Supplementary Data to Tropea et al. "Isolation of a circulating CD45^{-}, CD34^{dim} cell population and validation of their endothelial phenotype" (Thromb Haemost 2014; 112.4)

Suppl. Materials

Reagent	Supplier	Catalogue #
dextran	United States Biochemical	14465
Histopaque-1077	Sigma	H-8889
HUVECs	Clonetics	CC-2517
EBM2™ medium	Clonetics	CC-3156
EGM-2 bullet kit	Clonetics	CC-4176
normal mouse IgG	Caltag	10400
ACK	Lonza	10-548E
50 mm filters	Partec CellTrics	04-004-2327
RLT™ buffer	Qiagen	79216
Diff-Quick™	Dade Behring	B4132
UEA-1	Sigma	L4889
vWF	US Biological	V2700-01C
DAPI	Invitrogen	D3571
ProLong Gold™	Invitrogen-Molecular Probes	P36930
RNeasy Micro Kit	Qiagen	74004
RNA Nano Chips	Agilent 2100 Bioanalyzer	5067-1511
HeLa RNA	Ambion	7897G
OneStep RT-PCR	Qiagen	210210
E-Gels	Invitrogen	G501801
DNA ladder	Invitrogen	12373-031
Vacutainer® tubes	BD Worldwide	367841,366643,364606

Marker	Fluorochrome	Supplier	Catalogue #
Pilot			
CD31	FITC	BD	555445
CD146	PE	BD	550315
CD45	V450	BD Horizon	560367
CD34	APC	BD	BD Biosciences
CD146+ T cells & HUVEC Sorts			
CD45	Pacific Blue	Serotec	MCA87PB

CD3	FITC	BD	555332
CD14	PE	BD	347497
CD19	APC-Cy7	BD	557791
CD146	Alexa 647	Serotec	MCA2141A647
viability	7-AAD	BD	559925
CEC sorts			
CD45	V450	BD Horizon	560367
CD34	APC	BD	340441
CD146	PE	BD	550315
viability	7-AAD	BD	559925

Sample processing for cell sorting

Briefly, blood was centrifuged at 700g for 20 minutes at 4^{o}C with no brake. The plasma was removed without disturbing the buffy coat. Blood volume was restored with ice cold Hank's balanced salt solution without calcium or magnesium (HBSS-) containing 0.5% (w/v) bovine serum albumin and 1.5 mM EDTA. Samples were mixed and centrifuged again at 700g for 20 minutes at 4^{o}C with no brake. The upper phase was removed without disturbing the buffy coat and the remaining sample was mixed by gentle pipetting.

Immunofluorescent staining

For the demonstration of lectin and antibody binding, Cytospin™ slides were prepared and fixed with 1% formaldehyde for 30 minutes and washed twice with **phosphate-buffered saline** (PBS). TRITC-labelled *Ulex europaeus* (UEA-1) was added at a concentration of 10 μg/mL and incubated for 1 hour at 25°C and washed again with PBS. For von Willebrand factor (vWF) staining, cells were permeabilised with 0.1% Triton X-100 for 10 minutes at room temperature and blocked with 4% bovine serum albumin (**BSA)** and 0.2% Tween-20 in PBS for 1 hour. Slides were incubated with a 1:100 dilution of the FITC conjugated vWF and washed again with PBS. Slides were counterstained with 300 nM 4',6-diamidino-2-

phenylindole (DAPI) and washed again in PBS. Coverslips were mounted using ProLong Gold™ antifade reagent.

Ultramicro analytical immunochemistry

For ultramicro analytical immunochemistry, cell samples were centrifuged at 10,000g for 20 minutes at 4°C to pellet cells, which were then washed five times in 10 mM Tris, 400 mM sodium chloride, pH 7.5 before being lysed by re-suspension in double distilled water. Sodium deoxycholate (1%) was added and the pellets sonicated for 2 minutes on ice. Protease inhibitor cocktail VI (100 μL; Calbiochem, EMD Chemicals Inc.) was added and each pellet **freeze/thawed** five times to disrupt the cell integrity. Soluble membranes were recovered by centrifugation at 20,000g for 25 minutes and the proteins labelled by adding 5 μg of **AlexaFluor 633 laser dye (Molecular Probes/Invitrogen).**

