A Mouse Model of *De-novo* Collateral Formation Following Acute Myocardial Infarction: Dependence on CCR2-Positive Bone Marrow-Derived Cells

Hua Zhang and James E. Faber

Department of Cell Biology and Physiology and The McAllister Heart Institute, School of Medicine, University of North Carolina at Chapel Hill

Online supplemental methods and figures

Detailed materials and methods

Animals. C57BL/6 (B6), BALB/cByJ (BALB/c), C57BLKS/J (BLKS), A/J, SJL/J, C3H-He/J, DBA/2J, CBA/J inbred mice (Jackson Laboratories, Bar Harbor, MN) and CD1 outbred mice (Charles River, Wilmington, MA) were ~3-5 months-old male, except in the case of retrograde fill time which was measured in ~equal numbers of male and female mice. Guinea pigs (American Tricolor) and rats (Wistar and Sprague-Dawley) were male 3-4 months-old. Body weight determined by combining our published[7] and unpublished data for B6, BALB/c, A/J, BLKS (n=10-25): 24.2 ±0.8, 26.0±0.5, 26.5±0.8, 23.2±0.7. Arterial pressure and heart rate (tail cuff, conscious, habituated) for the first 3 strains: 129±2, 614±14; 111±2, 565±4; 135±3, 584±8.[25] None of the data presented in Results for these strains correlated with body weight, arterial pressure or heart rate.

Left anterior descending coronary artery ligation (LADX). LADX was performed under ketamine (53-67 mg/kg) with xylazine (4-7 mg/kg) plus light isoflurane (1.25% max) and 40% O_2, respectively) and rectal temperature maintenance at 37 ± 0.5°C. After endotracheal intubation and connection to a respirator (MiniVent 845, Germany; 120 strokes/min, 200 ul stroke volume) a 3 mm thoracotomy was made between the 3rd and 4th ribs. The LAD was ligated with 7-0 polypropylene monofilament ~3 mm below the edge of the left atrium just before the LAD is no longer evident under brightfield microscopy, or sham ligation with no knotting. Cephazolin (50 mg/kg sc and buprenorphine 40 ug/kg sc) were then administered. This distal LADX induces a small infarct volume with survival rates of ~99%. The ligation surgery required 10-15 minutes, followed by recovery with rectal temperature maintained. Survival was ~99% at all time-points examined. Proximal ligation ~1 mm below the atrial margin was also examined in B6 mice, wherein survival was ~50%.

Microangiography. Immediately after (day-0) and on day-1, -3, -7, -14 and -21 after LADX, heparin was injected (1000 units, ip). Five minutes later the thoracic aorta was cannulated retrograde and the vasculature cleared by perfusion at 100 mmHg pressure with PBS containing papavarine (1mM) and sodium nitroprusside (0.1 mM), followed by 10-15 sec of 1% paraformaldehyde (PFA) in PBS. Yellow Microfil® (8:1:1, latex:diluent:curing agent; Flow Tech, Carver, MA) was infused via the cannula while viewing the coronary circulation with a surgical microscope. Injection was continued until onset of venule filling (day-0, -1, -3) or until the LAD trunk below the ligation was filled to the point of ligation (day-7, -14, -21). After curing for at least 20 min while maintaining infusion pressure, the heart was fixed overnight in 2% PFA, dehydrated through an alcohol gradient (25, 50, 75, 95, 100%), and cleared in methyl salicylate during gentle agitation. The vasculature was imaged with a Leica M205 fluorescent stereomicroscope, and morphometry was performed with Image J software here and for other procedures indicated below. Collateral number and lumen diameter were obtained directly from regions between the opposing crowns of the right and left coronary and septal artery trees. Diameters were corrected for shrinkage of latex caused by post-processing.

Retrograde fill-time. 7 days after LADX the coronary vasculature was dilated and fixed as above. Microfil (1:1, latex:diluent) was then infused at 66 mmHg pressure, with video collected from the start of infusion until the Microfil reached the ligation point. Differences in retrograde fill-time among animals are inversely proportional to differences in conductance of the neo-collateral network.

Microsphere infusion. 7 days after LADX the coronary vasculature was dilated and fixed, as above. Then, during perfusion with PBS at 100 mmHg pressure, 1.8 x10^6 fluorescent microspheres (10 um, 580/605 nm, Invitrogen, F8834) were rapidly injected through a T-connector near the cannulation point. Perfusion was continued for 1 min, followed by 1% TTC (2,3,5-triphenyltetrazolium chloride in PBS) for 3 min. The heart was then placed in TTC at 37^{o}C for 10 min, fixed in 2% PFA overnight, and embedded in OCT. Sixty micron sections were cut from apex-to-base and microspheres were counted with microscopy in the infarct (white area) and non-infarct zones (red area) through the whole thickness of the section. Density was calculated as microsphere number per volume of the two regions, respectively. Differences in the ratio of the densities in the infarct versus non-infarct zones reflect differences in conductance of the neo-collateral network.

Infarct volume. The heart was arrested in diastole by injection of 150 mM KCl through the tail vein. Evans blue (2% in PBS) was injected through the thoracic aorta to delineate infarct versus non-infarct regions while viewing under a surgical microscope. The heart was then chilled for 20 min at -20^{o}C and cut into 1 mm sections that were stained in 1% TTC for 20 min and fixed with 1% PFA. Sections were imaged and infarct (white) and non-infarct (purple) volumes determined.

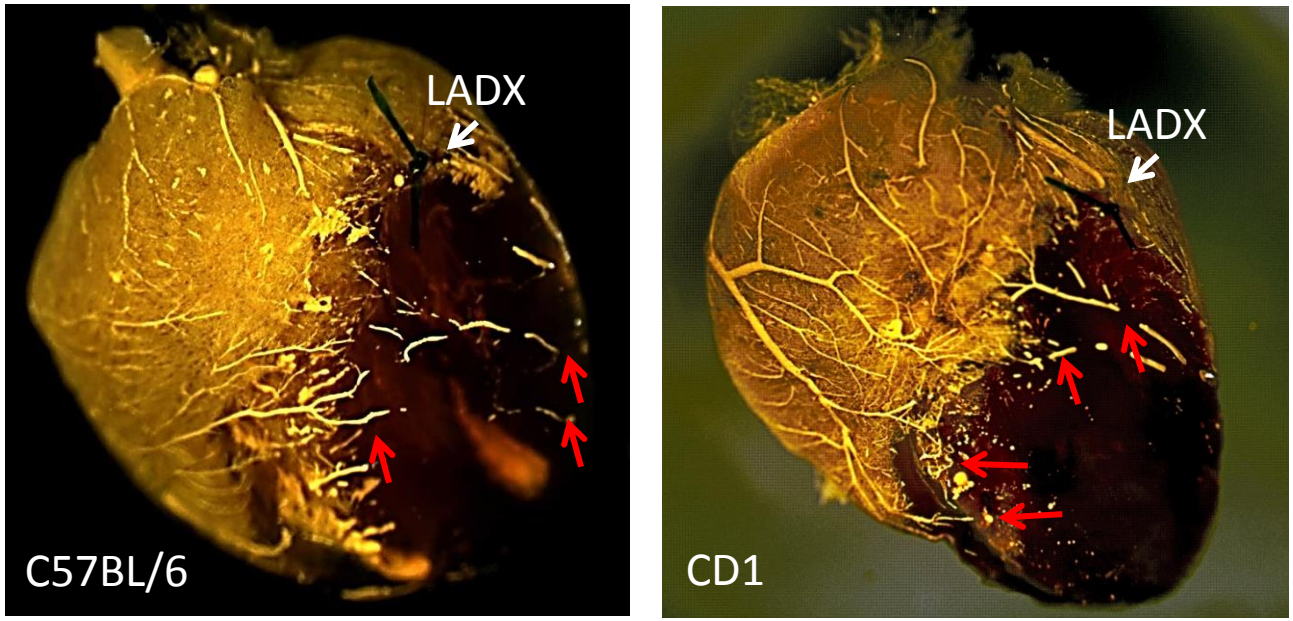
dP/dt analysis. Seven days after LADX, mice were anesthetized with 1.0-1.25 % isoflurane. A pressure sensing catheter probe (Millar Instruments, SPR-1000, cat # 841-0001) was pre-soaked in distilled water at room temperature for 15 minutes before calibration according to the manufacturer's protocol. The right carotid artery was ligated proximal to the carotid sinus, followed by arteriotomy and insertion of pressure and secured with 5-0 silk suture. The probe was then slowly advanced across the aortic valve into the left ventricle. Cardiac cycles were recorded for 5 minutes using a PowerLab 4/30 module and analyzed with LabChart 7 (AD Instruments, Colorado Springs, CO).

Histology and immunohistochemistry. After filling of collaterals with Microfil^R as described above, the heart was fixed in 1% PFA:50% glycerol overnight at 4^{o}C, and then changed to 75% glycerol. When the tissue was semi-transparent, tissue explants containing a collateral(s) were dissected, cryoprotected in 30% sucrose, imbedded in paraffin or OCT, and sectioned at 6 um. Antibodies for CD45, CD34, αSMA, EGFP, were from Abcam, caspase, other (Cambridge, MA). Isolectin-GS-IB4-Alexa568 was purchased from Invitrogen.

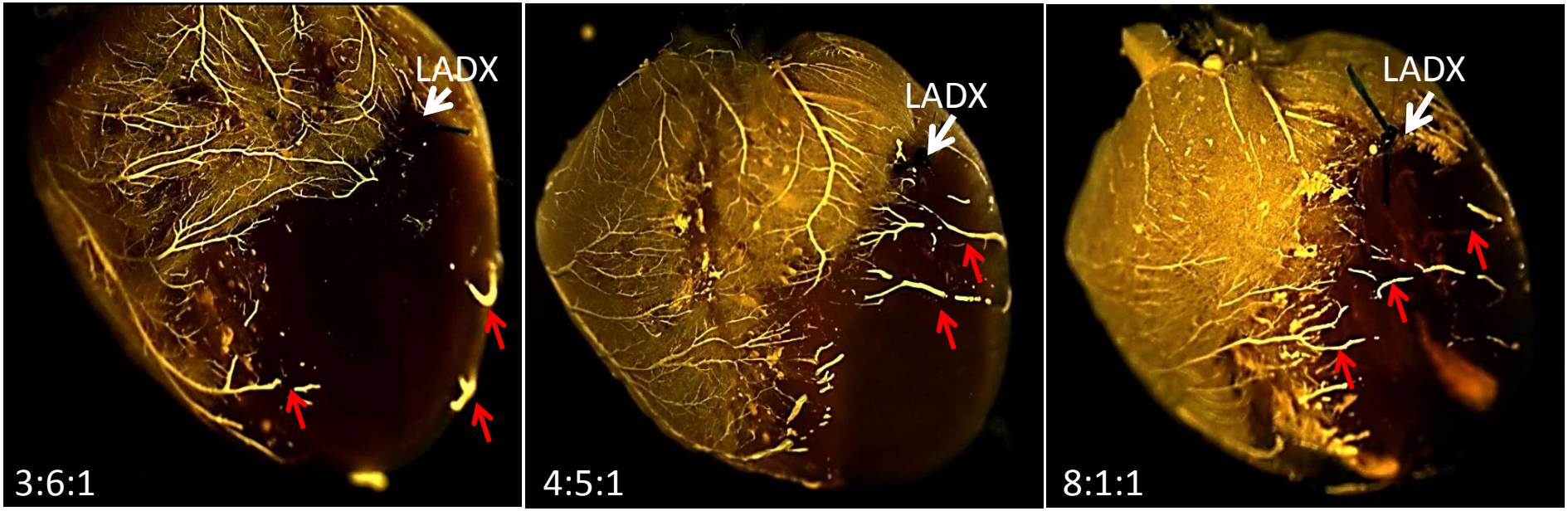
Cell proliferation. EDU (5-ethynyl-2′-deoxyuridine, 16 mg/kg, ip) was injected at day-2 and day- 4 after LADX. After treatment as above for histology, 1-3 neo-collaterals per mouse were sectioned every 200 um (10 sections per collateral) and EDU incorporation was detected by Click-iT Imaging kit (Invitrogen) with Alexa-488. Endothelial cells were labeled with isolectin-B4-568. The number of EDU^+ and EDU^- ECs were summed for the 10 sections per collateral and the average percent positive was obtained for each mouse.

