SUPPLEMENTARY APPENDIX

Hematopoietic stem and progenitor cells use podosomes to transcellularly cross the bone marrow endothelium

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Multi-photon imaging of vascular permeability

Vascular permeability in mice was detected with dextran using fluorescent microscopy. See for details online supplement. Mice were injected with 200 µl PBS containing the vascular marker GS-I (19, 20)(final concentration 30 µg/ml; Vector Labs), and fluorescently-labelled fixable dextran 10kD and/or 500kD (final concentration 30µg/ml; Thermo Fisher Scientific). This was allowed to circulate for 5 minutes before the mice were euthanized, after which tissue (BM, heart, lung, brain, liver, spleen) were dissected and fixed for 4 hours in 4% paraformaldehyde. The tissues were kept in PBS at 4°C before whole mount imaging using multiphoton microscopy. For measuring permeability upon VE-cadherin blocking, similar procedure was followed, yet four hours prior to injection with dextrans and GS-I, mice were injected with a VE-cadherin blocking or IgG1 isotype control antibody (13) (6 µg/mouse; clone 75; BD Transduction Laboratories). For measuring permeability upon irradiation, mice were irradiated (4 or 10 Gy, depending on the experiment) 4 hours prior to dextran injection. Where indicated, mice were also injected with VE-cadherin blocking antibody directly after irradiation as described above. Multiphoton imaging was performed on a Leica SP8 system equipped with a Spectraphysics Insight Deepsee laser and a 25x 0.95NA water immersion objective (Leica Fluotar VISIR). Excitation wavelength was tuned to 880nm, and NDD HyD detectors were used for detecting emission for FITC (525/50nm) and Texas Red (585/40nm). For 3-colors sequential scanning was performed detecting far red (AlexaFluor633) at 650-700nm using a regular PMT. Imaging was performed at 12-bit, at a frame size of 1024x1024 pixels (pixel size: 0.435 x 0.435µm), scanning at 300Hz and an interplanar distance of 0.50-1.00µm. Microvascular permeability was assessed using ImageJ (http://imagej.nih.gov/ij/), and was determined by calculating the fluorescence intensity of dextrans outside the microvessel (extraluminal area) in the various tissues. Extraluminal area was defined using a one cell layer cutoff (approximately 6um), as was assessed experimentally by measuring diffusion distance at baseline by performing line scanning (not shown). To correct for loss of intensity at greater tissue depth, fluorescence intensity of the dextrans was normalized to intensity value of GS-I vascular staining. All values were background-corrected. The mean ratio was calculated per time point and plotted.

Murine HSPC homing assay

Murine BM cells were obtained by crushing femurs and tibiae with a mortar and pestle, and the cell suspension was filtered through a 40-µm cell strainer. Murine c-kit+ HSPC were isolated with c-kit microbeads (StemCell Technologies). HSPCs were thoroughly washed in PBS and 2 x 10^6 cells were injected intravenously into recipient mice irradiated with a dose of 5 Gy. 16h after HSPC transfer, recipient mice were euthanized and homing of HSPC to BM, spleen, lung, and liver was determined with flow cytometric analysis. Gating strategy is shown in supplemental figure S3D.

Flow cytometry

Murine BM cells were obtained by crushing femurs and tibiae with a mortar and pestle. Single splenocyte suspensions were prepared by crushing the spleen through a 70-µm cell strainer with the plunger of a syringe. Single cell suspensions were stained for lineage markers (CD4 (clone GK1.5), CD8 (clone 53-6.7), B220 (clone RA3-6B2), CD11b (clone M1/70) , Gr-1 (clone RB6-8C5) and Ter119 (clone TER-119)), Sca-1 (clone D7), c-kit (clone 2B8), CD45.1 (clone A20) and CD45.2 (clone104) for 30 minutes at 4°C. Antibodies were obtained from eBioscience, unless indicated otherwise. Flow cytometric analysis was performed on a FACSCanto II (BD Biosciences) and 2 – 3 x 10^6 cells per sample were acquired.

