**Cell isolation and culture**

Adult buffy coat samples were obtained from the blood bank at Massachusetts General Hospital. Umbilical cord blood samples were obtained from the Pediatric Research Institute, University of St. Louis, MO, according to guidelines established by the Human Investigation Committee (IRB Protocols#2003-P-000588). The mononuclear blood cells (MNBCs) were isolated and cultured as described previously with minor modifications\(^1\). Briefly, mononuclear cells were isolated by centrifugation over Ficoll plus density gradient solution. The MNBCs were then cultured on collagen I coated dish (BD Bioscience, Bedford, MA) with EGM-2MV medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum. The medium was changed every 2 to 3 days. Once endothelial colonies were formed, the cells were then passaged and transduced with retrovirus vector encoding enhanced green fluorescent protein (EGFP) gene, as previously described\(^2\). All experiments were performed with cells less than 6 passages. C3H10T1/2 (10T1/2) (American Type Culture Collection, Manassas, VA) were grown and maintained in Eagle’s Basal medium (BME) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine.

**Immunocytochemistry and immunohistochemistry**

PB- and CB- EPCs were grown on chamber slides until confluent. The cells were washed with phosphate buffered saline (PBS) and then fixed by incubating them in ice-cold methanol for 30 minutes. The chamber slides were then washed with PBS to remove all traces of methanol. To block non-specific binding, the chamber slides were incubate in PBS with 3% bovine serum albumin (BSA) for 1 hour followed by overnight incubation with primary antibodies at 4°C. The following primary antibodies were used at 1:200 dilution: CD31 (Dako), VE-Cadherin (Pharmingen), von Willebrand Factor (Dako), Tie-2 (R&D Systems), and Ki-67 (Dako). The next day, the chamber slides were washed with PBS and incubated with appropriate Cy3-conjugated secondary antibodies (Jackson Immunoresearch). The chamber slides were then washed and mounted in Vectashield containing DAPI (Vector Laboratories).

Mice with CB-EPCs implanted in a gel in the cranial window were sacrificed and immediately perfused with 4% paraformaldehyde intracardially to remove the red blood cells and to fix the tissue. The gels were then extracted and incubated in 4% paraformaldehyde for 3 hours and then washed with PBS. The gels were initially blocked for endogenous mouse IgG with Vector Mouse on Mouse Kit (Vector Laboratories) for two hours following manufacturer’s protocol. The gels were then incubated overnight at 4°C in blocking solution containing 3% BSA, 5% normal rabbit serum, and 0.3% Triton X100 in PBS. The next day, primary antibodies (CD31 (Dako) and VE-Cadherin (Pharmingen)) were diluted in blocking solution at a dilution of 1:400 and were added to the gel. After overnight incubation with primary antibodies at 4°C, the gels were washed extensively with PBS to remove the unbound antibodies. The gels were then incubated with Cy3-conjugated rabbit anti-mouse secondary antibodies at 1:400 dilution for 3 hours at room temperature. After washing with PBS, the gels were mounted in Vectashield and imaged with confocal microscope (Model#BX61WI, Olympus, Tokyo, Japan) using 20X/0.95 NA water objective. Image was acquired with Fluoview FV500 (Olympus).

**Apoptosis assay**

PB- and CB-EPCs were grown in chamber slides to confluent. The chamber slides were then washed with PBS to remove all traces of serum and growth factors. EGM2 basal media
containing no serum or growth factors were then added to the chamber slides. After 48 hours of incubation, the chamber slides were then washed with PBS and fixed in ice-cold methanol for 30 minutes. The EPCs were then assayed for apoptotic cells with ApopTag® Red In Situ Apoptosis Detection Kit (Chemicon) following manufacturer’s protocol.

**Preparation of tissue-engineered vessel construct**
The EGFP-labeled endothelial progenitor cells (EGFP-EPCs) and 10T1/2 cells were co-cultured in a collagen gel and implanted onto a mouse cranial window (6 to 8 weeks old male SCID mice) as previously described. One million EGFP-EPCs (EPC-alone group) or $1 \times 10^5$ EGFP-EPCs and $2 \times 10^5$ of 10T1/2 (EPCs + 10T1/2 co-implantation group) were suspended in 1 ml solution of rat-tail type 1 collagen (1.5 mg/ml) (BD Biosciences, Bedford, MA) and human plasma fibronectin (90 µg/ml) (Sigma) in 25 mM Hepes (Sigma) buffered EGM-2MV medium at 4°C. pH was adjusted to 7.4 by using 1N NaOH (Fisher Science, NJ). The cell suspension was pipetted into a single well of a 12 well plates and warmed to 37°C for 30 min to allow polymerization of collagen. Once the collagen gel had solidified, one ml of warmed EGM-2MV medium was added into the well and the cell culture plate was then placed overnight in an incubator maintained at 37°C and 5% CO₂. The next day, a skin puncher (4-mm diameter) was applied to the collagen gel construct to create a circular piece and it was then implanted into a cranial window of a severe combined immunodeficient (SCID) mice.