Bone marrow transplantation. Adult 3 month-old recipient B6 or $CCR2^{-/-}$ mice received irradiation (two 650 rad exposures separated by 4 hrs). One day later, 5x10^6 bone marrow cells obtained from the femur and tibia of donor mice (B6, $CCR2^{-/-}$ or EGFP transgenic mice) were injected iv. Mice were studied 6 weeks later.

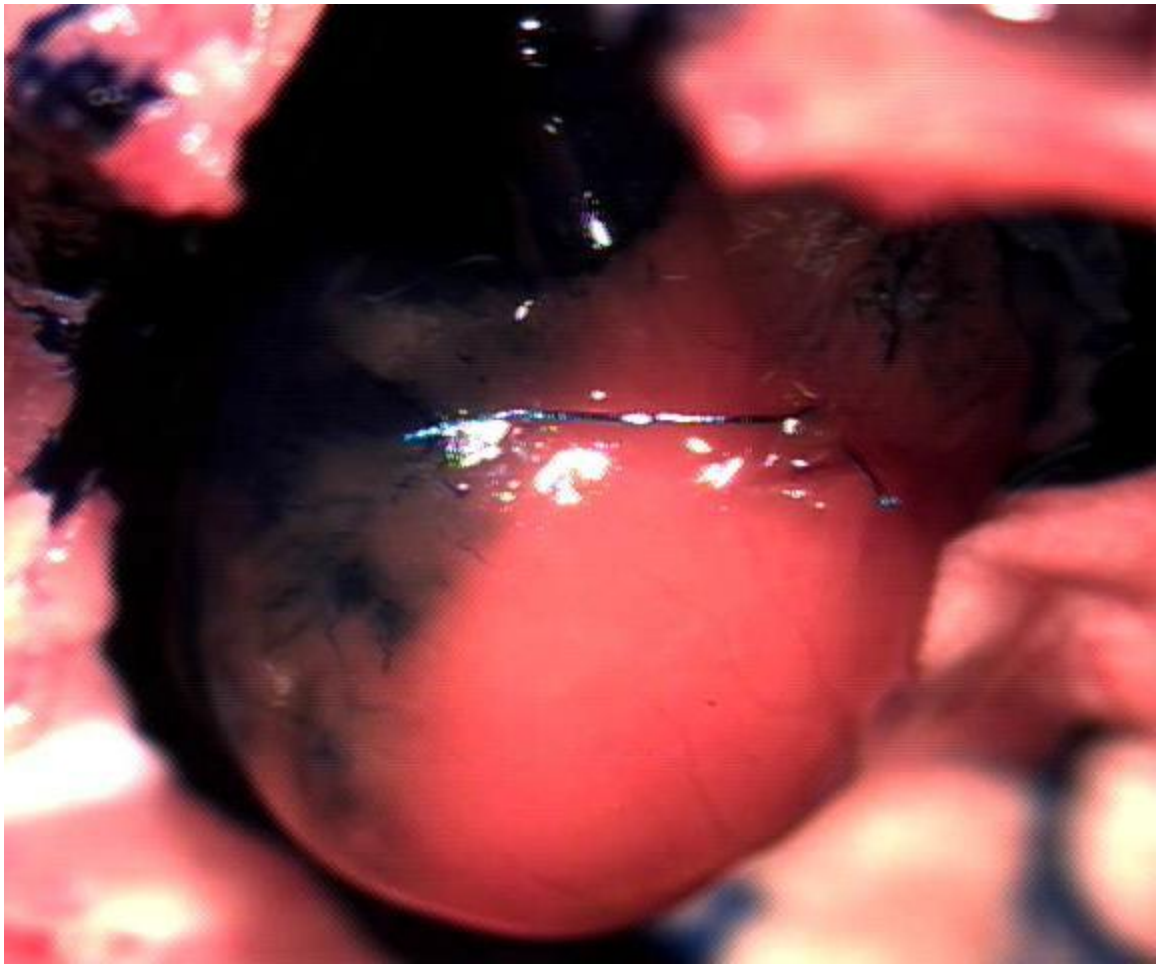
Statistics. ImageJ software was used for image analysis and statistical treatment. All values are expressed as mean ± SE. Data were tested with *t*-tests, linear regression, ANOVA, Bonferroni (significance at $P<0.05$). Where possible, data were collected with investigator blinded to mouse strain.



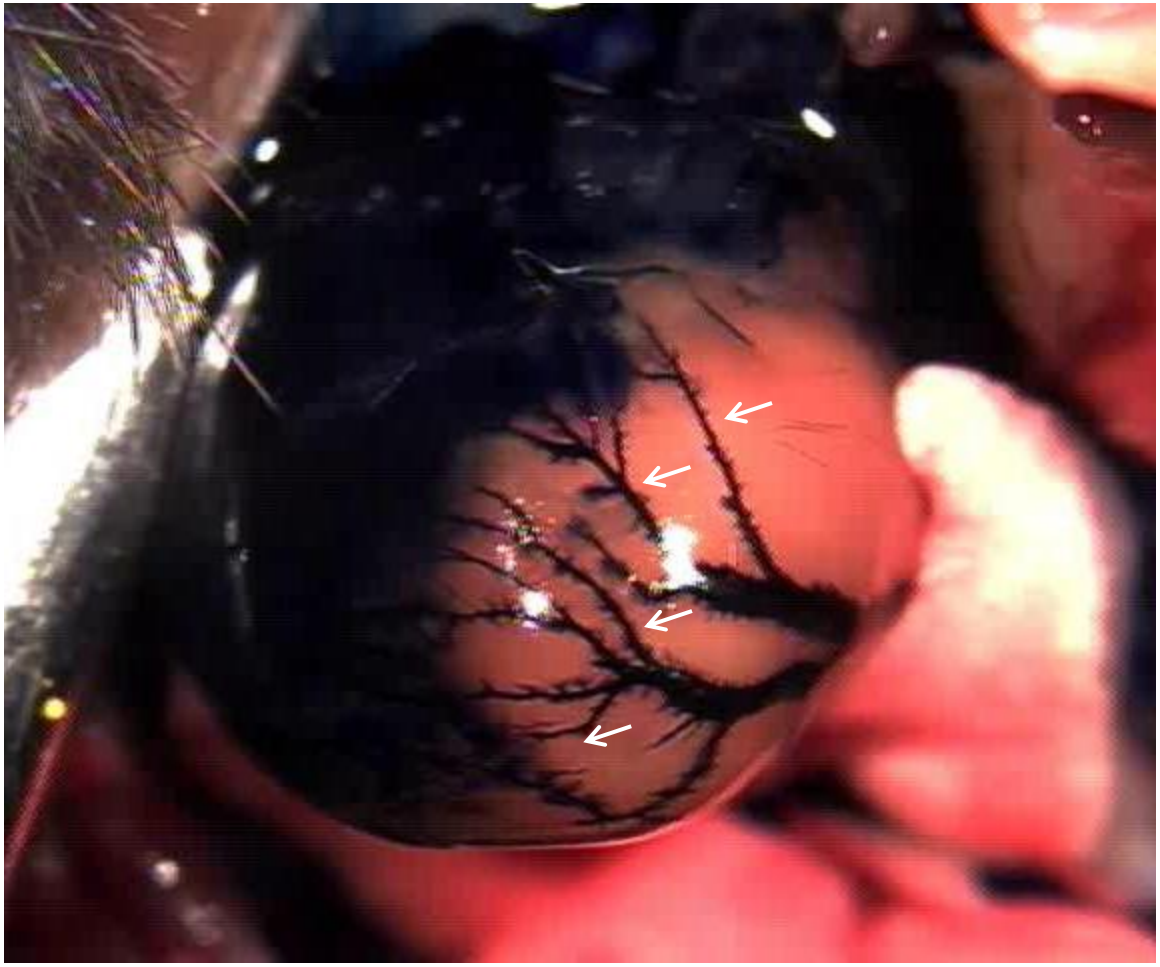
Online Figure 1. No native coronary collaterals were found after acute LAD ligation in different strains of ~3 months-old mice, even after overfilling to cause Microfil^{R} to fill capillaries and begin to fill venules. After maximal vasodilation and fixation per Methods, Microfil^{R} (8:1:1) was infused at a pressure sufficient to fill capillaries and begin to fill epicardial venules and veins that (red arrows), followed by optical clearing. Note especially dense capillary filling in the normal (non-infarct) zones. The results were confirmed for n $\geq$ 5 mice for inbred strains C57BL/6 (B6), BALB/cBy, C57BLKS, A/J, SJL, C3H-He, CBA, DBA/2 and n = 3 for outbred strain CD1.



