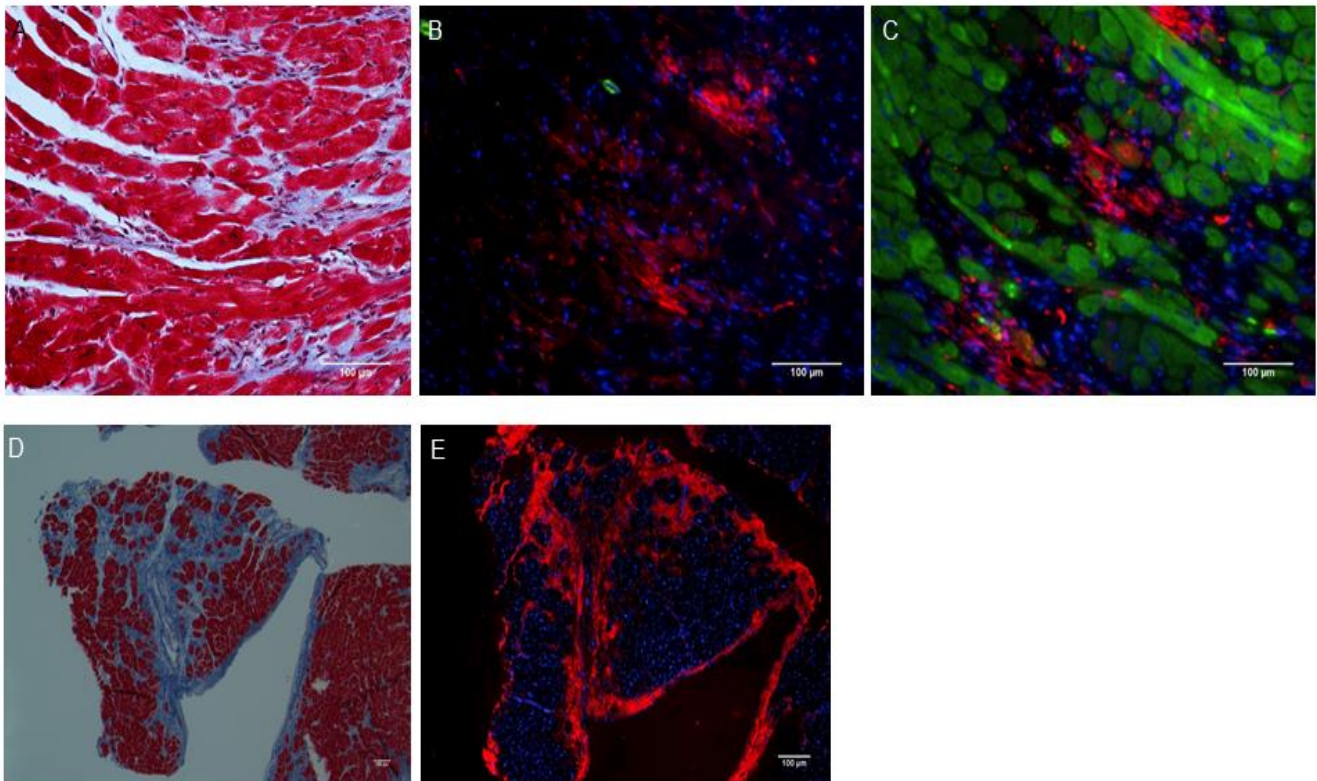# Online Supplement

**Cimini M et al. 126967-INS-RG-TR-2**

Figure S1

Supplementary Figure S1: Podoplanin expression in murine and human fibrotic hearts: Thin cardiac sections obtained from mice hearts (**A-C**) treated with Angiotensin II were stained with Masson's trichrome staining (**A**) and indirectly immunolabeled with antibodies that recognize podoplanin in red (**B-C**), αSmooth muscle actin (**B**) and αSarcomeric actin in green (**C**), nuclei, blue. Fibrotic area from Angiotensin II treated animals express podoplanin. n=3/group. (**D-E**) Thin cardiac sections obtained from explanted ischemic human hearts were stained with the Masson's trichrome staining (**D**) and (**E**) indirectly