SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

Male immune deficient non-obese diabetic (NOD/SCID) mice (NOD.CB17-*Prkdc*^{scid}/J) (Jackson Laboratories, Bar Harbor, ME) were used at the age of 8-10 weeks. Note these mice are not diabetic, but lack functional T and B cells. All mice were maintained in filter top cages and received sterilized food and acidified water. All experimental protocols were approved by the Harvard Center for Animal Research and Comparative Medicine.

Human Peripheral Blood CD34^{+} Cell Purification, Characterization, and Tracking

Human peripheral blood CD34+ stem/progenitor cells were selected from granulocyte colony stimulating factor (G-CSF) mobilized apheresis products obtained from normal healthy adult donors (catalog# mPB026, AllCells, Berkeley, CA). Briefly, G-CSF (10μg/kg/day) was administered to donors for 5 consecutive days to mobilize CD34+ cells from the bone marrow into peripheral circulation. Donors underwent apheresis on days 5 and 6 to collect mobilized peripheral blood mononuclear cells. CD34+ cells were highly enriched from the apheresis product using ISOLEX 300i Magnetic Cell Positive Selection System (version 2.5, Baxter Healthcare, Deerfield, IL, USA) according to the protocol provided with the instrument's User Manual. Purified cells were characterized by flow cytometry (see below). Enriched, selected cells were maintained in RPMI (Invitrogen) with 0.5% human serum albumin at 25ºC and used within 48h. To test

viability, aliquots of 2 x 10^5 cells were labeled with 7-AAD (20μg/ml, 20min, 4ºC in 100μl PBS), washed with FACS buffer (PBS/5%BSA), and then analyzed by flow cytometry. To test for clonogenic capacity, the colony forming unit assay (CFU) was performed using modifications to methods previously described [1]. Purified CD34+ stem/progenitor cells were diluted in Iscove's MDM (IMDM) with 2% FBS (Stem Cell Technologies, Cat # 07700 Vancouver, Canada) to a concentration of $5x10^3$ cells/ml. The primary dilution was further diluted in MethoCult GF+ H4435 Medium (Cat # 04445, StemCell Technologies, Vancouver, Canada) (1:10) to a final cell concentration of 500 cells per 35mm dish and cells were cultured in quadruplicate per sample and reported as an average. Fourteen days later, the CFU were scored using bright field microscopy for the presence of Colony Forming Unit-Granulocyte Macrophage (CFU-GM), Colony Forming Unit Erythroid (CFU-E), Burst Forming Unit-Erythroid (BFU-E) and Colony Forming Units with both GM and Erythroid colonies (CFU-GEMM). CFU-E: Colony-forming unit-erythroid produces a colony containing 1 to 2 clusters with a total of 8-200 erythroblasts. BFU-E: Burst-forming unit-erythroid produces a colony containing > 200 erythroblasts, usually present in > 2 clusters. CFU-GM: Colony-forming unit-granulocyte, macrophage produces a colony containing > 40 granulocyte and macrophage cells. CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte produces a colony containing erythroid cells as well as 20 or more granulocyte, macrophage and megakaryocyte cells.

In some experiments, in order to track HSCs following systemic administration, human CD34+ cells were labeled with the green fluorescent tracer 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen) using 1μg/10^7 cells for 30 minutes at 25ºC in 10ml of RPMI. Excess CMFDA was quenched after centrifugation (250x*g* 5min) by resuspending in 10ml 5%BSA (ultrapure) (Sigma) in RPMI. After further centrifugation cells were resuspended in 1%BSA/RPMI (12.5 x 10^6/ml). CMFDA labeling did not yield any changes in viability detected using 7-AAD (not shown).

Animal Model

Ischemia-reperfusion injury of the kidney was modified from methods previously described [2]. In brief, on day 0, kidneys of anesthetized male mice (8-10wks) were exposed through surgical incisions to the flanks, and at core temperature of 36.8-37.3^{o}C a surgical clamp was placed across the renal artery and vein of either one or both kidneys. The kidneys were confirmed to become dusky, then replaced in the retroperitoneum for 27minutes (unilateral model) or 25 minutes (bilateral model). Clamps were removed and reperfusion to kidneys was confirmed visually, and wounds closed. To test the effect of human HSCs, these mice with unilateral IRI kidney injury were divided into two groups. In the treatment group (n = 6-10/group), on days 1 and 2 after kidney injury, 200µl of cell suspension containing 2.5 x 10^6 human CD34+ cells labeled with CMFDA was infused intravenously through the tail vein. In the control group, mice were only given vehicle. Mice were sacrificed on days 3, 5, 7, 14 and 28 IRI of the kidney.

