show that the best and almost exclusive source for murine EPCs are the myeloid progenitors in the BM. This myeloid character of EPCs is in line with a recent study from Dammel et al showing that CD34+CD14+ cells in PB are a major source of EPCs. Translating our results to the human situation suggest that further purification of human CD34+ cells to include only CD34+ (immature myeloid marker)/CD34+ myeloid progenitors, but exclude contaminating cells that may yield unwanted side effects, could be of clinical relevance. Further experiments have to determine whether human myeloid progenitor cells from BM or cord blood provide a superior source of EPCs.

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Disclosures
None.

References


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Animals.

C57BL/6J, and FVB wildtype mice 6-22 wk of age were obtained from Harlan (Horst, The Netherlands). eNOS-GFP transgenic mice were generated as described previously\textsuperscript{27} and bred at the Central Animal Department of the Erasmus MC (Rotterdam, The Netherlands) under the institutional guidelines.

Isolation and differentiation of murine EPC, DC and Mph

Single-cell BM suspensions, were prepared by flushing femora and tibiae with medium. Mononuclear cells were isolated by ficoll density gradient centrifugation. Unless mentioned otherwise, EPC cultures were plated at a density of 1 \( \times \) 10\( ^6 \) cells per cm\(^2\) on 24-well plates (Nunc) coated with 10 µg/ml fibronectin (Sigma) and cultured up to 7 days in M199 medium supplemented with 20% FBS (Invitrogen), 0.05 mg/ml Bovine Pituitary Extract (Invitrogen), antibiotics, and 10 units/ml heparin (Leo Pharma BV). Several different culture conditions were tested for optimal EPC culture condition\textsuperscript{14,16,18}. Two different coating materials fibronectin (10 µg/ml) and gelatin (2%, Sigma) were tested with either BPE-containing medium described above\textsuperscript{18} or endothelial basal medium (EBM) (Clonetics) supplemented with endothelial growth medium SingleQuots and 20% FCS\textsuperscript{16} with or without extra addition of Vascular Endothelial Growth Factor (VEGF, 100 ng/ml, Peprotech)\textsuperscript{14}. Experiments using eNOS-GFP tg BM revealed that the combination of BPE containing medium and fibronectin-coated surfaces are the most optimal conditions for generating the highest number of GFP+ EPC
after 7 days of culture. These EPC culture conditions are used throughout the study, unless mentioned otherwise.

Mph and DC were cultured from total BM isolates in 10 cm Petri dishes (BD Biosciences) at a density of $2 \times 10^6$. For DC culture we used 20 ng/ml recombinant murine GM-CSF (Biosource) and for Mph cultures we used 10 ng/ml recombinant murine M-CSF (Peprotech). For activation, EPC, Mph and DC were incubated with 50 ng/ml Lipopolysaccharide (LPS, Sigma) at day 6. After overnight incubation at 37°C, the culture supernatants were collected and frozen for cytokine measurements and the cells were harvested for mixed leukocyte reactions (MLR).

An often used mouse brain derived mature endothelial cell line; bEnd3 was used for phenotypic comparisons as well as lung EC isolated from eNOS-GFP transgenic mice. The lung EC were harvested by collagenase treatment of murine lung tissue. Single cell suspensions were cultured with BPE containing medium (as described) on fibronectin coated flasks and the cells were cultured for 4-6 passages.

HUVEC were isolated by trypsin treatment of umbilical cords and cultured with BPE containing medium for 3 passages.

*Antibodies and conjugates for cell sorting, flow cytometric and immunohistochemical analysis*

Undiluted culture supernatants of the hybridomas ER-MP12 (anti-CD31), ER-MP20 (anti-Ly-6C), F4/80, MECA-20 (mouse endothelial cell antigen-20) were
directly used for staining\(^2^8\). Phycoerythrin- (PE-) labeled anti-CD11c, biotinylated anti-MHC class II and anti-CD14 were purchased from BD Biosciences.