Confocal microscopy of murine BM sections

Bones from VE-cadherin/α-catenin mice were mounted directly in tissue tek embedding compound (Sakura Finetek) and snap frozen in liquid nitrogen. Bones from VE-cadherin-GFP mice were treated for 4 hours with 2% PFA prior to mounting in tissue tek and snap freezing. 8µm cryosections were prepared using the CryoJane® Tape-Transfer System (Leica). Sections were air-dried, sections from unfixed bones were fixed for 10 minutes in dehydrated acetone and all sections were blocked with 5% BSA/PBS. Antibodies used for immunolabelling of BM sections were CD45.1 (clone A20; Biolegend) and CD144 (VE-cadherin; clone BV13; Biolegend). In some cases, sections were counterstained with DRAQ5 (ThermoFisher Scientific). Sections were mounted with Mowiol and imaged using a confocal microscope (Leica TCS SP8)

Human HSPC migration assays

Cord blood (CB) was collected according to the guidelines of Eurocord Nederland and $CD34^+$ cells were isolated as previously described (21). Generation of HBMEC cell lines was previously described (22), and HBMEC were cultured in complete EGM-2 MV medium (Lonza). For static migration assays, HBMEC were cultured until confluency on coverslips or 5 µm Transwell insert filters, stimulated for 16h with 10 ng/ml IL-1β (Preprotech), and washed 2 times with assay

medium (IMDM (Lonza) containing 10% FCS). In Transwell migration assays, 100 ng/ml recombinant human CXCL12 (Strathmann Biotech) was added in assay medium in the lower compartment. In some conditions, HBMEC were pre-treated with 10µg/ml α-VE-cadherin antibody (clone 75; BD Transduction Laboratories) for 30 minutes, and α-VE-cadherin antibody remained present during the transendothelial migration assay. In some conditions, HBMEC were pre-treated with NEM (300 µM; Sigma) or equal dilutions of vehicle (EtOH) for 1 minute and washed 2 times with assay medium. Subsequently, HBMECS were co-cultured with $CD34^+$ HSPC for 3 hours. In static migration assays, cells were fixed using 4% PFA for 10 minutes, washed in PBS and coverslips were mounted with Vectashield with DAPI (Vector Labs) on microscope slides. Para- or transcellular migration of HSPC was determined using a confocal microscope (Leica TCS SP8). In Transwell migration assays, migration of HSPC to the lower compartment was quantified by flow cytometry using Cyto-Cal Count Control beads (ThermoFisher Scientific).

Physiological flow assays

Physiological flow experiments were performed as previously described (23). Briefly, HBMEC and HUVEC were cultured in a FN-coated ibidi m-slide VI0.4 (ibidi, Munich, Germany), HBMEC were stimulated for 16h with 10 ng/ml IL-1β (Peprotech) and HUVEC were stimulated for 16 hours with 10 ng/ml TNFα (Peprotech). $CD34^+$ HSPC and freshly isolated neutrophils were resuspended in HEPES medium (20mM HEPES, 132mM NaCl, 6mM KCL, 1mM CaCL2,1mM MgSO4, 1.2mM K2HPO4, 5mM glucose (all from Sigma-Aldrich), and 0.4 % (w/v) human serum albumin (Sanquin Reagents), pH7.4) and were incubated for 30 min at 37 °C. Ibidi flow chambers were connected to a perfusion system and exposed to 0.2 - 0.5 ml/min HEPES shear flow. $CD34^+$ HSPC or neutrophils were subsequently injected into the perfusion system and real-time leukocyte-endothelial interactions were recorded for 20 min by a Zeiss Observer Z1 microscope using a 40x numerical aperture (NA) 1.3 oil immersion objective. Velocity of crawling leukocytes and distance travelled over the endothelium were manually quantified using the ImageJ plug-in Cell Tracker.

Podosome formation assays

For immunofluorescence of podosome formation, $CD34^+$ HSPC were grown on fibronectin-coated 12-mm coverslips. As a control, human DCs were generated from peripheral blood mononuclear cells as described previously (24) and also grown on fibronectin-coated 12-mm coverslips. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma) for 30 minutes to induce podosome formation. After treatment, the cells were washed in cold PBSA (PBS, 0.5% (w/v) BSA, 1 mM CaCl2, 0.5 mM MgCl2) and fixed in 4% (v/v) formaldehyde in PBS (supplemented with 1

mM CaCl2, 0.5 mM MgCl2) for 10 min. After fixation, the cells were permeabilized in PBS-T (PBS + 1% [v/v] Triton X-100 and 10% [v/v] glycerol) for 4 min, followed by a blocking step in PBS supplemented with 2% (w/v) BSA. The cells were incubated with primary and secondary antibodies as indicated, and after each step washed 3 times in PBS-A. Coverslips were mounted with Vectashield with DAPI (Vector Labs) on microscope slides and imaged with a confocal microscope (Leica TCS SP8).