Quantification of Renal Function and Circulating Human Cytokines

To evaluate renal function, mice with bilateral IRI kidney injury (d0) were randomly divided into two groups. The treatment group (n = 10) received 2.5 x 10^6 human HSCs by intravenously tail vein infusion on days 1 and 2. The control group (n = 16) received vehicle only. Plasma creatinine was analyzed from plasma samples were taken from the tail vein on days 1, 2, 5, 7, 14 and 28 after injury using Methods previously described [2]. Plasma samples were also assessed for the presence of angiogenic cytokines by human cytokine array (Ray Biotech, Inc.)

Tissue Preparation, Immunostaining, Imaging and Quantification of Injury and Repair

Mice were perfused with ice cold PBS then organs fixed in PLP solution for 2h followed by 18% sucrose 16h, then preserved in OCT (-80^{o}C) [3], or tissue for light microscopy was fixed in 10% neutral-buffered formalin for 12h, transferred to 70% ethanol, then processed for paraffin sections (3mm) and sections and stained with periodic acid-Schiff (PAS) or picrosirius red stain [3]. Immunofluorescence labeling was performed on 5mm cryosections. To detect infused human cells in kidneys, spleen and heart, either antibodies against human leukocyte antigens with no cross-reactivity to mouse antigens were used or fluorescence of CMFDA was used (up to d7). The following antibodies were used employing methods described elsewhere [2, 3]: anti-human leukocyte antigen class I (HLA)-ABC (FITC, 1:200, eBioscience), anti-human CD45 (FITC, 1:200, eBioscience), rat-anti-human CD45 (1:200, Abcam), anti-human CD3 (FITC, 1:200, eBioscience), anti-human CD31 (FITC, 1:200, eBioscience), rabbit anti-human vWF (1:200, Abcam), anti-human CD146 (FITC, 1:100, Abcam), biotin-anti-human CD34 (1:100, Abcam), rat-anti-mouse cross react with human CD11b (1:100 eBioscience), biotin-anti-human CD133 (1:100, Miltenyi), rabbit-anti-human-CD133 (1:100, CellSignaling), goat-anti-human KDR (1:100, R&D Systems), and rabbit-anti-human KDR (1:100, NeoMarkers), followed by rabbit-anti FITC (1:200, Invitrogen), anti-rat Cy3 or anti-rabbit Cy3 or anti-goat Cy3 (1:400, Jackson Immunosresearch) or anti-avidin Cy3 (1:3000, Jackson Immunosresearch). To label mouse vasculature using rat-anti-mouse CD31 (1:200, eBioscience), which does not cross-react with human antigen was

applied, followed by anti-rat Cy3 (1:400, Jackson Immunosresearch). Detection of the Ki67 antigen and detection of apoptotic cells by T.U.N.E.L was performed as previously described [3]. Sections were post-fixed with 1% paraformaldehyde (PFA), then mounted in Vectashield with DAPI. Peritubular capillary loss and tubule injury were determined by assessing anti-CD31-Cy3 labeled kidney sections or PAS stained paraffin sections respectively using a blinded scoring method as reported previously [4]. In brief, images were captured by digital imaging (X200) sequentially over the entire sagittal section incorporating cortex and outer medulla (10-20 images). Each image was divided into 252 squares by a grid. To calculate peritubular capillary loss, each square without a peritubular capillary resulted in a positive score; the final score presented as a percentage positive score. To assess the tubular injury, each square the presence of tubule injury (tubule flattening, necrosis, apoptosis or presence of casts) resulted in a positive score. The final score is the percentage of squares with positive score per image, which was averaged for all images from the individual kidney. Epifluorescence images were taken with a Nikon TE2000 microscope, CoolSnap camera (Roper Scientific, Germany) and processed using IP lab software (BD Biosciences, San Jose, CA). Confocal images were generated using a Nikon C1 D-Eclipse confocal microscope. Projection images were generated from 10 Z-stack images that were acquired at 0.1 mm steps. To allow comparison between sections, all confocal settings including were kept constant between sections.