Secondary antibodies FITC- or PE-labeled goat anti-rat IgG (G\(\alpha R\)-FITC or G\(\alpha R\)-PE) were purchased from Caltag Laboratories and BD Biosciences respectively. ER-MP12 was purified and biotinylated and ER-MP20 was labeled with FITC conjugate\(^2^9\). Biotinylated antibodies were detected with allophycocyanin-conjugated streptavidin (BD Biosciences). Directly PE-labeled murine antibodies directed to KDR, Sca-1, ckit and CD34 were purchased from BD Biosciences as well as the directly labeled PE isotype controls. Unlabeled MECA-32 and VE-cadherin (BD Biosciences) and isotype controls were labeled with PE-labeled goat anti-rat IgG PE (BD Biosciences) and Flt-1 (Santa Cruz) and its isotype control was labeled with PE-labeled goat-anti Rabbit IgG (BD Biosciences). For lectin staining, cells were stained with rhodamine labeled \textit{Bandeiraea Simplicifolia} lectin (BS-1 lectin 10\(\mu g/ml\), Vector labs). This labeling was performed in cell suspension for flow cytometric analyses and for immunohistochemical stainings. To that end, cells were attached to fibronectin coated-glass slides and incubated for half an hour and fixed with 3% paraformaldehyde. To visualize each cell nucleus, Hoechst (33258, Invitrogen) staining was performed according to the manufacturers’ protocol. To measure the uptake of Dil-labeled acetylated LDL (Molecular Probes) with flow cytometric analysis, cells were incubated (2.4 \(\mu g/ml\)) for 2 hours at 37\(^\circ\)C and counterstained with \textit{Ulex europaeus agglutinin} (UEA)-1 (10 \(\mu g/ml\), Vector), further referred to as Ulex, for 1 hour. Flow cytometric analyses expression were assessed using
FACScan (BD Biosciences) and analyses were quantified using CellQuest software (BD Biosciences).

**Cell sorting**

For cell sorting, BM-derived cells were labeled with ER-MP12^{bio} (anti-CD31) and ER-MP20^{fitc} (anti-Ly-6C)\(^{29}\). Before sorting (FACSVantage; BD Biosciences), cell suspensions were filtered over a 30-µm pore size sieve (Polymon PES) to avoid clogging of the nozzle. After sorting, the purity of the cell suspensions was checked by re-analyzing sorted samples, and purity exceeded 95%.

**Cytokine detection**

IL-10 and IL-6 ELISA kit (Biosource), and IL-12p40 and IL-12p70 ELISA kits (R&D) were used according to the manufacturers protocol.

**MLR assay**

Mixed leukocyte reactions (MLR) were done with allogeneic T cells from C57/Bl6 splenocytes. Cells were incubated with Abs recognizing CD11b, CD45 and MHCII and anti-rat IgG microbeads. Naïve T cells were obtained by negative selection using a magnetic cell sorter. Stimulated (LPS) or non-stimulated DC, Mph and EPC were irradiated sub-lethally. T cells (1.5 \(x\) 10\(^5\) cells/well) were added to varying concentrations of stimulator cells depending on the desired stimulator-responder cell ratio. Proliferation of T cells was measured after 4 days
by uptake of $^3$H-thymidine (1 µCi/well, DuPont-NEN) and expressed as counts per minute (cpm).

**In vitro angiogenesis assay.**

Conditioned media were obtained by replacing the medium of 6-day EPC cultures with serum-free EC basal medium-2 (Clonetics) supplemented with EGM-2 single aliquots (no vascular endothelial growth factor and basic fibroblast growth factor) and culturing the cells for an additional 16-20 h. EPC were counted and conditioned media were diluted to correct for cell numbers. After 14 h, tube formation by HUVECs was measured by staining the viable cells with Calcein-AM (5 µg/ml) (Molecular Probes). For quantification, total tube area was determined using images obtained with an inverted fluorescence microscope and the Scion Imaging software (Scion Corporation) and expressed in arbitrary units. To see the ability of EPC, DC and Mph to incorporate and/or participate in the formation of vessel-like structures, HUVEC were stained with PKH26 (Sigma), a red fluorescent cell linker dye, according to the manufacturer’s protocol. EPC, DC and Mph were stained with Calcein-AM and the labeled cells were applied on the in vitro angiogenesis assay kit in a ratio of 1:4 (EPC: HUVEC). After 14 hours, incorporation/participation of EPC, DC and Mph were evaluated with a confocal microscope (Carl Zeiss) using z-stack images.