VE-cadherin internalization assays

HUVEC were cultured on fibronectin coated glass coverslips and subjected to 4 Gy irradiation or left untreated. 30 minutes prior to irradiation, a FITC-labelled VE-cadherin antibody (polyclonal; eBioscience) was added to the medium. Cells were fixed at 10, 30, and 60 minutes after irradiation using 4% PFA for 10 minutes, washed in PBS, and stained with an AlexaFluor 647 labelled VE-cadherin antibody (clone 55-7H; BD BD Biosciences) without permeabilizing to stain the membrane fraction. Coverslips were mounted with Vectashield with DAPI (Vector Labs) on microscope slides and imaged with a confocal microscope (Leica TCS SP8). Images were analyzed using ImageJ by determining the colocalization coefficient of VE-cadherin on the membrane, and by determining the fluorescence intensity of VE-cadherin intracellularly.

Immunoprecipitation and western blot analysis

Immunoprecipitation and western blot were performed as previously described (25). Briefly, HUVEC were cultured in a 10cm^2 dish and subjected to 4 Gy irradiation for 1 h or left untreated. Cells were lysed in cold NP-40 lysis buffer and incubated with 0.5 µg of mAb to VE-cadherin (clone BV6, Millipore) and 50 µl of Dynabeads (Invitrogen) at 4°C with continuous mixing. Subsequently, beads were washed with NP-40 lysis buffer and boiled in SDS sample buffer containing 4% β-mercaptoethanol. Samples were analyzed by SDS-PAGE. Proteins were transferred onto a 0.2 µm nitrocellulose membrane (Whatman, Dassel, Germany), and incubated with specific primary antibodies against p120-catenin, beta-catenin, actin and phosphotyrosine (pY-20) (Abs from Transduction Labs) overnight at 4°C, followed by incubation with secondary HRP-labeled antibodies for 1 h at room temperature. Staining was visualized with an enhanced chemiluminescence (ECL) detection system (ThermoScientific, Amsterdam, The Netherlands). Alternatively, blots were incubated with IR-680- or IR-800-dye-conjugated secondary antibodies. The infrared signal was detected and analyzed using the Odyssey infrared detection system (Li-cor Westburg).

Statistics

Mean values plus or minus standard error of the mean (SEM) are shown, unless indicated otherwise. For group comparisons, data were tested for Gaussian distribution, after which a Student t-test (Gaussian) or Mann-Whitney U test (non-Gaussian) was used to compare individual groups; multiple groups were compared by ANOVA or Kruskall-Wallis tests, with Bonferroni or Dunn's post-hoc test, respectively. Statistics were performed using Graphpad Prism 5.0. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Legends Supplemental figures Rademakers et al.