Flow Cytometric Analysis and Cell Sorting

Isolex-enriched CD34 cells were analyzed using the following human antibody

combinations: anti-CD31-FITC (1:100, BD), anti-CD146-PE (1:100, BD), anti-KDR-FITC (1:100, R&D Systems), anti-CD45-FITC (1:100, BD), anti-CD140b-Alexa Fluor 488 (1:100, BD), anti-CD29-PE (1:100, BD), anti-CD105-FITC (1:100, R&D Systems), anti-CD34-PE (1:100, BD), anti-CD99-FITC (1:100, BD), anti-CD144-PE (1:100, R&D Systems), anti-CD38-FITC (1:100, BD), anti-CD14-FITC (1:100, BD), anti-CD64-PE (1:100, BD), anti-CD61-PerCP (1:100, BD) anti-CD133-APC (1:100, Miltenyi), anti-CXCR4-APC (1:100, BD), anti-CD90-APC (1:100, BD), anti-CD117-APC (1:100, BD), anti-VEGFR1-APC (1:100, R&D Systems), using methods previously described [2]. Full characterization of HSCs will be documented elsewhere (D.M. & A.C. unpublished). Single cells were prepared from kidney, spleen and bone marrow as previously described [3]. In brief, single cells (1x 10^5) from kidney, spleen and bone marrow were resuspended in FACS buffer and incubated with antibodies against human CD45 (FITC, 1:200, eBioscience) and mouse CD11b (PE, 1:200, eBioscience) for 30 minutes. After washing with FACS wash buffer, and resuspending in 200ul FACS buffer, cells were analyzed using BD FACSCalibur flow cytometer. The human HSCs labeled with CMFDA on day 2 after injection were sorted directly by FACS sorting using FACSaria [3]. Sorted CMFDA+ cells from kidney were immediately lysed and RNA purified using RNA Easy (Qiagen) system, for real time PCR. In some experiments ISOLEX purified HSCs were separated into CD146+ and CD146- subpopulations prior to systemic injection, by magnetic activated cell sorting using MACS cell separation system (Miltenyi Biotech, Auburn, Calif., USA) and methods previously described [5]. Briefly, following, incubation with anti-human CD146-FITC antibodies (1:20 dilution) on ice for 20 min. cells were labeled with anti-FITC IgG MicroBeads (Miltenyi Biotech; 1:5) washed applied to the MS column of MiniMACS magnetic separation kit followed by a second column.

Unlabeled, CD146- cells were collected, and then labeled CD146+ cells were collected separately after the magnetic field was removed. Cells were relabeled with anti-CD146-FITC antibodies and compared with unlabeled cells by flow cytometry.

Real Time PCR

Total RNA was generated from tissue and cells using a kit (RNA Easy Qiagen), according to the manufacturer's instructions. Purity determined by A260 to A280. cDNA was synthesized from 1μg of total RNA using iScript and primers comprising random hexamers and poly dT [3]. Real-time PCR of human and mouse samples was performed using an ABI7900HT sequence detection system (PerkinElmer Life Sciences, Boston, Applied BioSystems, Foster City, CA) in the presence of SYBR-Green (SYBR Green PCR kit; Qiagen) using methods previously described [6]. Primer/probe sets specific for human GAPDH, HPRT1, Angiopoietin 1 (ANGPT1), Fibroblast growth factor 2 (FGF2), Hepatocyte Growth Factor (HGF), Insulin-like growth factor 1(IGF1), interleukin-8 (IL8), Platelet-derived growth factor B (PDGFB), transforming growth factor β1 (TGFB1), Vascular endothelial growth factor (VEGF), TIE1, were from Sabiosciences. Equal amounts of cDNA were used for RT-PCR reaction and mixed with ready to use reaction mix (Sabiosciences). All of the reactions were performed in triplicate. Optimization of the real-time PCR was performed according to the manufacturer's instructions. For standard curve determination, we used a pool of all the samples, serially diluted in four $\log_2$ steps and run in parallel to the samples. The total volume of each reaction was 20 μl, containing 300nM forward and 300nM reverse primer and 125ng of cDNA. Appropriate negative controls were run for each reaction.