**Endocytosis assay.**
Uptake of dextran-FITC was done at 37°C and 4°C (negative controls) for 30 min. Cells were carefully washed and uptake of dextran was measured by flowcytometric analyses.

**Real-Time Quantitative-PCR (RQ-PCR)**

Total RNA preparations sorted cell populations were performed using the RNeasy kit (Qiagen) and the integrity of RNA was checked before further use (Bioanalyzer, Agilent). mRNA expression of human eNOS and two murine normalization genes (Actin and GAPDH) were analyzed using quantitative RT-PCR. cDNA was synthesized from total RNA samples using standard cDNA synthesis reagents and a 1:1 mixture of oligo dT\textsubscript{(12-18)} primers and random hexamer primers (Invitrogen). Quantitative analyses of the synthesized cDNA were performed with use of SYBR green I (Molecular Probes) in real-time PCR (Amplitaq Gold, Applied Biosystems), using an iCycler Thermal cycler (Biorad). Gene specific primer combinations were generated with Oligo Explorer (Gene link) and synthesized (Isogen). eNOS forward primer: GGCTCTCACCTTCTTCTG, reverse primer: ACCACTTCCACTCCTCGTAG. For normalization genes, primer sets GAPDH forward: ACTCCCACCTCTCACCTTC reverse: CACCACCTGTTGCTGTAG and also actin forward: GACTTCGAGCAGGAGATG reverse: GGTACCACCAGACAGCAC were used.

Samples were analyzed in triplicate and threshold cycle numbers and their SD were calculated using icycler v3.0a analysis software (Biorad) and further used to
calculate expression ratio's of the different samples in relation to both normalization genes.

EPC colony formation (CFU-EC)
To assess the property of EPC to differentiate to EC and to proliferate we have used an established CFU-EC assay (Endocult, StemCell Technologies). After 48 hours, non-adherent cells were collected and replated in fibronectin-coated 24-wells plates. After 3 days, GFP+ colonies were detected using fluorescence microscopy.

Statistical analysis.
Results are expressed as mean ± SD. Probability values of \( P < 0.05 \) were considered statistically significant (Student t-test).
B

BS-1 lectin

Counts

BL6 EPC day 7  90 %

BL6 Mph day 7  90 %

BL6 DC day 7  96 %

Rhodamine- intensity

Counts

CD11b-PE

94 %

96 %

98 %

PE- intensity
BM day 0

EPC day 7

bEnd3

CD31-APC

Ly6C-FITC

P4

FL2-height

eGFP

FSC

SSC

FSC
B

- **CD31<sup>hi</sup>/Ly6C<sup>hi</sup>**
  - ckit-PE: 63%
  - SCA-PE: 11%
  - CD34-PE: 7.5%
  - Flt-1-PE: 1.1%
  - KDR-PE: 4.3%
  - MECA32-PE: 1.0%
  - VE-Cadherin-PE: 1.2%

- **EPC: GFP**
  - PE-intensity: 3.5%
  - PE-intensity: 60%
  - PE-intensity: 3.0%
  - PE-intensity: 0.68%
  - PE-intensity: 0.68%
  - PE-intensity: 0.68%

- **bEnd3**
  - PE-intensity: 0%
  - PE-intensity: 79%
  - PE-intensity: 93%
  - PE-intensity: 32%
  - PE-intensity: 61%
  - PE-intensity: 73%
  - PE-intensity: 47%
C

CD31\textsuperscript{bio}-APC

EPC: GFP+

Counts

- 80 %

bEnd3

Counts

- 98 %

APC- intensity

CD11b-PE

Counts

- 50 %

- 15 %

PE- intensity

BS-1 lectin

Counts

- 92 %

- 89 %

Rhodamine- intensity

BS-1 lectin

BS-1 lectin
Fig I. Additional phenotypical characterization of EPC, Mph and DC cultured for 7 days. C57BL/6J BM was cultured under the appropriate conditions for all three cell types. Cells were harvested and stained for flow cytometric analysis with various antibodies. All antibody stainings (filled lines) were corrected with isotype controls (dotted line) and representative histograms are shown (5 experiments performed). Percentage of positive cells was calculated and staining of isotype controls were subtracted. No distinctive marker for EPC could be identified using these antibodies. Figure 1B, shows extra histograms for BS-1 lectin binding and for a myeloid marker CD11b.