immunolabeled with podoplanin antibody in red, nuclei, blue. Note that the infarcted/fibrotic tissue in blue (**D**) corresponds to the area in red (**E**) and is highly positive to podoplanin. n=5/ischemic cardiomyopathy hearts.
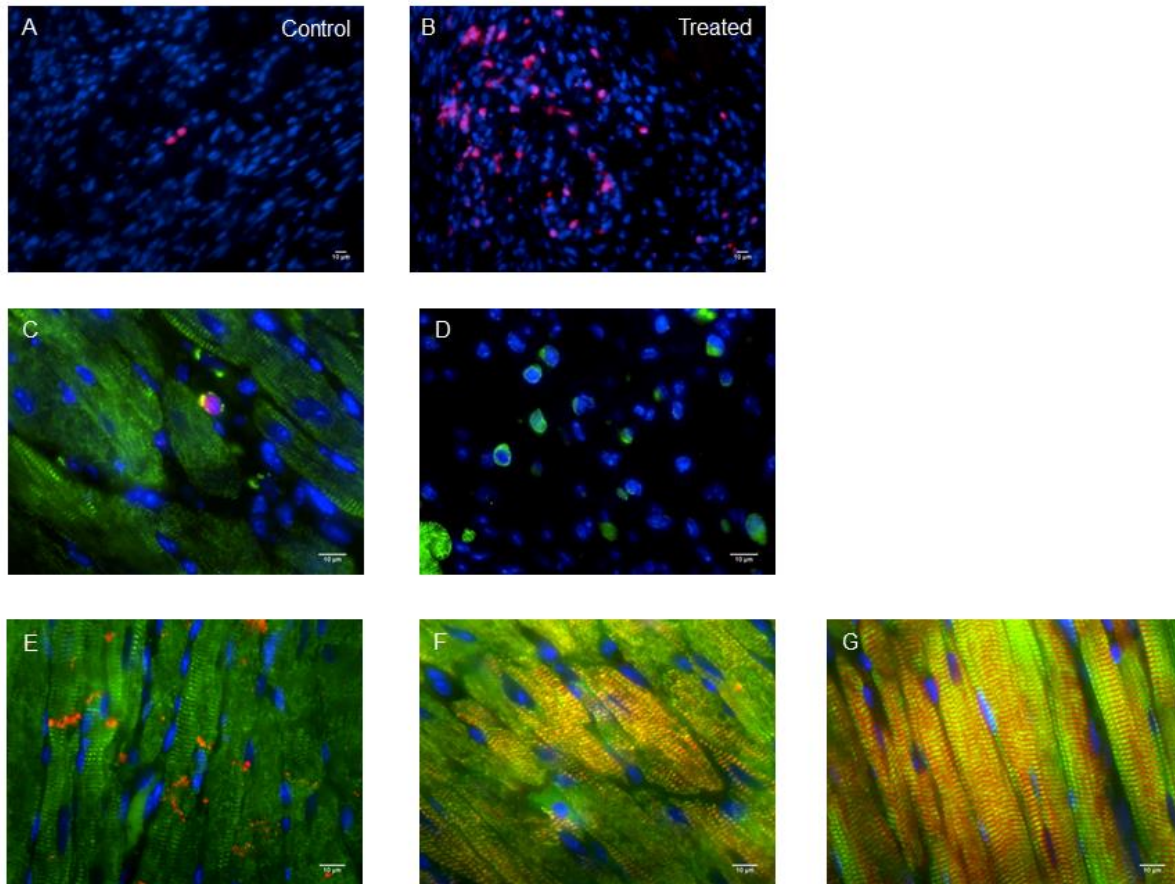
Figure S2
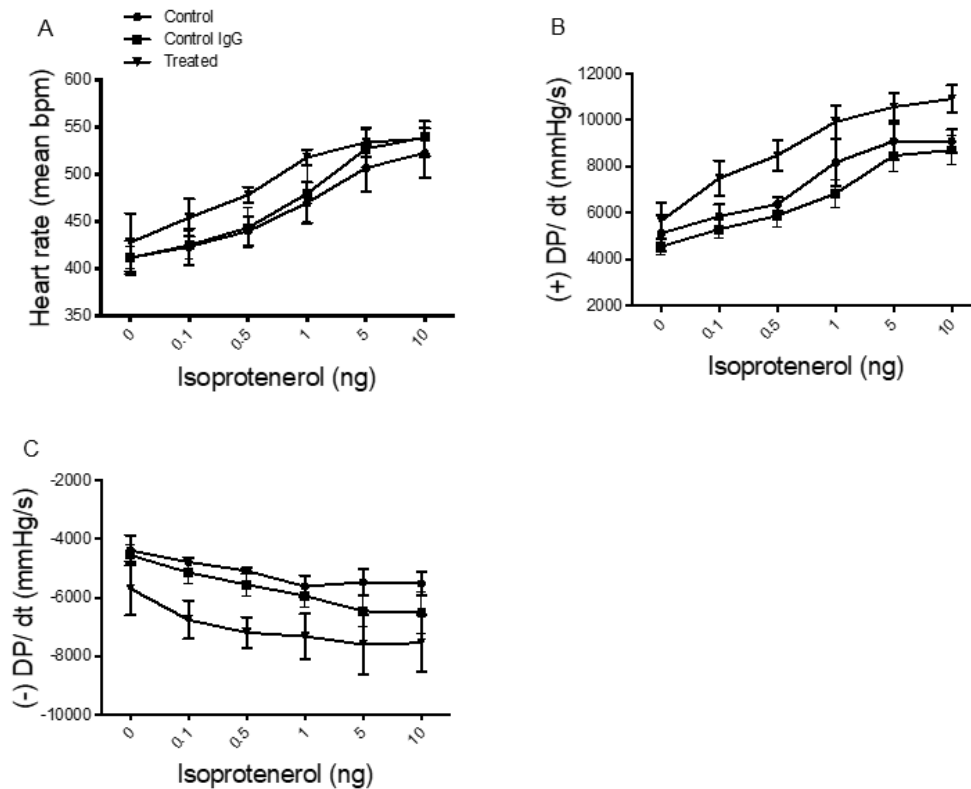
Supplementary Figure S2: Podoplanin neutralization increases heart contractility. (**A**) Heart rate, maximum (**B**) and minimum (**C**) Dp/dt, during the hemodynamic assessment showed an increase in contractility and relaxation in response to isoproterenol (0.1, 0.5, 1, 5, 10 ng/ml) in infarcted mice heart treated with podoplanin neutralizing antibody compared to the saline and IgG controls, at 30 days after