Cell Culture

Human ISOLEX purified HSCs were incubated ($2.5x10^6$) in 3cm diameter wells (Falcon) in 2ml of medium (X-vivo 10, Lonza) supplemented with SCF and Flt-3 ligand (20ng/ml each, Peprotech) for 48h. Supernatants were separated from cells by centrifugation and filtration then 1ml was administered to mice 24h post IRI (200µl IV and 800µl IP). In other experiments, spleens was harvested from mice 72h post HSC or vehicle infusion and splenocyte single cell preparation made [5]. $1x10^6$ cells were incubated in 3cm diameter wells in serum free RPMI for 12h. Supernatants were separated from cells by centrifugation and filtration. From the same mice, plasma was collected. Cytokine content from supernatants and plasma were analyzed by antibody cytokine array (Ray Biotech, Inc.).

Supplemental Tables

Table 1. Human CD34+ enriched mobilized peripheral blood stem cells (n = 6/group).

Markers	**CD34+**	**CD34+CD45-**	**CD34-CD45+**
Average	96.44	0.08	2.96
SD	0.84	0.13	0.89

Table 2. Minor subpopulations in human CD34+ enriched mobilized peripheral blood HSCs by cell surface markers (n = 6/group)

	CEP type markers				**CECs**
Markers	**CD34+ CD14+**	**CD34+KDR+ CD133+**	**CD34+KDR+ CD146- CD31-**	**CD34+CD133+ CD45+CD146+**	**CD34+CD133- CD146+CD31+**
Average	**0.05**	**0.30**	**0.05**	**0.07**	**0.05**
SD	**0.04**	**0.22**	**0.07**	**0.11**	**0.06**

Table 3. Analysis of recruitment to the kidney and *de novo* expression of hCD146+ in the kidney by hCD146+ and hCD146- subpopulations of ISOLEX purified CD34+ HSCs following IV injection and following recruitment to kidney at day 2 post IRI.

	hCD146- group	**CD146+ group**
hCD146+ on D0	**0.02%**	**7.34%**
hCD146+ on D2 in kidney	**98%**	**100%**
hCD146+cells/section	**155.3±56.4**	**166.7±40.4**