Fig II. Functional characterization of murine EPC compared to DC and Mph. (a) In vitro angiogenesis assay. Representative pictures of calcein-labeled tubular structures formed by HUVEC under conditioned media produced by EPC, DC or Mph. Quantitative analyses of the angiogenic capacity of the conditioned media were performed. Data are shown relative to non-conditioned media as control (-sup), which was set at 1.0. Only EPC were able to significantly (* P < 0.05) stimulate angiogenesis. (b) Incorporation into vessel like structures in vitro. The ability of EPC, DC and Mph to incorporate and/or participate in in vitro formed vessel structures was measured by using an in vitro angiogenesis setting. Thereby HUVEC were stained with PKH (red) and EPC, Mph and DC cultured for 7 days were stained with Calcein-AM (green). Cells were visualized by confocal microscopy and representative pictures show tubular EPC (arrowheads) while the morphology of DC and Mph did not change. (c) Endocytosis assay. The
capacity of EPC, DC and Mph to take up large dextran-FITC molecules was examined on ice (filled blue plot; control) and at 37°C (green line). EPC and Mph efficiently phagocytozed dextran, whereas DC showed less capacity above control levels (ice). (d) Mixed lymphocyte reaction. The proliferation of T-lymphocytes was measured by incorporation of ³H-thymidine at day 4 after initial contact with LPS-stimulated EPC, DC or Mph. The ³H-thymidine incorporation was counted and plotted in cpm. LPS stimulated EPC induced T cell proliferation to ~20% of the levels found by adding equal cell numbers of DC, but clearly showed stimulatory capacity in MLR compared to EPC only or T cell only. Values represent the means of triplicate measurements ± SD. This stimulatory capacity was slightly lower than the levels of LPS stimulated Mph. (e) ELISA’s were performed for IL6, IL10, IL12p70 and IL12p40 with conditioned media of non-stimulated (light bars) as well as LPS-stimulated (dark bars) EPC, DC or Mph. Values represent the means of triplicate measurements ± SD. The only detectable cytokines produced by EPC were IL12p70 and IL12p40. LPS stimulation increased IL12p70 production, but not IL12p40 production.

Fig III: Phenotypical characterization of EC differentiation. A panel of stem cell markers and EC markers was used to characterize three different populations of cells in EC differentiation. These gated cell populations were CD31high/Ly6Chigh cells in freshly isolated BM, GFP+ cells derived from a 7 day EPC culture of eNOS-GFP transgenic FVB mice and as positive EC control we used a murine brain endothelial cell line also known as bEnd3 cells (A). Harvested cells were stained with a panel of antibodies and representative histograms out of 3
experiments performed are shown (filled line). Isotype controls were used (dotted line) and the percentages of positive cells above this background were calculated (figure 2B). Panel C shows extra characterization of the GFP+ EPC cells compare to mature EC. CD31 expression as well as CD11b expression was analyzed. As expected, an up regulation of CD31 was observed and on the contrary a diminished expression of CD11b, a myeloid marker could be seen. BS-lectin showed strong binding on both cell types and this binding was confirmed with immunohistochemistry, showing a distinct pattern of lectin binding. Similar results were obtained with cultured lung mEC cells derived from lung tissue of eNOS-GFP tg mice, when gated on the eNOS (GFP+) fraction (data not shown). The expression of GFP, reflecting eNOS expression was about 2.5 times higher on mEC compared to EPC. We also observed a marked difference in the side scatter of mEC and EPC (300 vs. 500 respectively, data not shown).

Fig IV. Expression of eNOS mRNA in sorted cell populations. EPC were cultured for 7 days and GFP+ fraction was separated from the negative cells as shown in upper panel. Total RNA was extracted from the purified populations and eNOS mRNA expression was measured in both fractions relative to normalization genes GAPDH and Actin(not shown) using real time PCR techniques. Relative expression of eNOS normalized to GAPDH was calculated and is shown in lower left panel.