myocardial infarction (MI). The statistical analysis showed a trend ($p = 0.057$) in max and min Dp/dt after maximum dose of isoproterenol. n=5-7/group. Data are presented as mean±SEM. Two-way ANOVA analysis and Bonferroni post hoc test were performed among all groups.
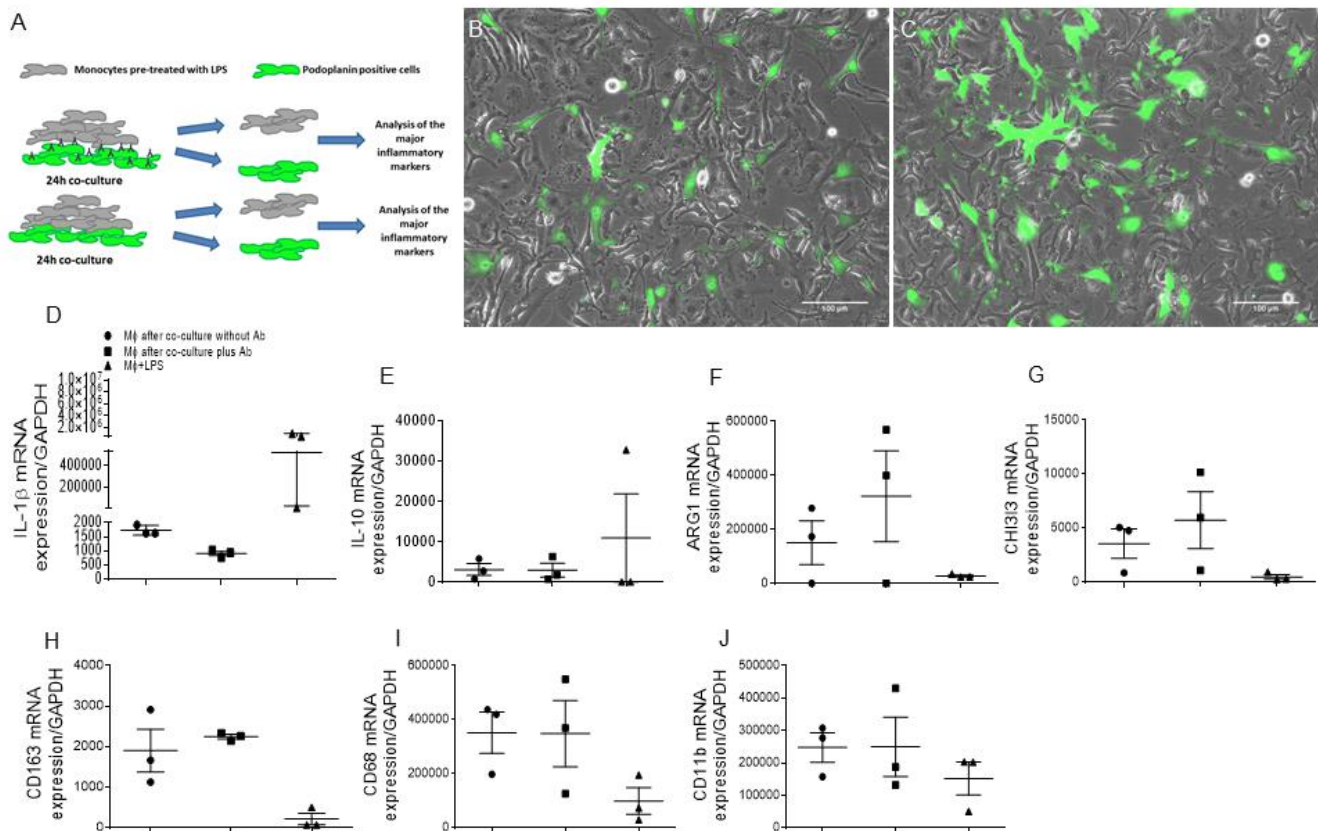
Figure S3
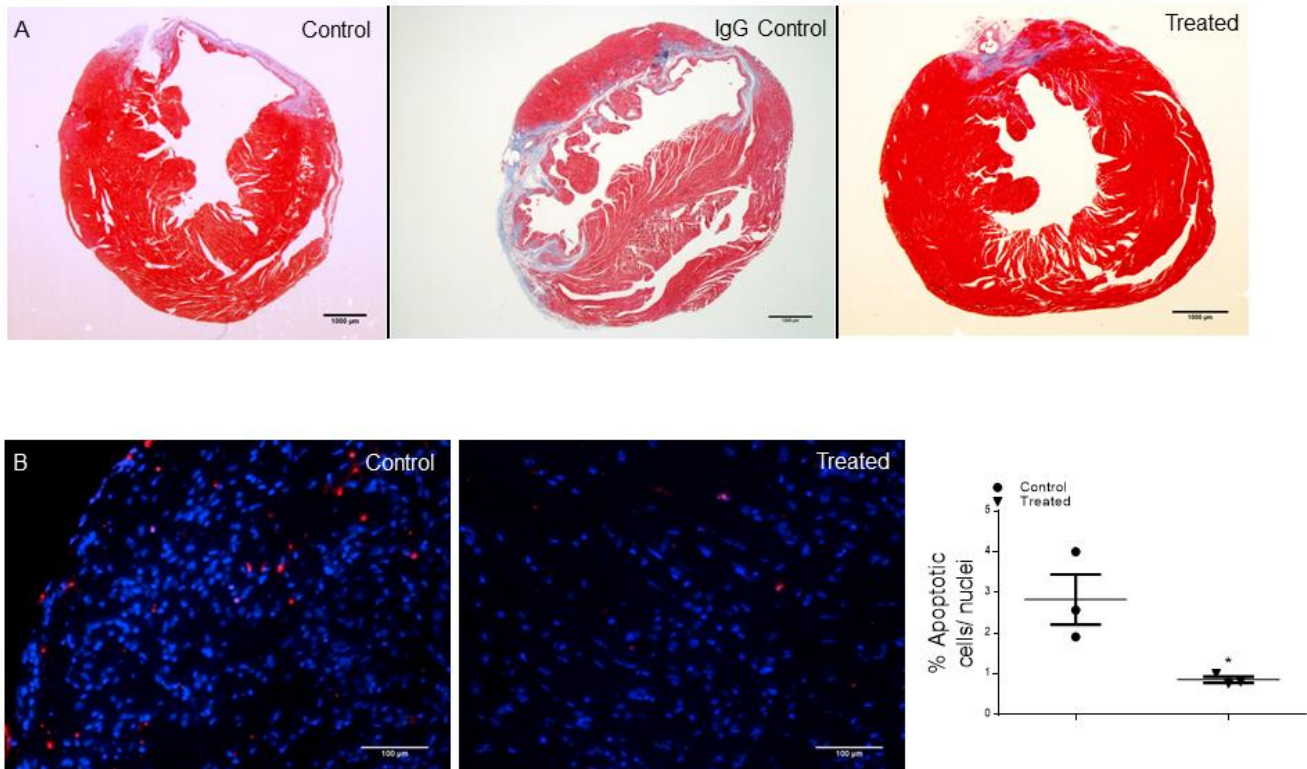
Supplementary Figure S3: Podoplanin neutralization reduced the cell death. (**A**) Thin cardiac sections of saline treated (left panel), IgG control treated (middle panel) and podoplanin neutralizing antibody treated (right panel) mice 30 days post-MI were subjected to Masson's trichrome staining. (**B,** Left panel) Thin cardiac sections from control (saline treated) and