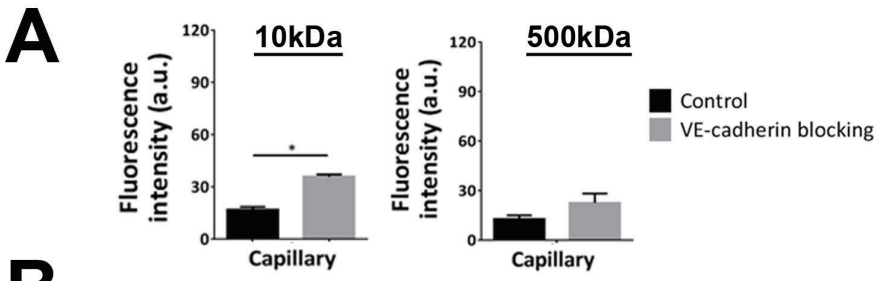
Supplemental figure 1: *The effect of irradiation on the VE-cadherin complex.* (**A**) Quantification of vascular permeability in capillaries of the heart after blocking VE-cadherin. (10kD: n=7-8 per group, 500kD: n=4 per group). (**B**) Schematic summary of experimental set-up for D-F. (**C**) HUVECs were cultured on FN-coated glass covers and pre-incubated with VE-cadherin antibody (red) prior to 4 Gy treatment or left untreated, as indicated; followed by additional VE-cadherin staining (green) on fixed but not permeabilized cells, making it possible to discriminate between internalized VE-cadherin and membrane VE-cadherin. (**D**) Quantification of membrane-bound VE-cadherin, showing that 4 Gy induces loss of membrane localization of VE-cadherin, measured by co-localization of both VE-cadherin antibodies, as explained in C, after 10 or 60 minutes of 4 Gy irradiation or CTRL conditions. (**E**) Lower graph shows quantification of internalized VE-cadherin, showing that 4 Gy induces VE-cadherin internalization after 60 minutes. Experiment was independently carried out at least three times. (**F**) VE-cadherin was precipitated from HUVEC cell lysates after 60 minutes of 4 Gy treatment or left untreated and analyzed for p120-catenin binding. Although similar amounts of VE-cadherin are precipitated in both conditions, no difference for p120-catenin binding is detected. Lower panels: VE-cadherin was precipitated from HUVEC cell lysates after 60 minutes of 4 Gy treatment or left untreated and analyzed for changes in tyrosine phosphorylation levels. As positive control, HUVECs were treated for 5 minutes with pervanadate. Although similar amounts of VE-cadherin are precipitated in all conditions, no difference for tyrosine phosphorylation of VE-cadherin is detected. As appositive control, pervanadate treatment massively increases the phosphotyrosine levels of VE-cadherin (left panel) and other proteins (right panel; total cell lysates). Actin is shown as protein control in the total cell lysates. Experiment was independently carried out at least three times.

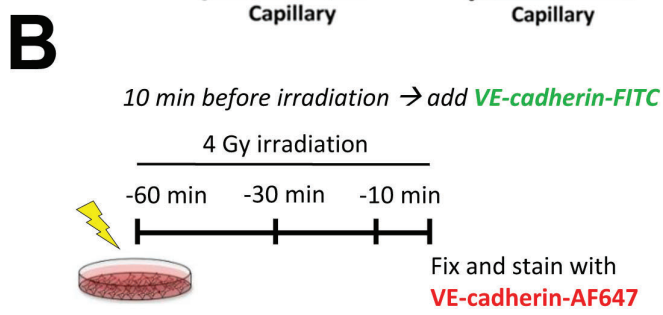
Supplemental figure 2: *The role of VE-cadherin in BM engraftment of HSPC after total body irradiation.* (**A**) Number of different leukocyte types (in percentage) present in the circulation after 1, 2 and 3 months of homing of HSPCs in control and anti-VE-cadherin antibody group (n= 4-5 per group, representative of two independent experiments). (**B**) Number of different leukocyte types present in the bone marrow (as indicated) after 3 months of homing of HSPCs in control and anti-VE-cadherin antibody group (n= 4-5 per group, representative of two independent experiments). (**C**) Number of different leukocyte types present in the circulation after 1 and 2 months of homing of HSPCs in control and anti-VE-cadherin antibody group (n= 4-5 per group,

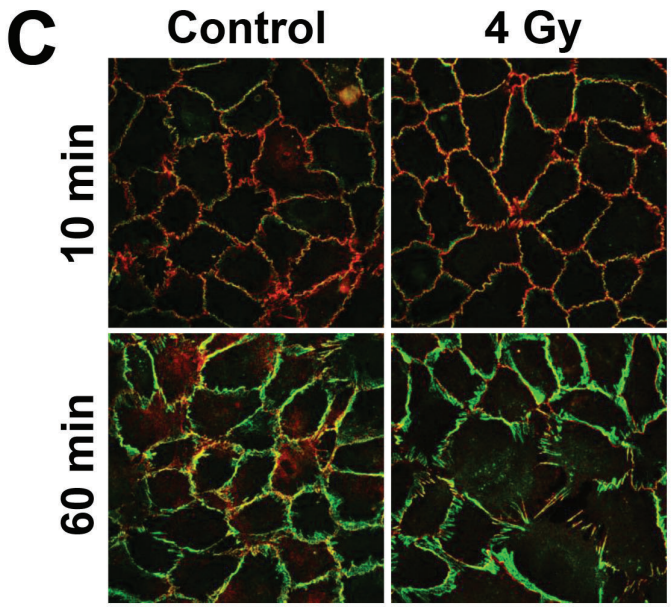
representative of two independent experiments). (**D**) Imaging of the bone marrow sections from HSPC homing experiments in WT and VE-cadherin-alpha-catenin chimera (VE-α-cat) mice (Figure 4D) showing no residual cells left in the bone marrow vessels, indicating that the detected cells were all homed into the bone marrow. Circles show occasional presence of cells in vessels. (**E**) Quantification of cell numbers present in the bone marrow vessels. No difference was detected. (**F**) Sixteen hours after administration of the VE-cadherin antibody, no permeability differences were detected anymore in both arterioles and sinusoids, indicting the reversible effects of blocking VE-cadherin using antibodies (n=2 per group).