Supplemental Figures and Figure Legends

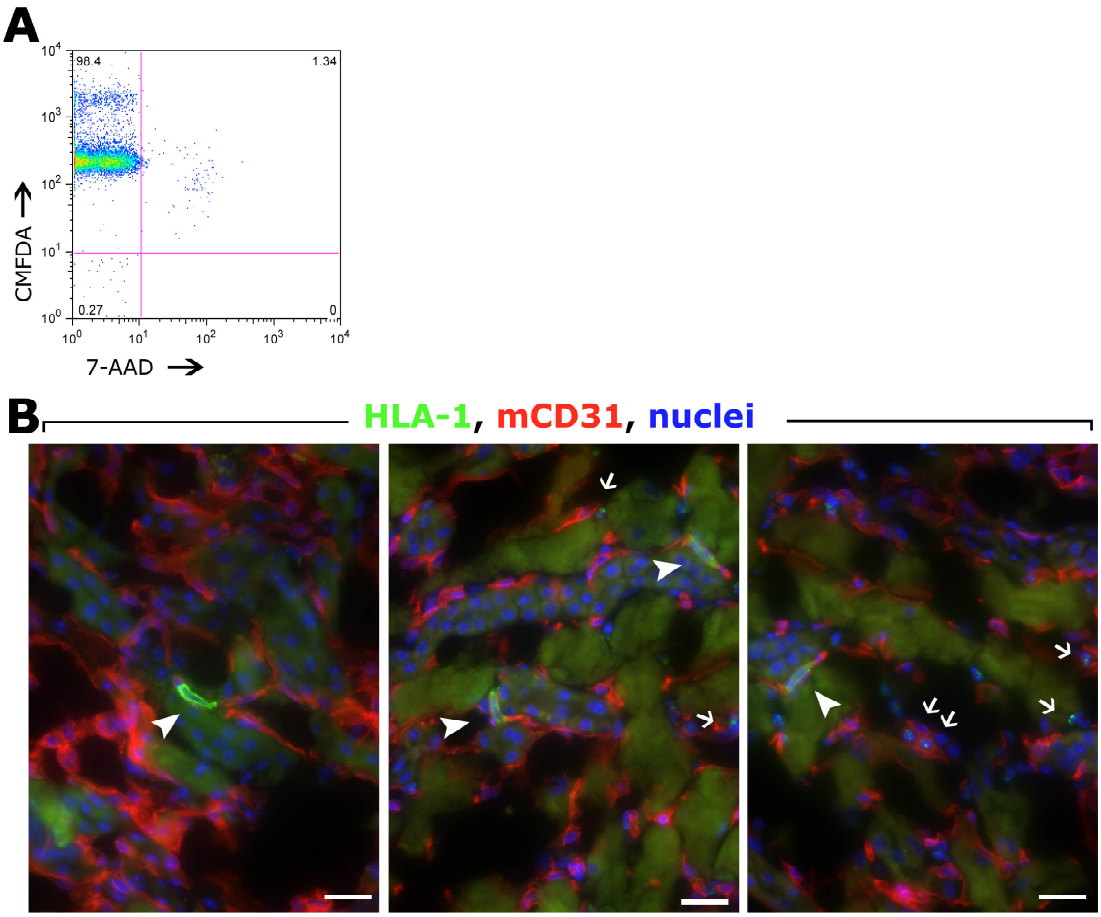

Figure S1. (**A**) FACS plot showing CMFDA labeled ISOLEX purified human CD34+ cells and uptake fluorescent viability marker 7-AAD. Note only 1.3% of CD34+ cells lack viability. (**B**) Fluorescence photomicrographs showing HLA-I human HSCs 12h after recruitment to the post IRI kidney showing expanded morphologies 'bridging' gaps in the disrupted capillaries (arrowheads) and recruitment within capillaries and in the interstitium (arrows). Marker = 50µm.

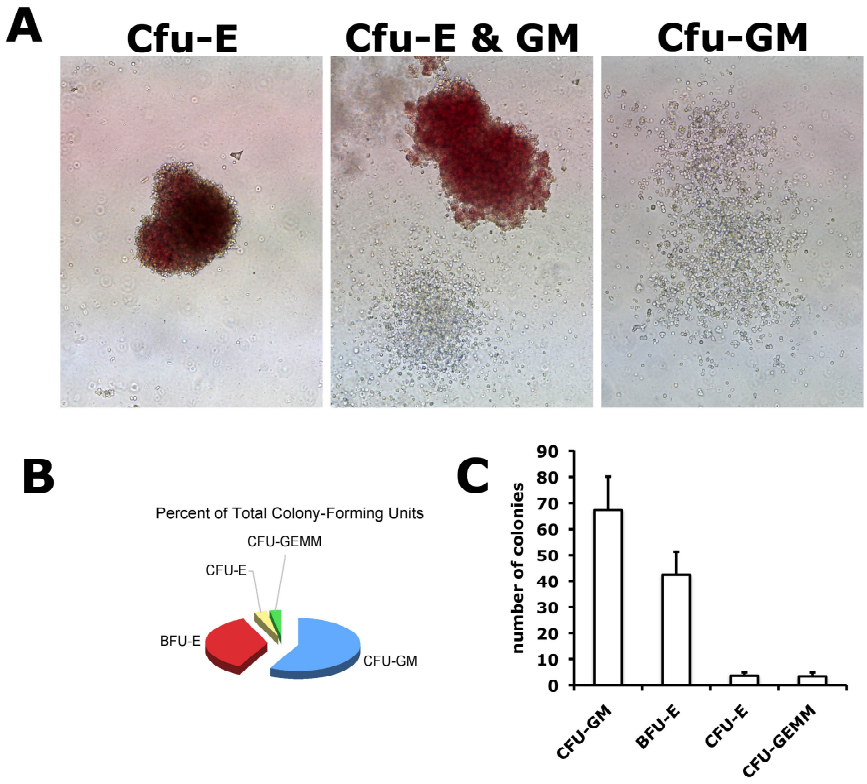

Figure S2. Evidence of multi-lineage clonogenic capacity of CD34+ ISOLEX purified cells. (**A**) Low power images of erythrocytic and granulocyte/monocyte colonies growing from single CD34+ cells. (**B**) Pie chart showing the proportion of colony types cultured from CD34+ cells and (**C**) graph showing the number of colonies per 500 HSCs plated.