podoplanin neutralizing antibody treated (**B,** right panel) infarcted mice hearts at 3 days after MI were directly stained for the Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) in red. Nuclei, blue. Treated animals showed a reduced cell apoptosis compare to the control animals at the same time point after MI. (**B,** graph) Quantitative analysis of the apoptotic cells (stained for TUNEL assay) showed a reduced cell death when the animals are treated with podoplanin neutralizing antibody compared to the saline. n=3/group. Data are presented as mean±SEM *P<0.05 saline control vs treated, scholastic t-test analysis and Bonferroni post hoc test was performed among all groups.
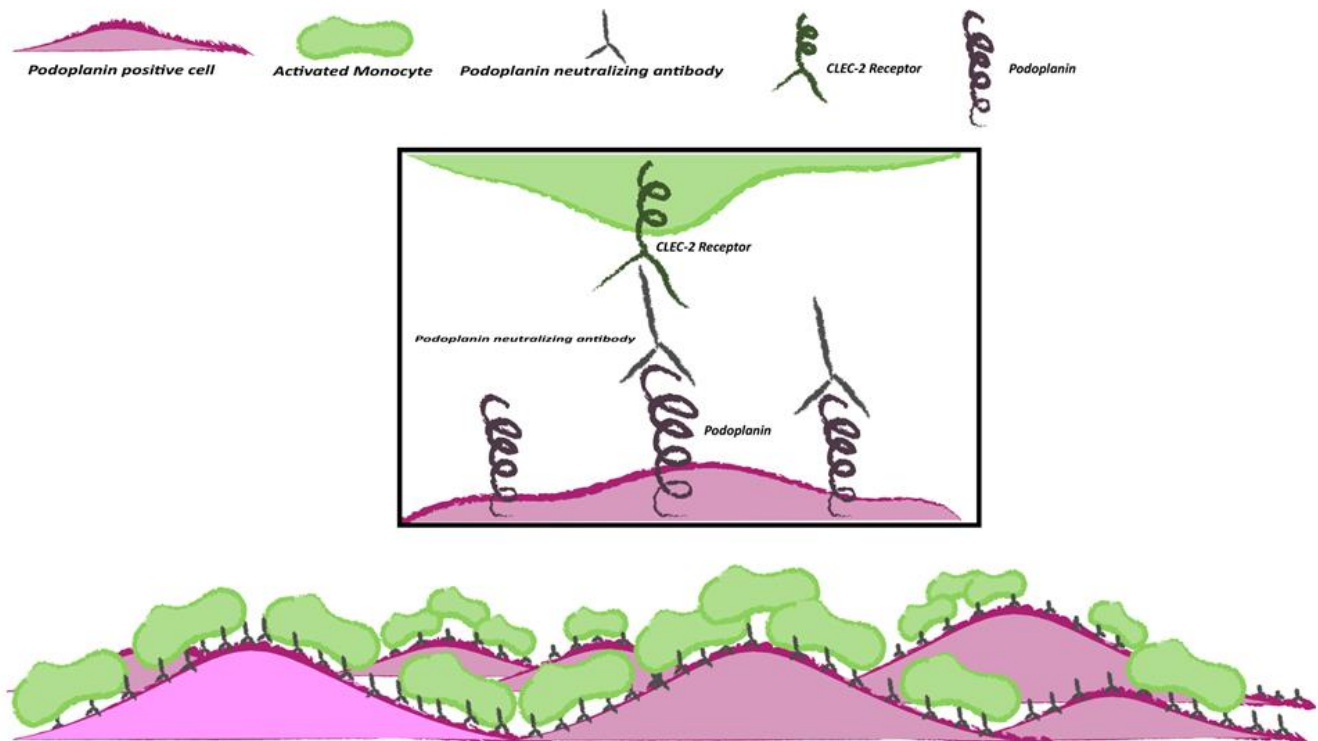
Figure S4