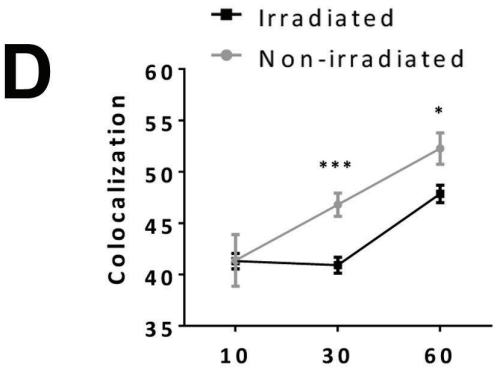
Supplemental figure 3: *Migration of leukocytes over endothelium and podosome formation.* (**A**) Migration tracks of cells under flow conditions on HUVEC (left) or HBMEC (right) show highly motile neutrophils. (**B**) Quantification of distance travelled on the apical surface of the endothelium in µm, or (**C**) average velocity (µm/s). Experiment was carried out independently at least three times. (**D**) Monocyte-derived dendritic cells were treated for 30 minutes with PMA showing the induction of podosomes, based on typical podosome markers: vinculin (green) in the circle and HS1 (magenta) and F-actin (red) in the core. (**E**) Orthogonal projection shows that podosomes localize at the basolateral surface with F-actin in red, vinculin in green and the nucleus in blue. Scale bar, 6µm. (**F**) Murine LSK^+ were treated for 30 minutes with PMA showing the induction of podosomes, based on typical podosome markers: α-actinin (green) and F-actin (red) in the core. Zoom shows magnification of box in upper panels. Scale bar, 25µm.

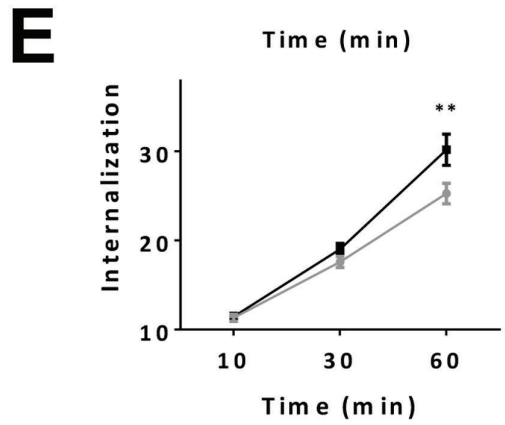
Supplemental figure 1.