**Visualization and analysis of tissue-engineered vessels**
The fate of the EGFP-labeled endothelial cells *in vivo* was tracked by intravital imaging with multi-photon laser scanning microscopy at various time points (modified Axioskop 50 microscope, Carl Zeiss, Germany and MaiTai Ti:Sapphire laser, Spectra-Physics, Mountain View, CA). Image was taken with 20×/0.50 NA water objective. Functional blood vessels were revealed by intravenous injection of 100 µl of tetramethylrhodamine-conjugated dextran (2,000,000 MW at 10mg/ml) via tail-vein. The same region of the gel was tracked at different time points for consistency. The perfused vessel length density was quantified by manual tracing of perfused blood vessels lined by EGFP-EPCs with a macro developed in-house in Matlab. For each animal, three stacks of images (333 µm by 250 µm) were taken at 5 µm interval in the z direction and an average gel thickness around 80 µm. For consistency, we chose to analyze the middle section of the gel by projecting the maximum intensity of the middle five stacks (20 µm in thickness). EGFP-EPCs derived cord-like structure with no blood flow was similarly quantified as unperfused blood vessel density.

**Vascular permeability measurement**
Vascular permeability to albumin was determined by intravital microscopy as described previously. Briefly, mice were injected with a bolus (100 µl) of 1% tetramethylrhodamine-labeled bovine serum albumin (Molecular Probes, Eugene, OR) in saline via the tail vein. Fluorescence intensity of the tissue was measured every two minutes for a total of 20 min by a photomultiplier (9203B, EMI, Rockaway, NJ) using a 20× objective lens. The effective vascular permeability (P) was calculated as follows: $P = (1-HT) \frac{V}{S} \left\{ \frac{1}{I_0 - I_b} \right\} * \frac{dl}{dt} + \frac{1}{K}$ where I is the average fluorescence intensity of the whole image, $I_0$ is the value of I immediately after the filling of all vessels by rhodamine-BSA and $I_b$ is the background fluorescence intensity. HT is the average hematocrit. V and S are the total volume and surface area of vessels within the tissue.
volume within the view field, respectively. The time constant of BSA plasma clearance (K) was $9.1 \times 10^3$ s.

**Analysis of leukocyte-endothelial interaction**

Leukocyte-endothelial interaction in the engineered vessels was determined as described previously \(^3\). Briefly, endogenous leukocytes were labeled with an intravenous bolus injection of 50 µl of 0.02% rhodamine-6G and then visualized with intravital microscopy. For each animal, 3 to 4 blood vessels were randomly chosen that resembled post-capillary venule based on width of diameter (20 to 40 µm) and blood flow pattern. Video images were recorded and 60 seconds of the recordings were analyzed offline. The number of rolling leukocytes and total flux of leukocytes was counted along a 100 µm segment of vessel. Rolling leukocytes were defined as cells interacting with and moving along the vessel wall at a velocity that was significantly lower than the centerline velocity. The ratio of rolling cells to total flux (rolling count) was used as an indicator of leukocyte rolling. Leukocyte rolling was determined at the baseline and after stimulation with IL1β (R&D Systems, Minneapolis, MN)\(^5,6\). For induction of systemic inflammation, mice were injected intraperitoneally with 100 ng of IL-1β for 4 hours.

**Red blood cell velocity analysis**

Red blood cell (RBC) velocities along the central axis of 15-25 µm diameter vessels in both host and engineered vasculature networks were measured using the line scan modality of the MPLSM to image RBC\(^3,7\). Mean RBC velocity was determined from the mean orientation angle of the RBC motion using a custom image segmentation program written in Matlab version 7.3 (Mathworks, Natick, MA).

**Statistical analysis**

All data were analyzed by ANOVA with the Fisher post hoc test. All data are reported as mean with standard error. Statistical significance was set at $P<0.05$.

**References**