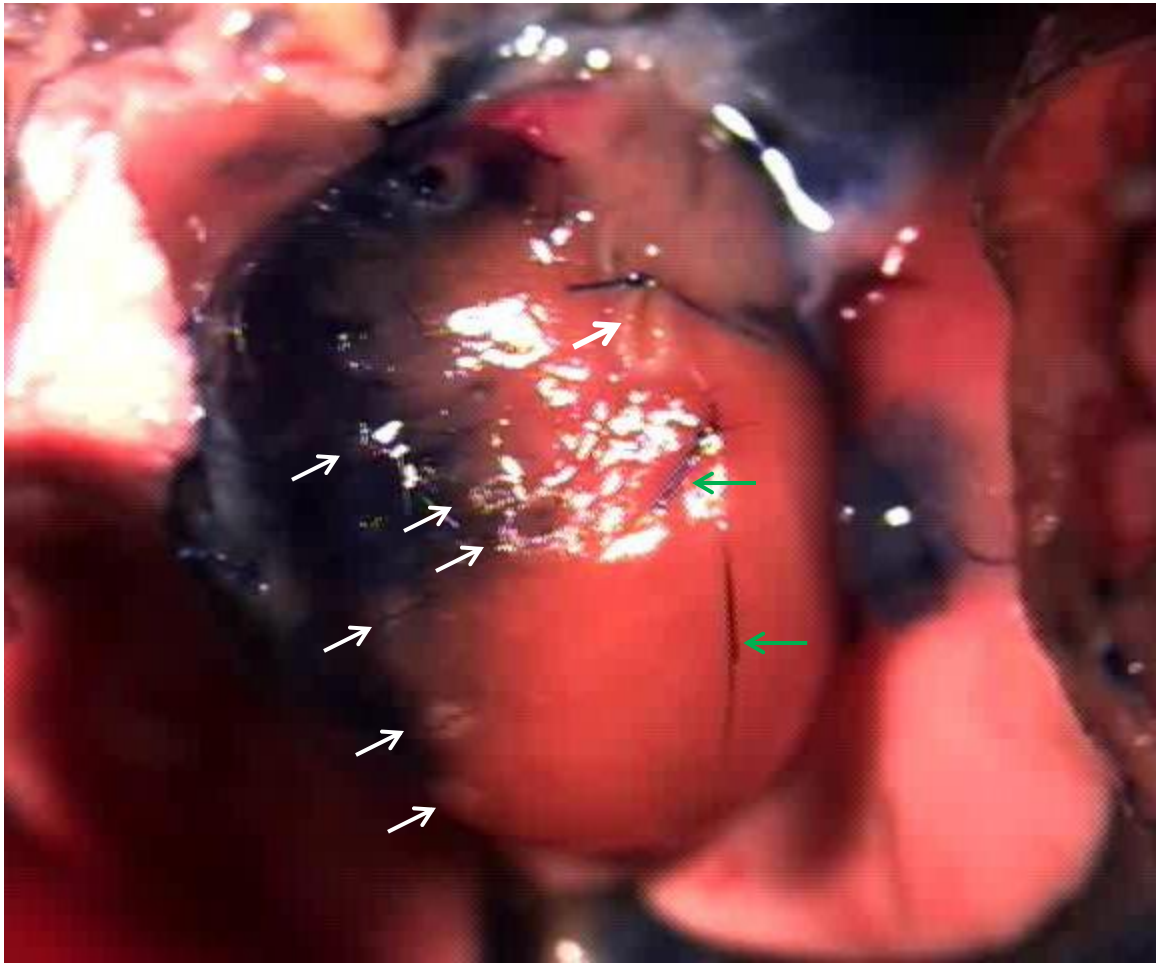
Online Figure 2. No native collaterals were found after acute ligation in three adult C57BL/6 mice filled with Microfil^{R} of different viscosities (latex : diluent : curing agent). After maximal vasodilation and fixation per Methods, Microfil^{R} was infused at a pressure sufficient to fill capillaries and begin to fill venules and veins (red arrows), followed by optical clearing. Images are representative of ≥ 3 mice for each viscosity. Note dense filling of capillaries in normal zone.



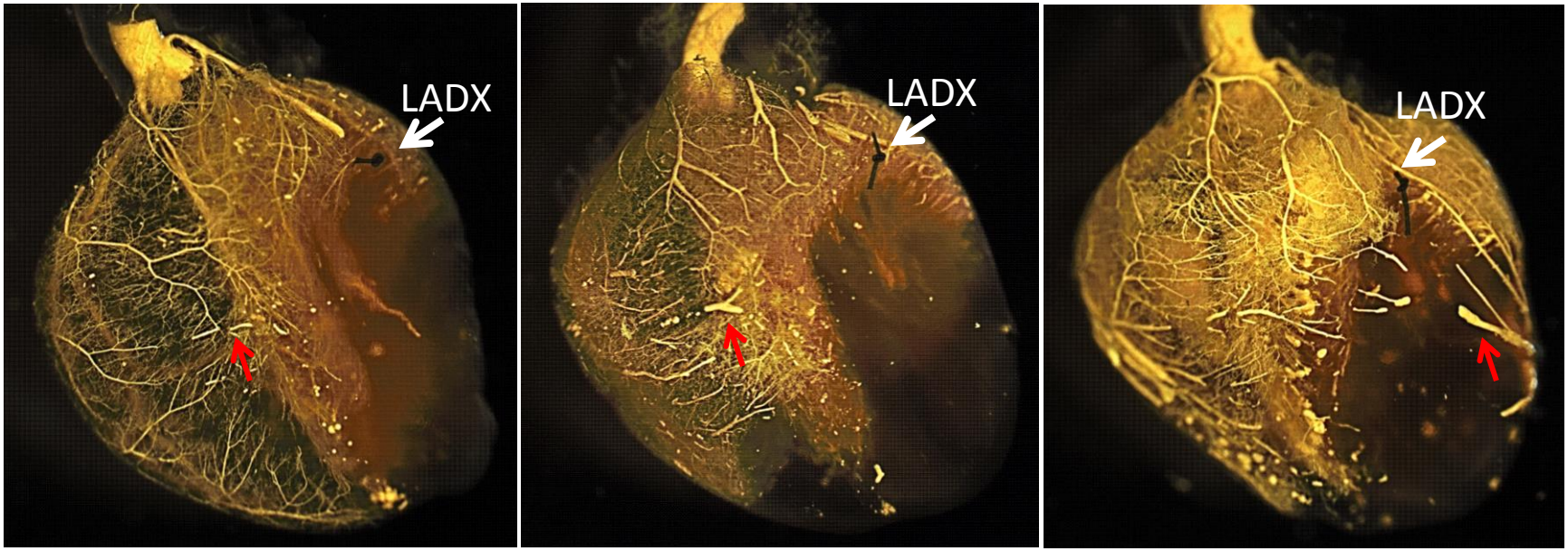
Online Figure 3. Video frame at the ~1 minute time-point of a 4-minute video (Movie-1) demonstrating no detection of native collaterals during infusion of Evans blue. A C57BL/6 adult mouse received LAD ligation (suture) and was immediately euthanized and perfused with vasodilators per Methods. After 10 sec of 2% PFA perfusion, 5% Evans blue (in PBS) was infused retrograde through the descending thoracic aorta at 35 mmHg pressure. The movie shows that the non-infarct zone filled with Evans blue within the first minute, followed by filling of several epicardial veins crossing the infarct zone. At the ~1'30" time point a deeper vein in the infarct zone also filled. At ~1'40", additional epicardial veins crossing the infarct zone are filled. Although the watershed zones between adjacent arterial trees share a plexus of interconnected capillaries, consist with previous reports (eg, Downey et al, Basic Res Cardiol 1986;81:336), after acute ligation the path of least resistance results in the dye (ie, blood) returning to the coronary sinus via the venous tree(s) of the adjacent territory rather than retrogradely perfusing the occluded LAD tree. This is also evident in Online Figures/movies 4 and 5.



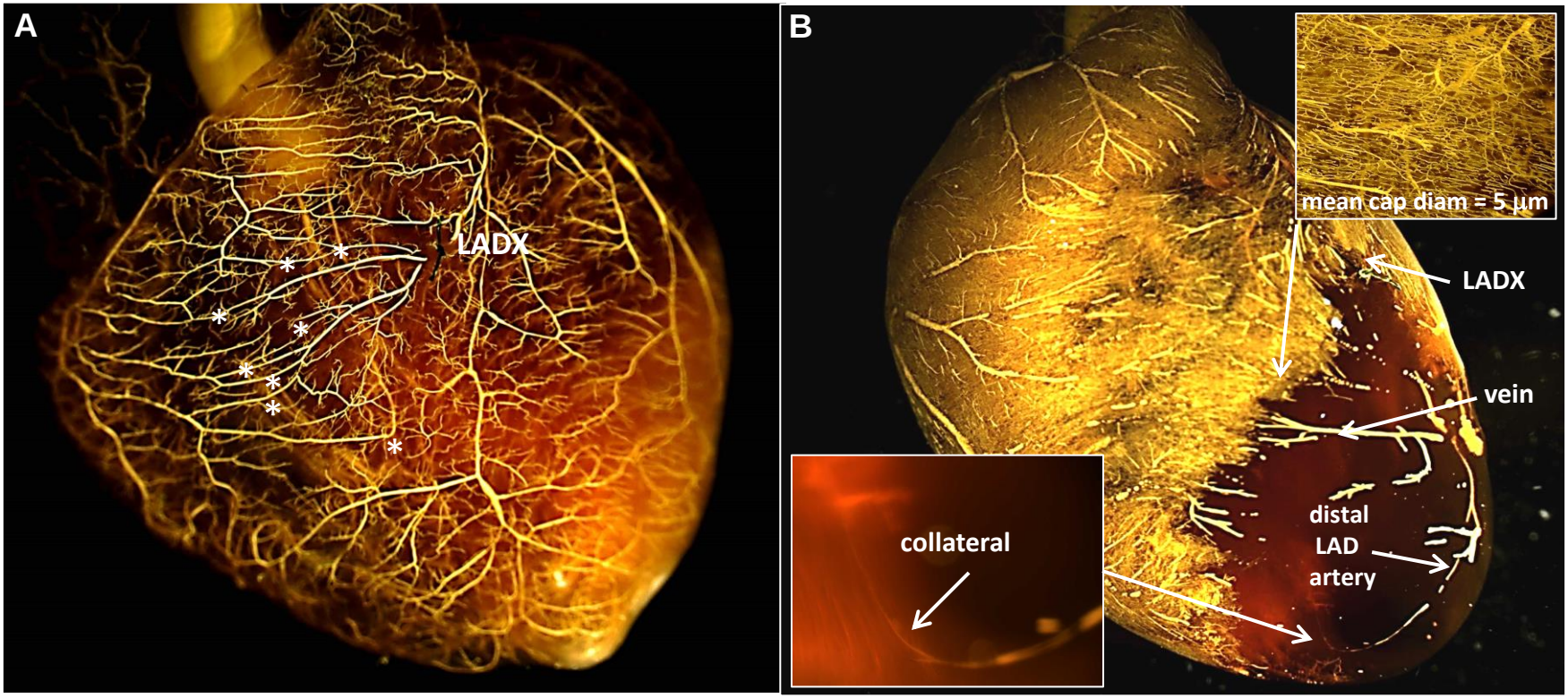
Online Figure 4. Video frame at ~1 minute time-point of a 1-minute duration video (Movie-2) demonstrating no detection of native collaterals during infusion of Evans blue. A C57BL/6 adult mouse received LAD ligation and was immediately euthanized and perfused with vasodilators per Methods. After 10 sec of 2% PFA perfusion, 5% Evans blue was infused retrograde through the descending thoracic aorta at 35 mmHg pressure. The movie shows that epicardial veins (arrows) crossing the infarct zone began filling at 12 sec.