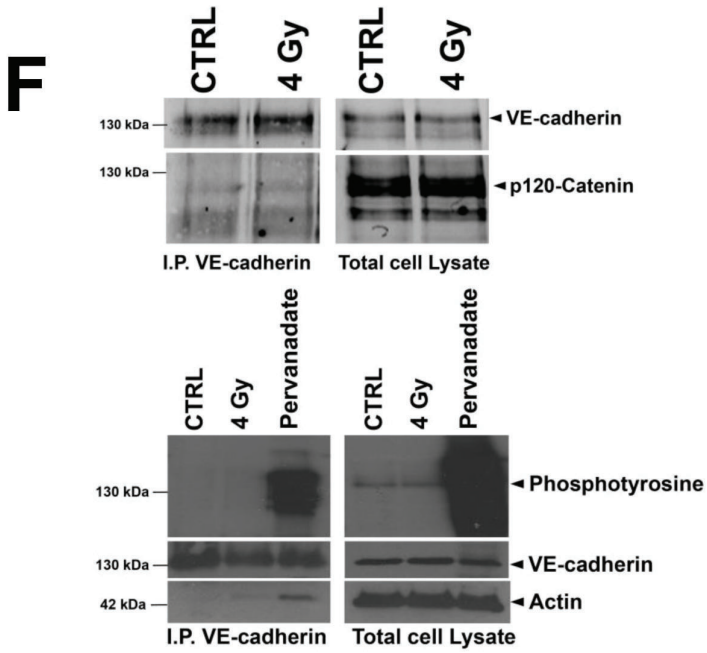




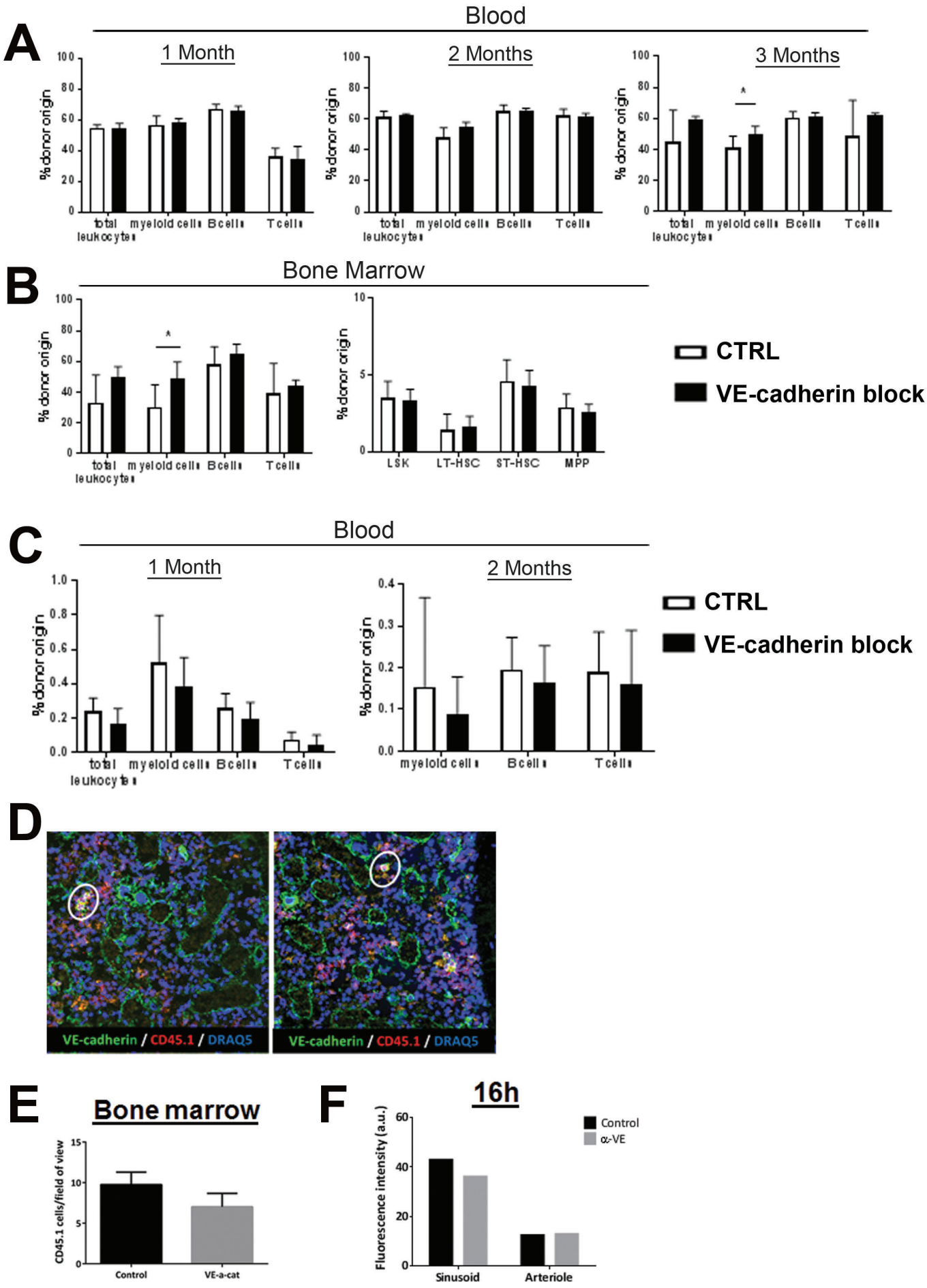








Supplemental figure 2.



Supplemental figure 3.