Supplementary Figure S4: Podoplanin neutralization enhances endogenous activation of progenitor cells after myocardial infarction (MI). (**A**) Thin cardiac sections of saline control and (**B**) podoplanin neutralizing antibody treated mice hearts at 7 days after MI were indirectly immunolabeled with NKX 2.5 in red, nuclei, blue. The panels A and B represent the low magnification of the corresponding main figures in Fig.5, A. **(C)** Thin cardiac sections of podoplanin neutralizing antibody treated infarcted mice hearts at 7 days after MI were indirectly immunolabeled with NKX 2.5 in red and troponin T in green, nuclei, blue. The NKX 2.5 positive cells were found disperse in the border zone (BZ) of the MI. (**D**) Thin cardiac sections of treated infarcted mice hearts at 7 days after MI were indirectly immunolabeled with αSarcomeric actin in green, nuclei, blue. Small cells positive for αSarcomeric actin were found disperse in the BZ and the ischemic area of MI. (**E-G**) Thin cardiac sections of podoplanin neutralizing antibody treated mice hearts at 30 days after MI were indirectly immunolabeled with connexin 43 (**E**) in red, troponin T (**F**) in red, troponin I (**G**) in red, and αSarcomeric actin in green, nuclei, blue. (E-G) The photomicrographs represent the remote area (RA) of the samples, used as internal control for the staining. n=5/group.