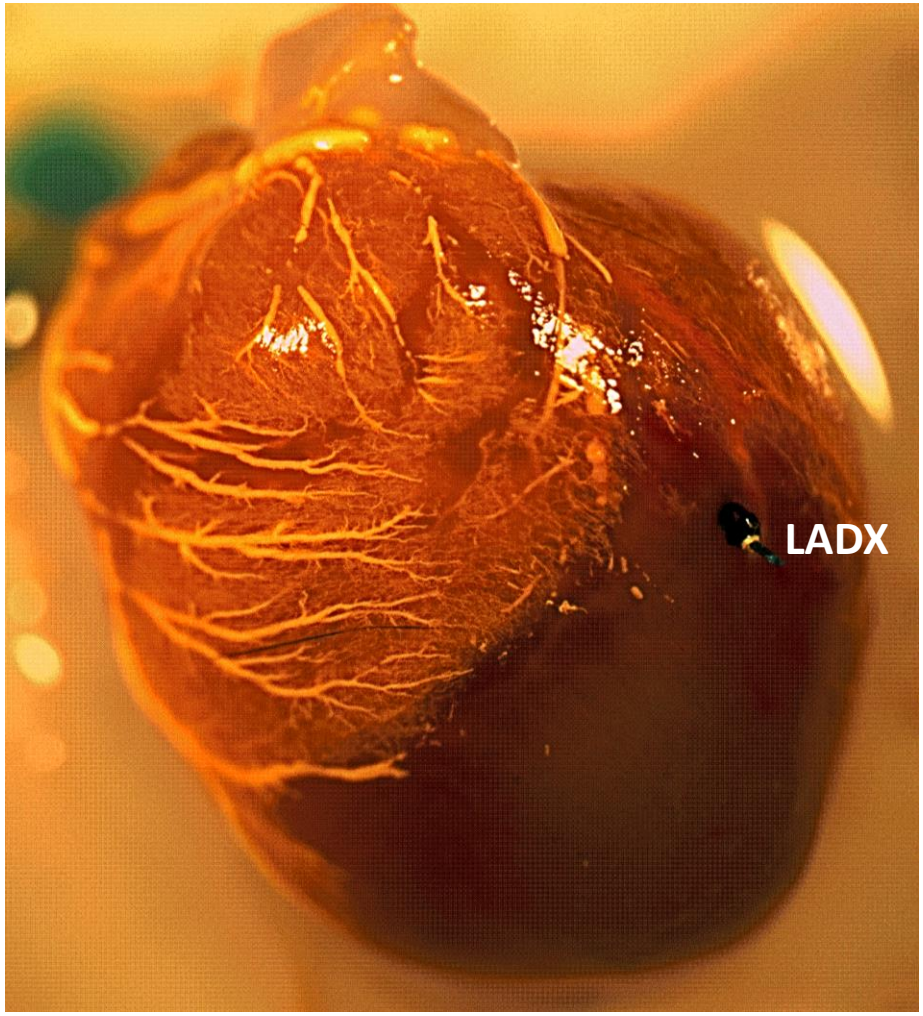
Online Figure 5. Video frame at ~4-minute time-point of a 6'45" duration video (Movie-3) demonstrating no detection of native collaterals during infusion of Evans blue. A C57BL/6 adult mouse received ligation (suture) and was immediately euthanized and perfused with vasodilators per Methods. After 10 sec of 2% PFA perfusion, 5% Evans blue (in PBS) was infused retrograde through the descending thoracic aorta at 35 mmHg pressure. The movie shows real-time cauterization of epicardial veins at the borders of the infarct zone during infusion (white arrows) to prevent filling of them as they begin to cross the infarct zone. Green arrows, 2 adherent hairs.



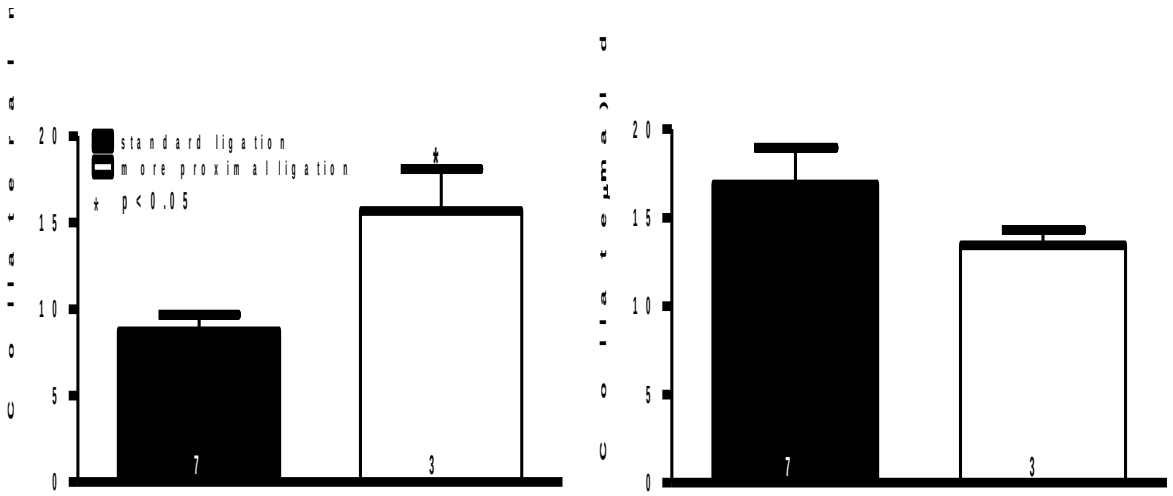
Online Figure 6. No native coronary collaterals were found after acute ligation in three 3-week-old C57BL/6 mice. After maximal vasodilation and fixation per Methods, Microfil^{R} (8:1:1) was infused at a pressure sufficient to fill capillaries and begin to fill epicardial venules and veins (red arrows), followed by optical clearing. These results show that absence of collaterals in adults is not from loss of collaterals that are present in the neonate but subsequently lost during growth to adulthood due to the rapid increase in resting heart rate and thus short intervals of diastolic coronary blood flow.



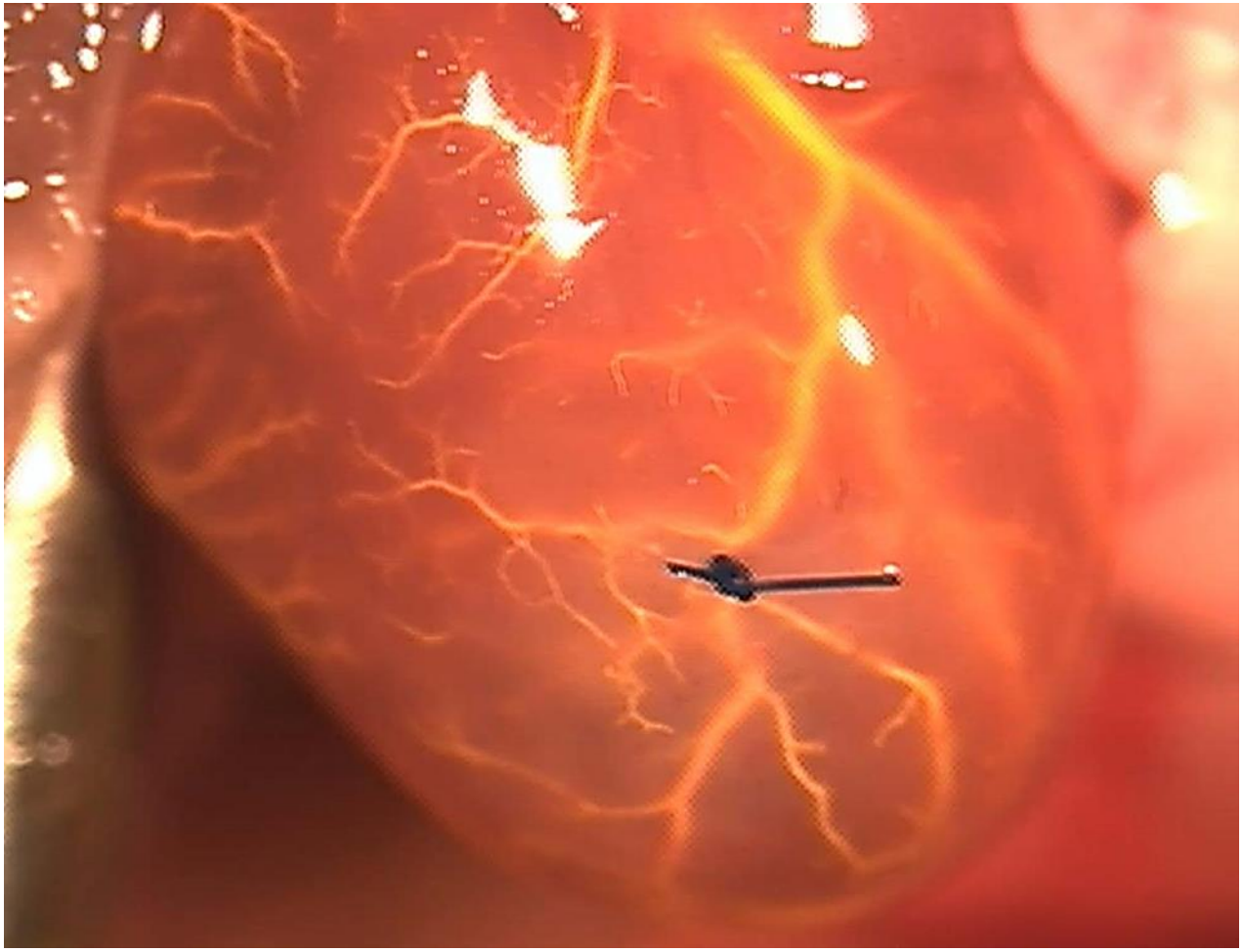
Online Figure 7. The coronary circulation in guinea pig (A, American Tricolor strain) and rat (B, Wistar strain) immediately after ligation of the mid-LAD (LADX), using the same methods as in Figure 1, identifies abundant collaterals in guinea pig and few in rat. A, Stars identify sub-epicardial collaterals; deeper collaterals to the territory below the ligation (territory at risk) cannot be quantified without dissection away of the epi- then myocardium; following dissection, this animal has 31 collaterals of 46 µm mean lumen diameter to the territory at risk. **B,** Wistar rat with one collateral supplying the territory at risk (insert, 9 µm diameter at arrow). 0-2 collaterals were found supplying the territory at risk in other Wistar and Sprague-Dawley rats. Note presence of large unfilled region (territory at risk) below the ligation in rat (B) with 1 collateral, compared to guinea pig (A) with 31 collaterals. B, Microfil^{R} was infused at lower viscosity to fill capillaries (inset) and begin to fill venules and veins (also see Online Figures 1,2). Data are representative of ≥ 4 each of guinea pigs, Wistar and Sprague Dawley rats, both genders. These data, together with the methods and results described in the legend for Figure 1, demonstrate that the casting methods used in this study fill collaterals when present.