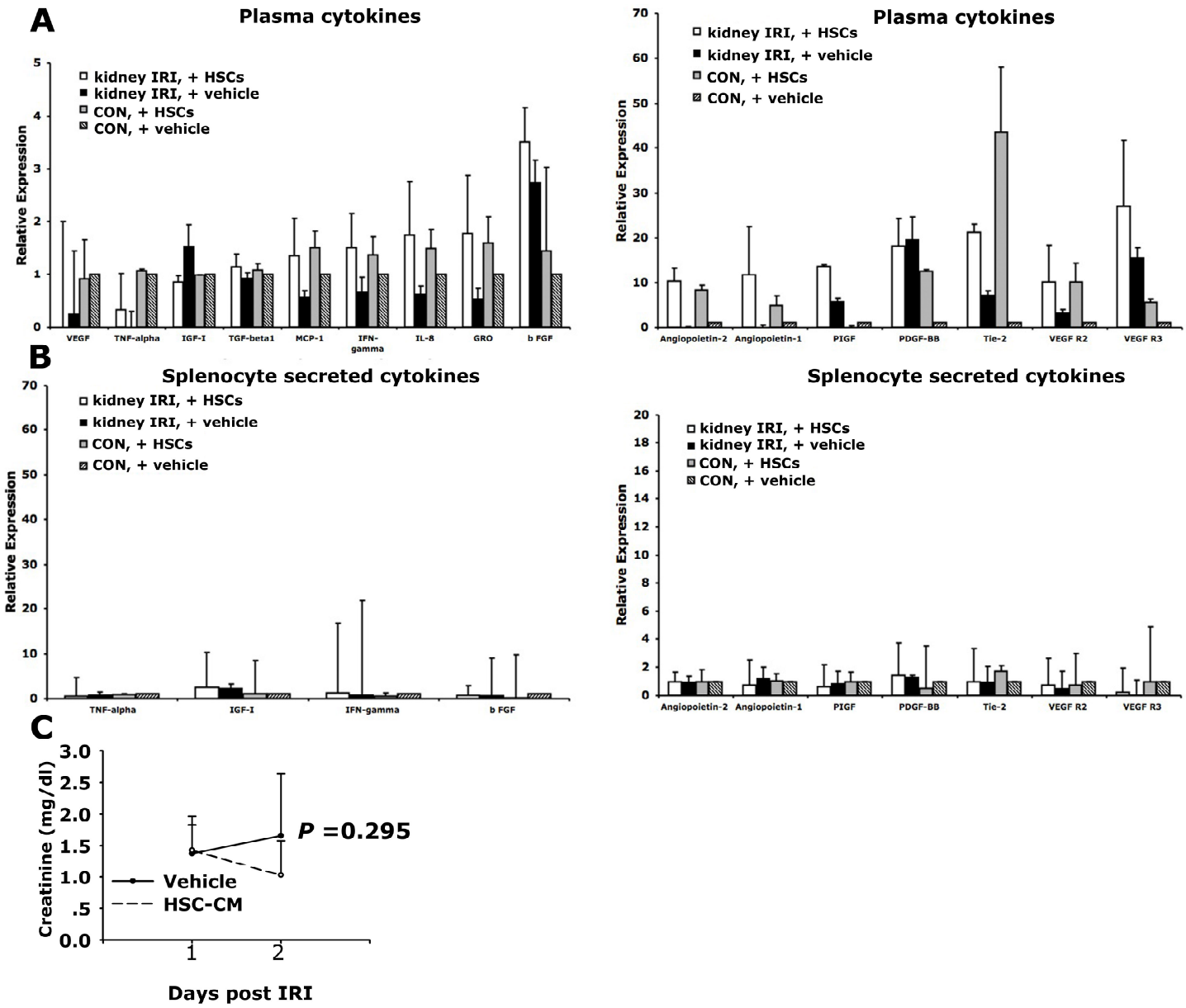

Figure S3. Angiogenic cytokine production by HSCs is dependent on recruitment of HSCs to the injured kidney. **(A-B)** Detection of human angiogenic cytokines in plasma of mice **(A),** or in supernatant following 12hr culture of splenocytes **(B),** collected from mice d3 after IRI mice or from healthy mice infused with either HSCs ($2.5x10^6$) or vehicle on d1. Cytokine levels are means with SE and are relative to healthy mice with vehicle injection (n=3 per group). **(C)** Plasma creatinine levels from d1 and d2 in mice subjected to bilateral IRI on d0 and infused on d1 with 1ml of conditioned medium from cultured HSCs ($2.5x10^6$) or vehicle. The difference between groups at d2 is not significant (n=7 per group).

Supplemental References

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