A series of different antibodies were covalently immobilised into the channels of an FC_R50.332.2 glass microfluidic chip (Micronit BV, Enscheda, The Netherlands). The chip contained 30 channels in a serpentine array and a different antibody was immobilized into the first 21 arms of the array. The inlet of the array was connected via an automated injection port (Upchurch Scientific, Oak Harbor, WA), equipped with a 500 nL sample loop, to a SP100i syringe pump (World Precision Instruments, Sarasota FL) set to deliver a flow rate of 0.01 mL/minutes. A 500 nL sample was introduced via the injection port and pumped through the array, allowing immobilized antibody in each column to capture and retain its specific analyte, while the remainder of the sample passed onto the next column. Once the sample had completely passed through the chip, the array was flushed for 10 minutes with phosphate buffer and scanned. Detection of each analyte was achieved via laser-induced

fluorescence (LIF), using a laboratory-built scanning LIF detector, equipped with a 650 nm diode laser (Edmund Scientific, Barrington, NJ) and a fibre optic spectrometer (Ocean Optics, Dunden, FL). The detector head scanned across the column array and was programmed to record three measurements per column (the leading edge, centre, and far edge of each capillary channel). A complete scan of all 21 channels took 30 minutes. During this time the fluorescent signal, generated by the labelled analyte bound to the immobilised antibodies in each channel, were collected by the detector. The signals were relayed via the spectrometer to a computer and analysed by spectrometer software (OOIBase32, Ocean Optics). In this way, each sample was subjected to extraction by 21 different antibodies, the analytes retained in each column being compared to standard curves, constructed by subjecting recombinant standards to the same extraction procedure.

RNA isolation and analysis

Total RNA was purified from Fluorescence Activated Cell Sorting (**FACS)** sorted samples using RNeasy Micro **Kit following** the manufacturer's directions. Human elutriated monocytes were used as controls for the RNA isolation method. Total RNA concentration was determined using Nanodrop ND-1000 with concentrations of at least 10 ng/ul considered accurate. RNA integrity was evaluated using RNA Nano Chips. HeLa RNA was used as the chip control.

PCR of FACS Sorted Cells

Multiplex PCR #1 consisted of CD45, GAPDH and CD144 while #2 was used to detect CD105, vWF and CD31 (Table 1). Gene combinations were chosen that gave the same level of expression when compared to the single gene PCR.

Table 1: PCR primers used for PCR multiplex assays

Gene	Accession#	Forward primer	Reverse primer	Amplicon Size (bp)
CD45	Y00638.1	GGA GCT GGA GGA CAC AGC ACA	TCG AAT GTG GAA ACC ATG CCT AGC	784
GAPDH	BC001601.1	TCG AAT GTG GAA ACC ATG CCT AGC	ACA GTT TCC CGG AGG GGC CA	629
CD144	NM001795.3	GCG TCC GTG CCT GAG TCG TC	TCC TGG TAG TCG CCC CGC AA	440
CD105	BC014271.2	CCC CGA GAG GGA CGA GGT GAC	GTC ATG GCG TCG TCG GCA CA	999
vWF	NM000552.3	ATG CCC ACG TGT GTG CCC AG	TGA CGG TCG CTT CCG GTC CT	695
CD31	NM000442.4	TGC CAC GGG AGC TGC AAA GAC	TCC GCG GCC GAG AAA ACT CC	592

Qiagen OneStep RT-PCR kit was used according to the manufacturer's instructions with the following modifications. Total RNA (1 ng) was used as the template, primers were used at a final concentration of 0.4 μM, the denaturation time was 1 minute, and an annealing temperature of 62^{o}C was applied for 45 seconds across a total of 33 cycles. RNA isolated from cultured Human Umbilical **Vein Endothelial Cells** (HUVECs) and human mononuclear cells were used as controls. Bands were visualised on 1.2% E-Gels. E-Gel low range quantitative DNA ladder was used to quantitate the amount of DNA in each band from the PCR. Images were obtained on the Carestream Gel Logic 2200 PRO Imager and quantitated with the Carestream Molecular Imaging Software v. 5.0.