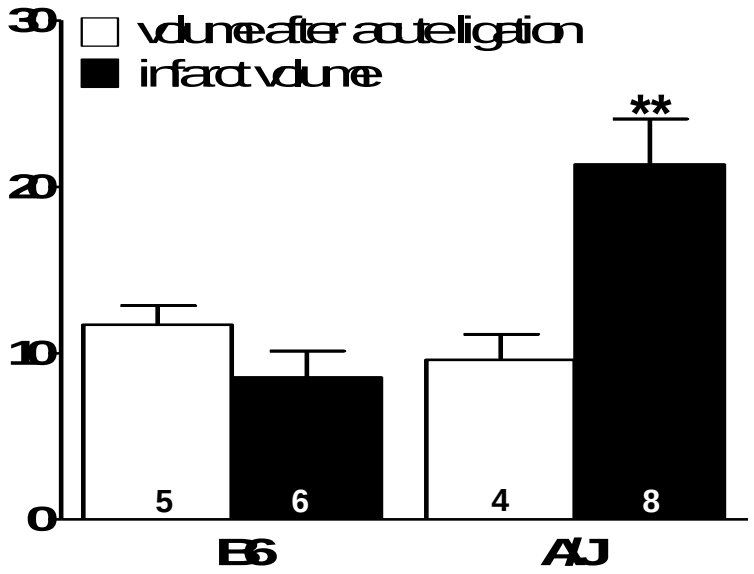
Online Figure 8. No native collaterals were found 24 hours after acute ligation (LADX) in adult C57BL/6 mouse. After maximal vasodilation and fixation per Methods, Microfil^{R} was infused at a pressure and viscosity sufficient to fill capillaries and epicardial venous trees that are evident. Image is representative of 5 mice.



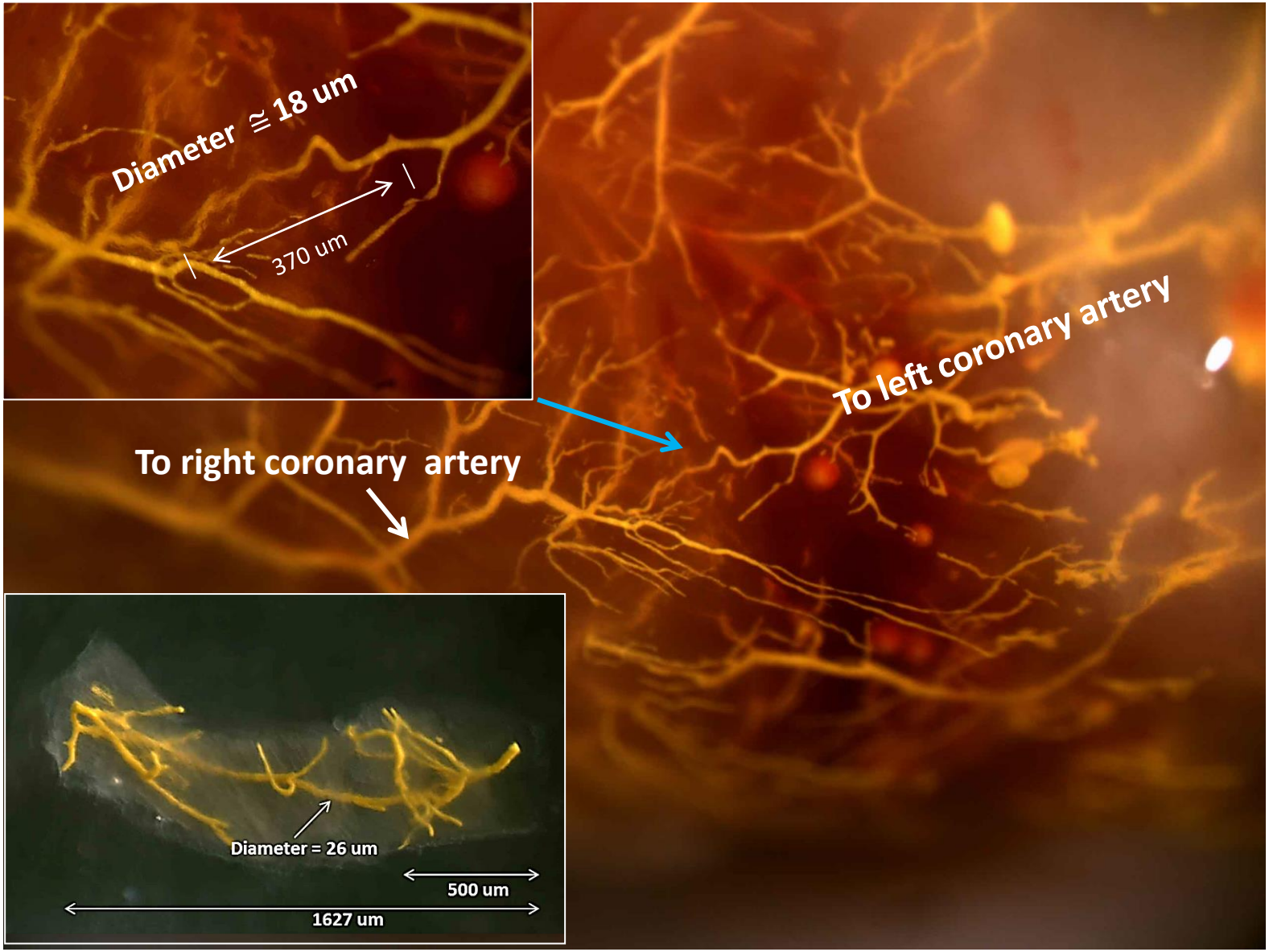
Online Figure 9. More proximal LAD ligation causes greater neo-collateral formation. B6 mice were examined 1 week after LAD ligation at a more proximal site (~1 mm below atrial margin) than the ligation used elsewhere in this study (~3mm below atrial margin). Data in filled bars are from Figure 3. N = number of animals. Approximately 50% of mice with this proximal ligation did not survive to day-7. No native collaterals were observed immediately after this proximal ligation, in agreement with our results elsewhere in this study using distal ligation.



Online Figure 10. Video frame at the 35 second time-point of a 35 second video (Movie-4) demonstrating method for determining the time required to fill the main trunk of the LAD in a retrograde manner back to the point of ligation ("retrograde fill-time"), as a measure of relative resistance of the neo-collateral network. Two weeks after LAD ligation (suture), the adult C57BL/6 mouse was euthanized and perfused with vasodilators, followed by paraformaldehyde per Methods. Microfil^{R} (5:4:1) was infused through the descending thoracic aorta at 66 mmHg. The video shows retrograde filling, via neo-collaterals that are not visible at the magnification used, of branches of the LAD tree (crossing the infarct zone in the frame). Retrograde fill-time is the time in seconds between start of perfusion (time-zero) and the time at which the Microfil^{R} just reaches the ligation point.



Online Figure 11. Territory (ie, volume after acute ligation) of the LAD artery tree downstream of the LAD ligation does not differ between C57BL/6 (B6) and A/J strains; infarct volume measured 1 week after LAD ligation is larger in A/J mice that form fewer neo-collaterals (see Figure 4). B6 and A/J mice received ligation and maximal dilation, followed by infusion of 2% Evans blue into the coronary circulation via the thoracic aorta, fixation with 2% PFA, and sectioning through the long axis of the left ventricle into 1 mm slices, per Methods. The volume after acute ligation is calculated as non-Evans blue stained volume / left ventricle volume x 100. Territory did not differ between the two strains. In separate mice, infarct volume was determined with TTC staining per Methods (these values for infarct volume are also shown in Figure 3). Infarct volume is higher in A/J mice, in associated with reduced neo-collateral formation and greater retrograde fill-time (Figure 4). **, $p < 0.01$ versus B6 infarct volume. N, number of mice.



Online Figure 12. Optically cleared tissue prepared for dissection of neo-collaterals for subsequent sectioning and histochemistry. 3 months-old-B6 mouse, 1 week after LADX, maximally dilated, fixed with 1% paraformaldehyde 50% glycerol overnight at 4°C, followed by 75% glycerol for a second day, then 30% sucrose and embedding in OCT. The blue arrow identifies a neo-collateral. Lower insert, tissue block dissected from the left ventricle free wall, per Methods, showing a neo-collateral cross-connecting a distal arteriole of the LAD and right coronary artery trees, 1 week after LAD ligation. Neo-collaterals often will have not acquired tortuosity at 7 days after LADX, but this will usually be present by 14 days (see Figure 2D).

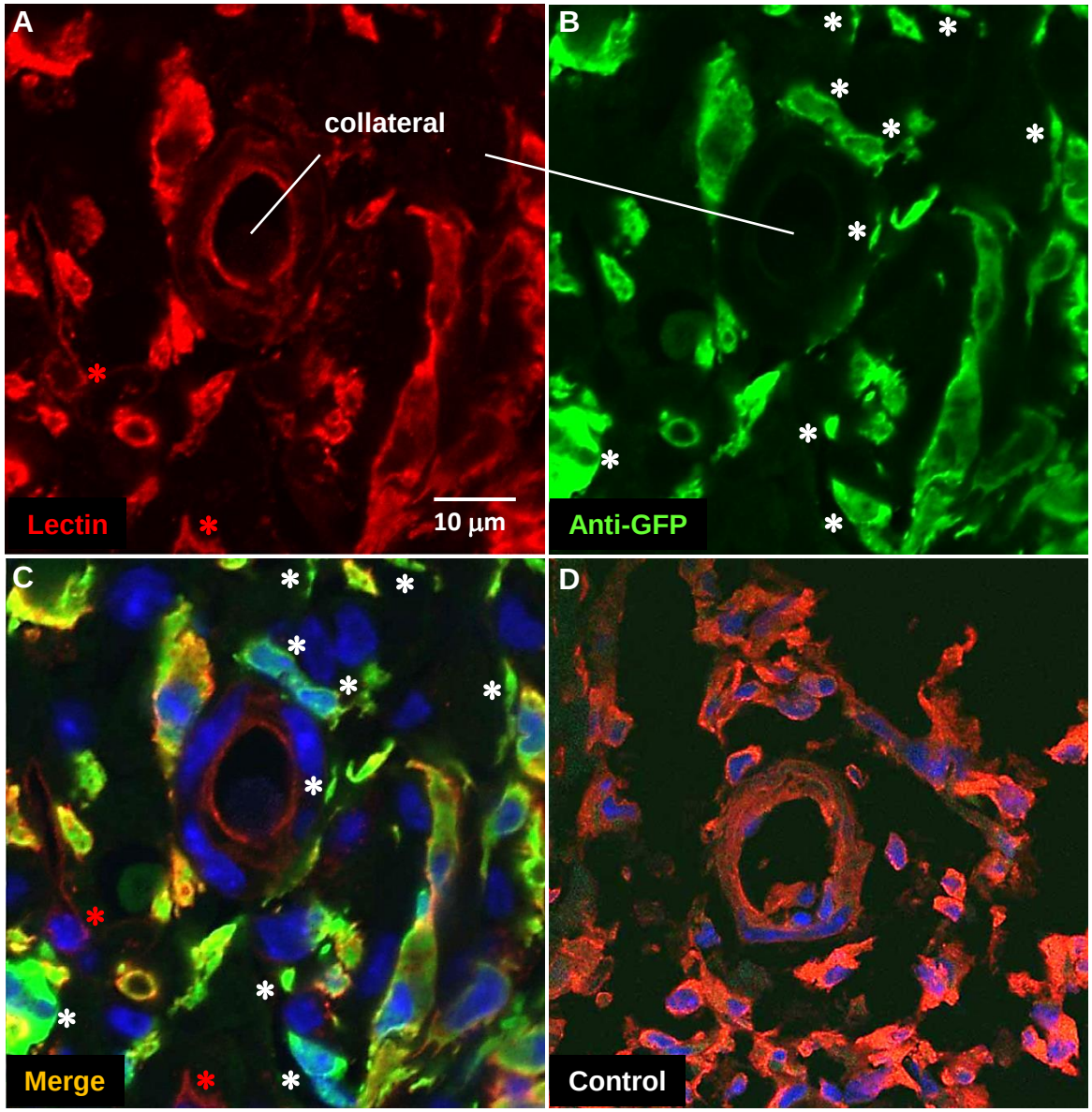


Figure 13A-D. Bone marrow-derived cells around neo-collateral (11 μm lumen diameter) 7 days after LAD ligation of C57BL/6 mouse that had received bone marrow transplant from a constitutive ubiquitous GFP$^+$ mouse. Isolectin-B4$^+$ endothelial cells of a collateral (**A**) that is surrounded by cells that are mostly lectin$^+$/GFP$^+$ (**A-C**). Less-abundant lectin$^-$/GFP$^+$ cells (white stars) and lectin$^+$/GFP$^-$ (red stars) also present. **D**, Adjacent section treated same as A-C but lacking anti-GFP. Blue in C,D = DAPI (nuclei). Data are representative of ≥ 3 B6 mice. See also Online figure 14. Magnification bar shown in panel A is same for other panels.