Figure S5

Supplementary Figure S5: Activated monocytes co-cultured with podoplanin positive cells display anti-inflammatory characteristics. (**A**) Schematic representation of experimental design. Podoplanin positive cells (in green) isolated from global GFP mouse hearts two days after myocardial infarction (MI) were treated or not with podoplanin neutralizing antibody (dark grey) and co-cultured for 24 hours with LPS activated monocytes (in grey). The neutralizing antibody inhibited the binding of podoplanin (ligand) with its receptor CLEC-2 expressed on activated monocyte/macrophages. After 24 hours of co-culture the cells where detached and the two populations where separated via GFP sorting. q-PCR analysis for pro and anti-inflammatory cytokines and markers was performed. (**B-C**) Representative image of GFP/podoplanin positive cells co-cultured with activated monocytes without (**B**) and with(**C**) the presence of podoplanin neutralizing antibody. q-PCR analysis of monocytes co-cultured with podoplanin positive cells in the presence of podoplanin neutralizing antibody show reduced level of (**D**) IL-1β and (**J**) CD11b mRNA. mRNA levels of anti-inflammatory monocyte lineage markers (**F**) ARG-1, (**G**) CHI3I3, (**H**) and CD163 were increased. The mRNA expression of IL-10 (**E**) and CD68 (**I**) did not change. Data are presented as mean±SEM of three independent experiments p< 0.05, one-way ANOVA analysis.

Figure S6

Supplementary Figure S6: Representative cartoon of co-culture of podoplanin cells and activated monocytes/macrophages. Cultured podoplanin positive cells (in pink) were treated with neutralizing antibody (grey). The neutralizing antibody inhibited the binding of podoplanin with its receptor CLEC-2 (dark green) expressed on activated monocyte/macrophages (green).