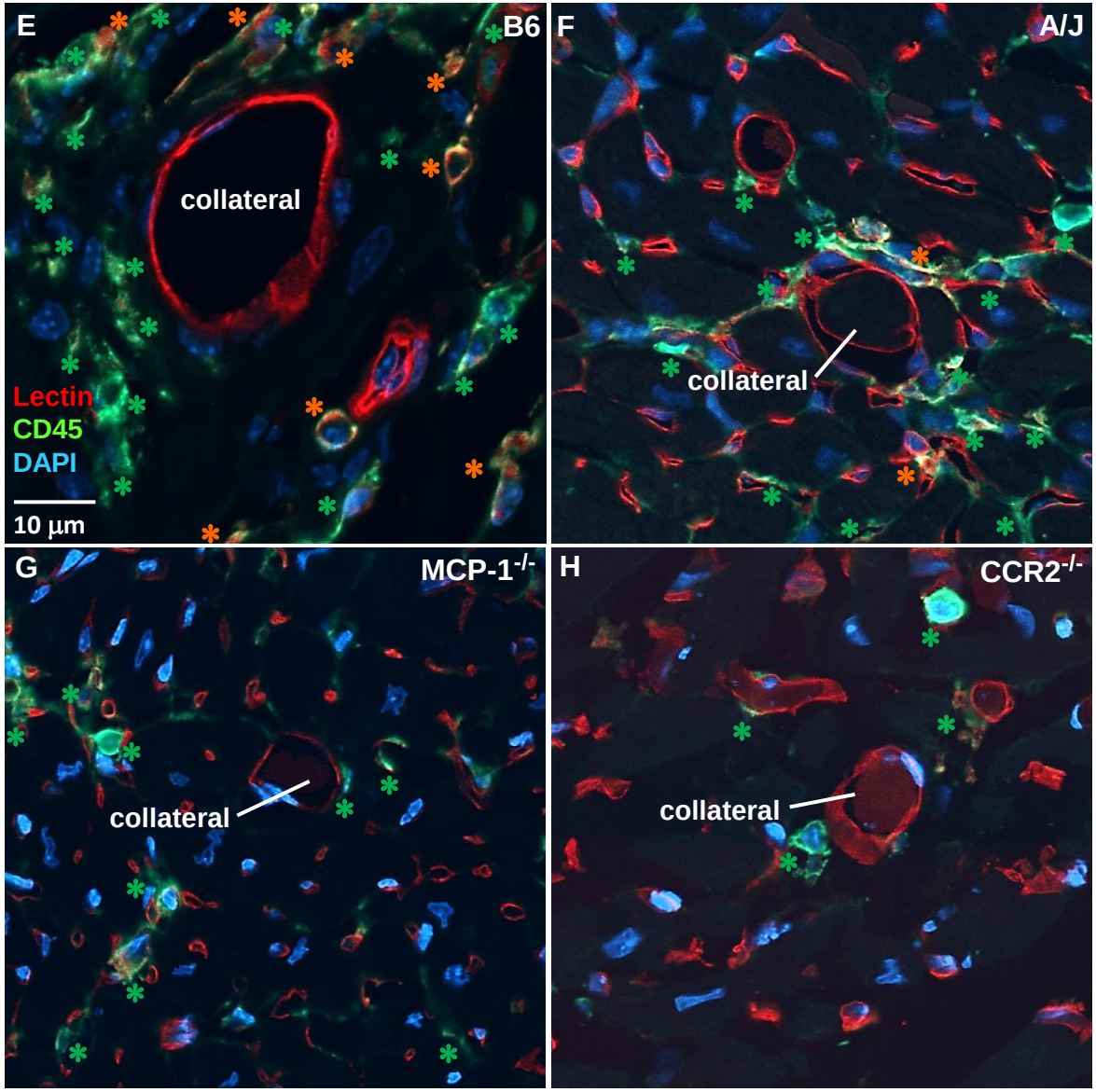
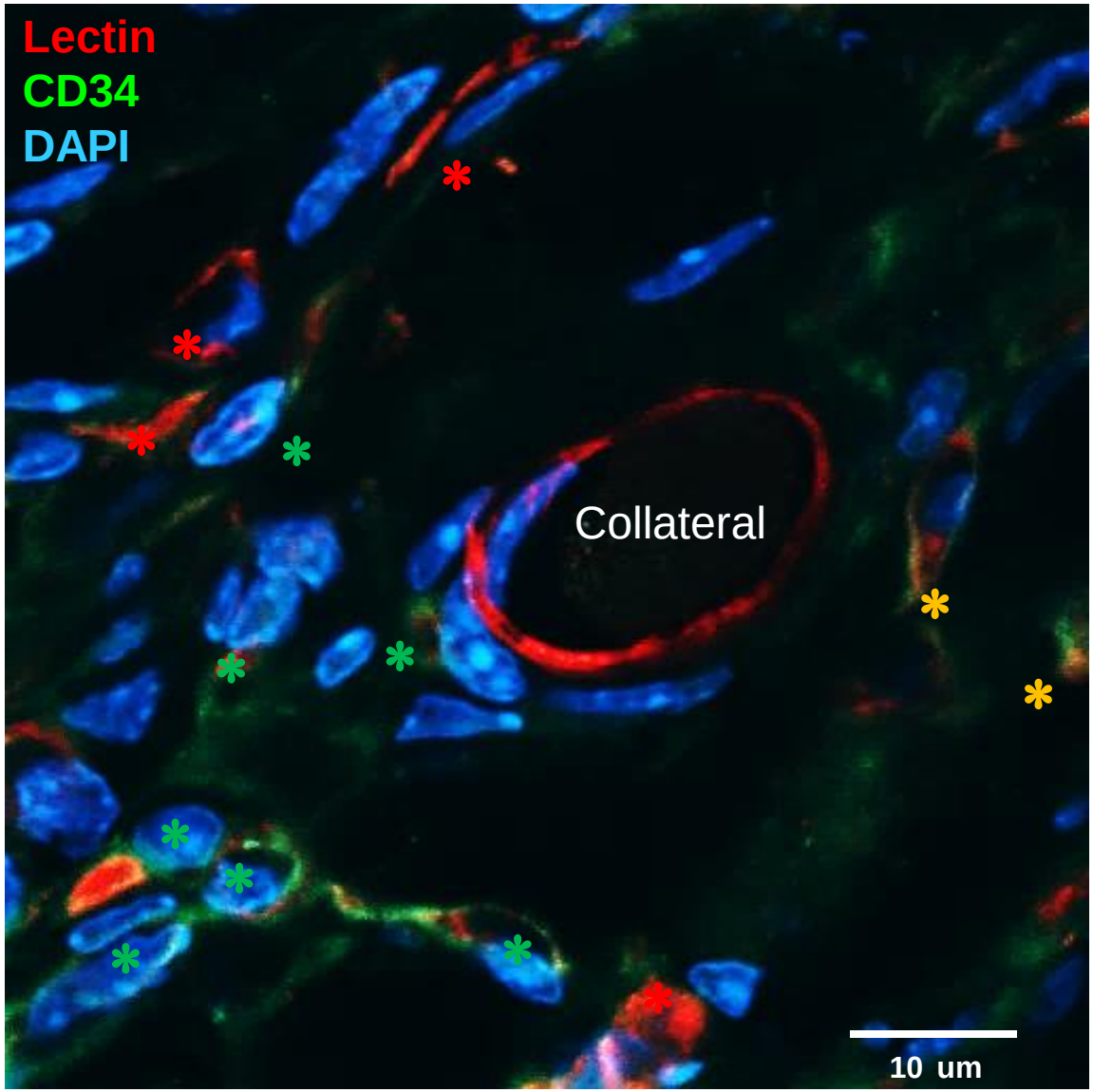
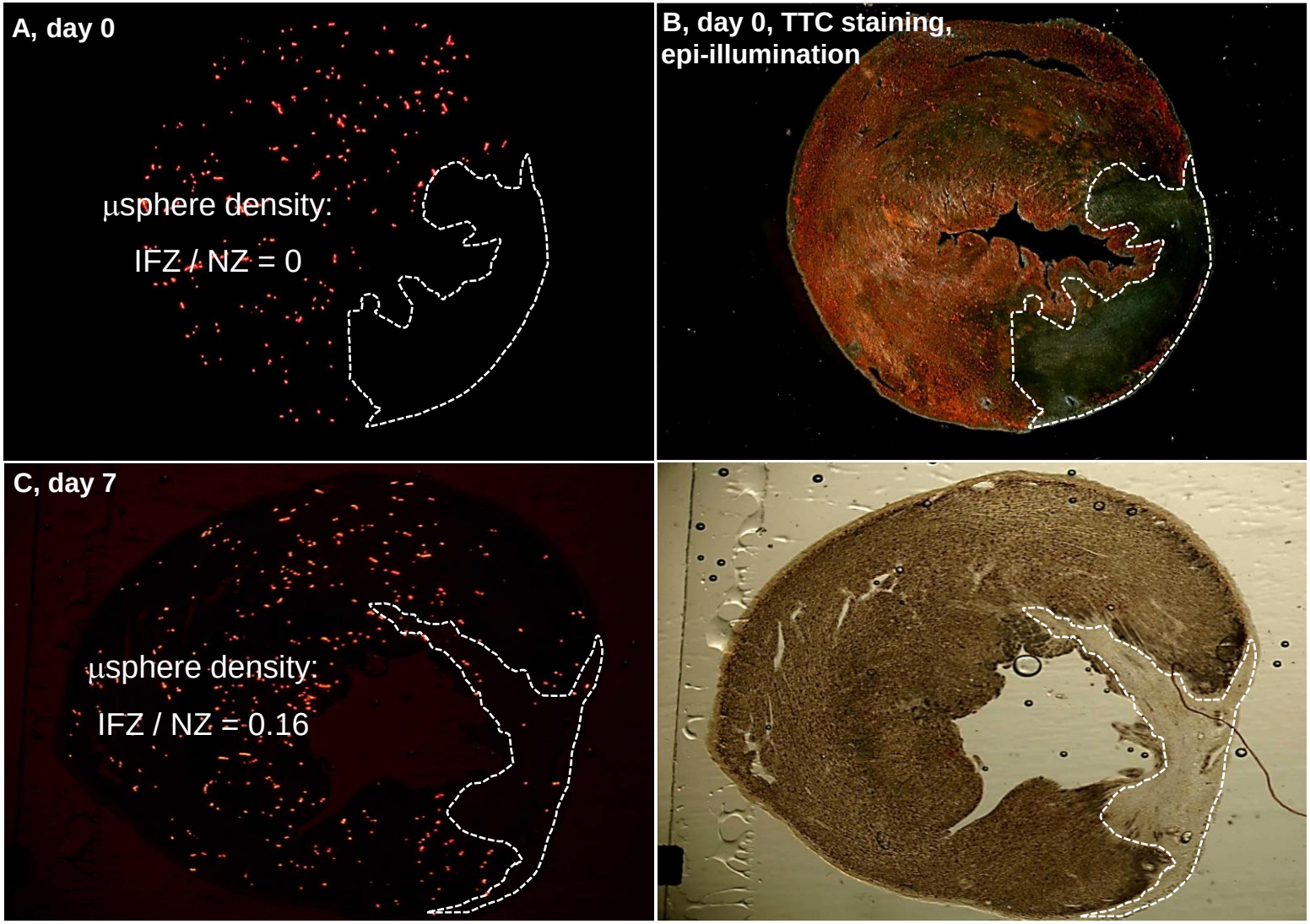


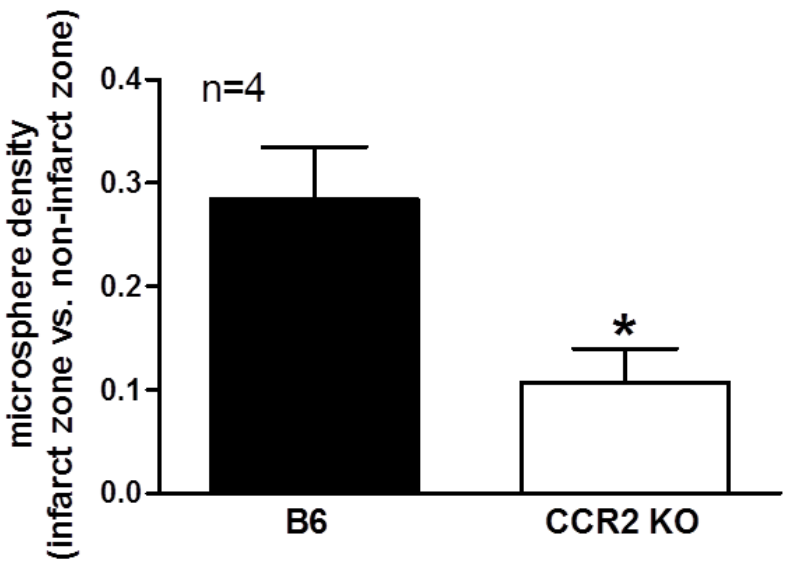
Figure 13E-H. CD45$^+$ cells around neo-collaterals 1 week after LAD ligation. **E,F**, C57BL/6 (B6) and A/J mice have similar abundance of CD45$^+$/lectin$^-$ cells (green stars) and CD45$^+$/lectin$^+$ cells (orange stars), while MCP-1$^{-/-}$ and CCR2$^{-/-}$ mice have fewer of both cell types (**G,H**). Lectin$^+$/CD45$^-$ cells, presumably mostly ECs of capillaries and larger vessels, are also present. Data are representative of ≥ 3 mice of each strain.



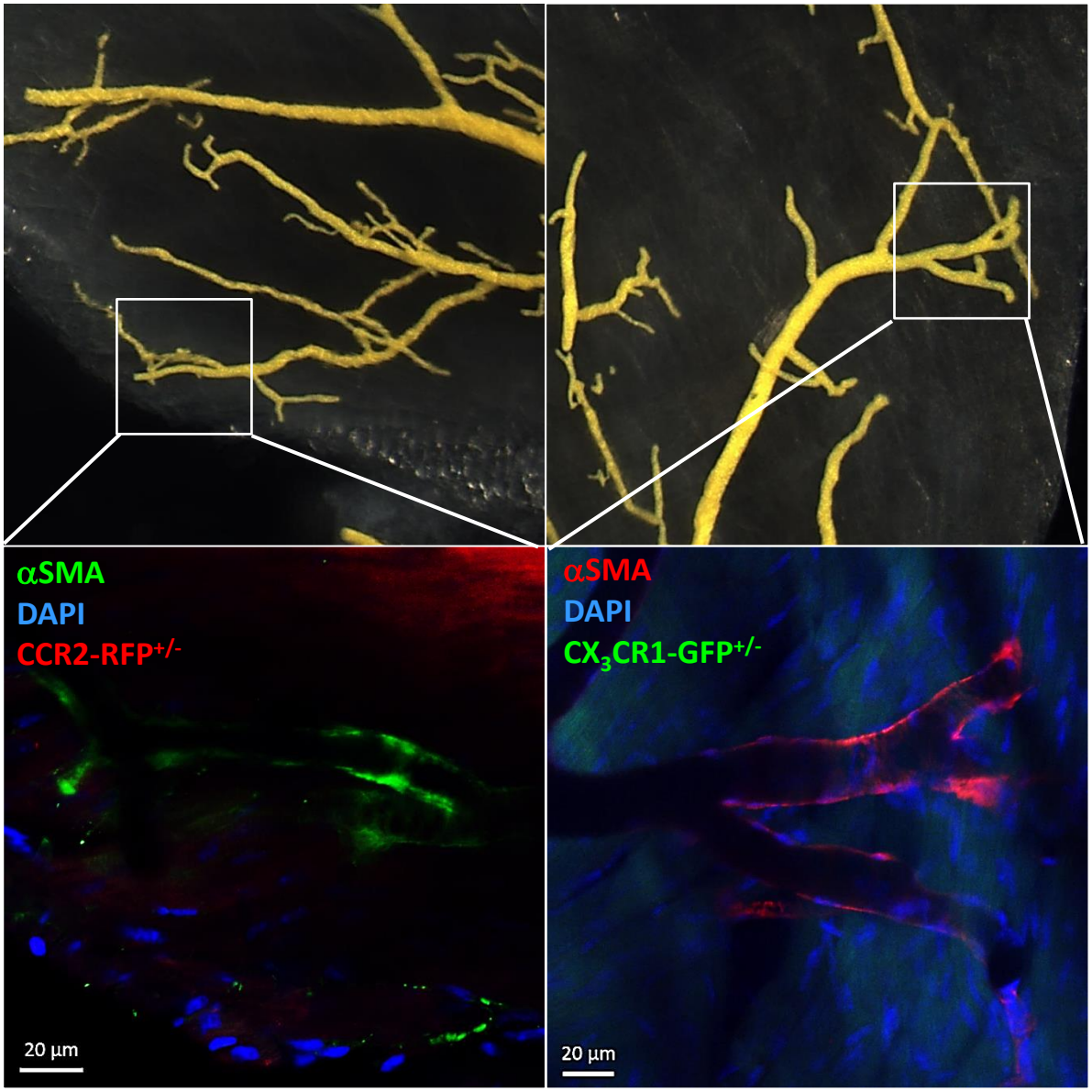
Online Figure 14. CD34^{+}/ Lectin^{-} cells in perivascular region of neo-collateral (green stars). C56BL/6 mouse 7 days after LAD ligation. Isolectin-B4^{+} endothelial cells line the collateral that also is surrounded by lectin^{+}/ CD34^{-} cells (red stars). Lectin^{+}/ CD34^{+} possibly hematopoietic stem cells also present (orange stars). Blue = DAPI (nuclei). Image is representative of ≥ 3 mice. No non-specific binding seen when primary or secondary antibody treatment withheld.



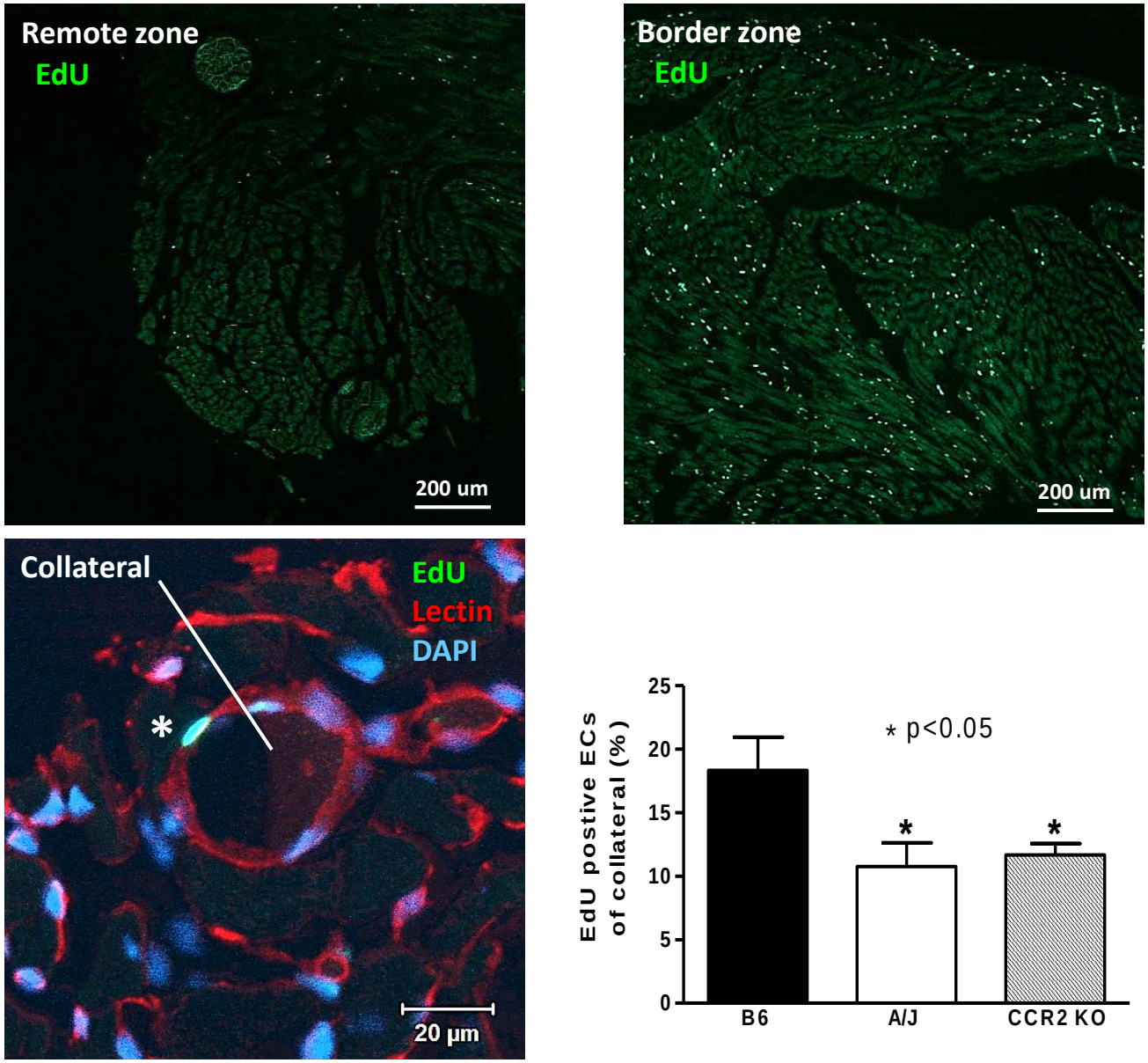
Online Figure 15. Microspheres (10 μm diameter, 605 nm fluorescence) were perfused retrograde into ascending aorta immediately or 1 week after LAD ligation following maximal dilation and fixation per Methods; 60 μm sections were then stained with TTC. Microsphere density was calculated per area of infarct zone (IFZ), ie, area circumscribed by white line / non-infarct zone area (NZ, remaining area of left ventricle). Density values are an average of 10 to 12 sections. Group-wise data are shown in Online Figure 16.