Supplementary Tables

Table S1: List of antibodies, reagents and sources

Primary Antibodies				
Antigen	*Manufacturer*	*Catalogue number*	*Host species*	*Applications*
Anti-Biotin MicroBeads	Miltenyi	130-090-485		
CD163	Abcam	Ab182422	rabbit	IHC, FC
CD206	eBioscience	12-4321-81	rat	FC
CD68	Abcam	Ab31630	mouse	IHC, FC
CD86	Abcam	Ab213044	mouse	FC
Clec-2	RnD Systems	AF1718	goat	WB
Connexina 43	Abcam	ab11370	rabbit	IHC
Fibronectin	Abcam	ab2413	rabbit	IHC
Green Fluorescent Protein	Invitrogen	A10259	chicken	FC
Lineage Cocktail	Miltenyi	130-092-613	mouse	FC
LYVE-1	Abcam	ab14917	rabbit	IHC, FC
NKX 2.5	Bioss	Bs-2054R	rabbit	FC
PECAM-1	RnD Systems	AF3628	goat	IHC
Podoplanin	Santa Cruz	Sc-134483	rabbit	IHC, FC
Podoplanin	RnD Systems	AF3244	goat	IHC, FC
Podoplanin (biotinylated)	RnD Systems	BAF3244	goat	IHC, FC
Podoplanin (human)	RnD System	AF3670	sheep	IHC
Troponin I	Santa Cruz	Sc-15368	mouse	IHC
Troponin T	Thermo Scientific	MA-12960	mouse	IHC

αSmooth Muscle Actin	Sigma-Aldrich	A2547	mouse	IHC
aSarcomeric Actin	Sigma-Aldrich	A2172	mouse	IHC

Non-immune IgGs

Description	*Manufacturer*	*Catalogue number*	*Host species*	*Applications*
APC-IgG2a control	BD Pharmingen	553991	rat	FC
PE-IgG2a control	eBioscience	12-4321-81	rat	FC

Secondary Antibodies

Reactivity	*Manufacturer*	*Catalogue number*	*Conjugate*	*Applications*
Rabbit	Life Technologies	A21206	Alexa Fluor 488	IHC, FC
Rabbit	Life Technologies	A31572	Alexa Fluor 555	IHC, FC
Mouse	Life Technologies	A21202	Alexa Fluor 488	IHC, FC
Goat	Life Technologies	A21432	Alexa Fluor 555	IHC, FC
Sheep	Life Technologies	A21436	Alexa Fluor 555	IHC
Mouse	Jackson ImmunoResearch	715-545-140	Alexa Fluor 488	IHC, FC

Table S2: List of primers used in the study

Primer (Mouse Gene) Name	Forward 5'-3'	Reverse 5'-3'
ARG-1	AAGCCAGGGACTGACTACCTTAAA	TGATGCCCCAGATGGTTTTC
bFGF	GTCACGGAAATACTCCAGTTGGT	CCGTTTTGGATCCGAGTTTATACT
CD11b	CGCACCCAGGTCTTTGGA	CCACGCAGTCCGGTAAAATT
CD163	CAGGACAGCCAGCTAAATGGA	ATGAAGATGCAGCTGTGGTCAT
CD68	TGGCGGTGGAATACAATGTG	GAGATGAATTCTGCGCCATGA
CH3I3I3	TTCTGAATGAAGGAGCCACTGA	ATTGTCATAACCAACCCACTCATTAC
EGF	GCTACGAAGGAGACGGGATCT	TGCAGGCGGCATTCTCA
IL-10	CAGCCGGGAAGACAATAACTG	CCGCAGCTCTAGGAGCATGT
IL-1b	CTACAGGCTCCGAGATGAACAAC	TCCATTGAGGTGGAGAGCTTTC
Ly6C	TGCTATGGAGTGCCAATTGAGA	GAGCAATGCAGAATCCATCAGA
PDGFa	CTCGAAGTCAGATCCACAGCAT	CTCAGCCCCTACGGAGTCTATC
PDGFb	ACCTCGCCTGCAAGTGTGA	TGCTCCCTGGATGTCCCA
TNFa	GGCTGCCCCGACTACGT	AGGTTGACTTTCTCCTGGTATGAGA
VEGF-A	AGGCTGCTGTAACGATGAAG	TCTCCTATGTGCTGGCTTTG
VEGF-C	CAG CAA GAC GTT GTT TGA AAT TAC A	GTG ATT GGC AAA ACT GAT TGT GA
VEGF-D	GCAAATCGCGCACTCTGA	TGGCAAGACTTTTGAGCTTCAA