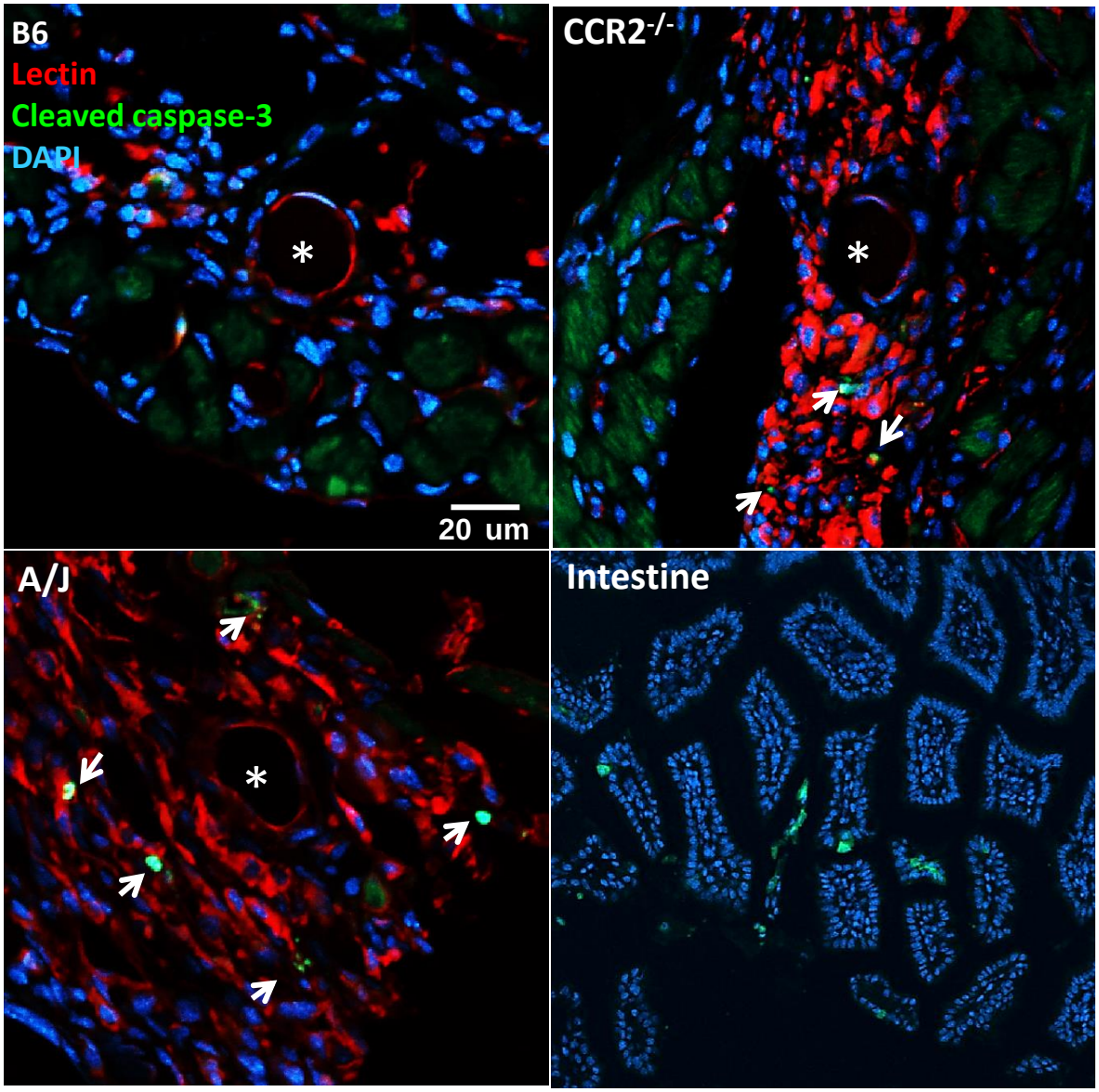
Online Figure 17. Reduced collateral-dependent perfusion in CCR2$^{-/-}$ mice 1 week after LAD ligation. Methods described in Online Figure 16.



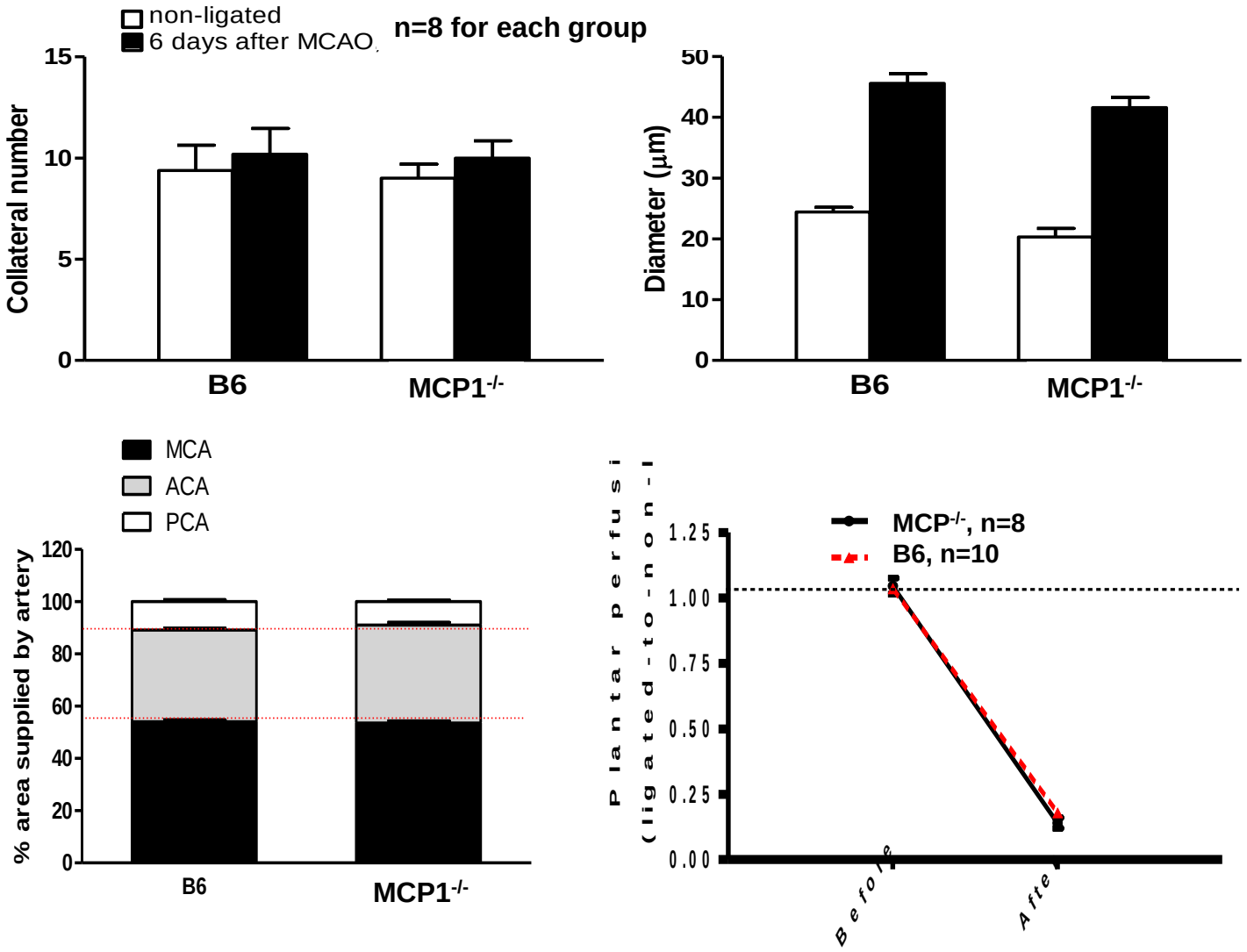
Online Figure 17. Whole mount images of $CR2^{RFP/+}$ and $CX_3CR1^{GFP/+}$ B6 reporter mice show no positive cells in the vicinity of arterioles excized from remote zone 5 days after LAD ligation (see Figure 7). αSMA^+ mural cells surround arterioles. Images representative of 5 mice.



Online Figure 18. Endothelial cell proliferation (white star) in neo-collaterals is less in A/J and CCR2$^{-/-}$ mice. The fewer EdU^{+} ECs in neo-collaterals 7 days after LAD ligation in these strains is consistent with the smaller number and diameter of neo-collaterals and other deficits shown in previous figures that occur in these strains. N = 3 of each strain, 10 sections examined per mouse. Upper panels show much more abundant proliferation in border zone than remote zone.



Online Figure 19. No cleaved caspase-3^{+} apoptotic cells detected in wall of neo-collaterals (white star); several are evident (white arrows) in surrounding area at a density seen elsewhere in the border zone. 7 days after LAD ligation. Results shown are representative of 3 mice of each group examined (10 sections examined per mouse) . Myocytes showed weak autofluorescence. Small intestinal villi served as a positive control.



Online Figure 20. Native collaterals in brain or hindlimb of MCP1$^{-/-}$ mice not different from wildtype controls (B6). No effect of MCP1$^{-/-}$ deletion on number or average diameter of pial collaterals between middle cerebral artery (MCA) and anterior cerebral artery (ACA) trees (white bars in upper panels). No effect on perfusion immediately after unilateral femoral artery ligation (bottom right panel), suggesting no effect on collateral extent in the hindlimb. Area of the cerebral artery trees unaffected by MCP1 deletion (lower left panel). Unlike in hindlimb after femoral artery ligation,[1,6,22] remodeling (increase in diameter) of pial collaterals after permanent middle cerebral artery occlusion (MCAO) was not reduced in MCP1$^{-/-}$ mice (upper panels, black bars). Methods used were per R Sealock et al, Circ Res, 2014. Values are mean ± SEM for n number of mice.