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Interleukin-3 greatly expands non-adherent endothelial forming cells with pro-angiogenic properties

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Abstract
Circulating endothelial progenitor cells (EPCs) provide revascularisation for cardiovascular disease and the expansion of these cells opens up the possibility of their use as a cell therapy. Herein we show that interleukin-3 (IL3) strongly expands a population of human non-adherent endothelial forming cells (EXnaEFCs) with low immunogenicity as well as pro-angiogenic capabilities *in vivo*, making their therapeutic utilisation a realistic option. Non-adherent CD133$^+$ EFCs isolated from human umbilical cord blood and cultured under different conditions were maximally expanded by day 12 in the presence of IL3 at which time a 350-fold increase in cell number was obtained. Cell surface marker phenotyping confirmed expression of the hematopoietic progenitor cell markers CD133, CD117 and CD34, vascular cell markers VEGFR2 and CD31, dim expression of CD45 and absence of myeloid markers CD14 and CD11b. Functional experiments revealed that EXnaEFCs exhibited classical properties of endothelial cells (ECs), namely binding of *Ulex europaeus* lectin, up-take of acetylated-low density lipoprotein and contribution to EC tube formation *in vitro*. These EXnaEFCs demonstrated a pro-angiogenic phenotype within two independent *in vivo* rodent models. Firstly, a Matrigel plug assay showed increased vascularisation in mice. Secondly, a rat model of acute myocardial infarction demonstrated reduced heart damage as determined by lower levels of serum creatinine and a modest increase in heart functionality. Taken together, these studies show IL3 as a potent growth factor for human CD133$^+$ cell expansion with clear pro-angiogenic properties (*in vitro* and *in vivo*) and thus may provide clinical utility for humans in the future.

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Introduction

Endothelial progenitor cells (EPCs) circulate in peripheral blood and migrate to a site for vascular formation and repair (Asahara et al., 1997). The pro-angiogenic nature of EPCs is of therapeutic interest for a range of medical conditions, including wound healing (Cho et al., 2003), limb and myocardial ischemia (Eizawa et al., 2004; Laing et al., 2007; Schmidt-Lucke et al., 2005; Werner et al., 2005) and diabetes (Loomans et al., 2004), where low numbers of circulating EPCs have been linked to disease. To overcome this problem, EPCs as a cell-based therapy have been widely pursued and involves transplantation of healthy and functional cells to replenish damaged cells and repair injured tissue. Notably, pre-clinical and pilot clinical studies have yielded poor results suggesting that treatment with EPCs is yet to be fully realised and that significant modifications are clearly required (reviewed in Sen et al., 2011).

Studies suggest that there are three different types of EPCs; namely the adherent 'early EPCs'(Hill et al., 2003; Hur et al., 2004; Ito et al., 1999), the adherent 'late outgrowth' EPCs (Ingram et al., 2004; Lin et al., 2000) and the 'non-adherent EPCs' that we and others have recently characterised (Appleby et al., 2012; Janic et al., 2010; O et al., 2011). While all three populations demonstrate the capacity to improve neovascularisation in preclinical models, they differ with respect to their capacity to differentiate into endothelial cells (ECs) and to physically form new blood vessels in vivo. 'Early EPCs' are cells with haematopoietic characteristics and share features with immune cells, particularly CD14 expression of monocytes/ macrophages and are obtained from short-term cultures of 4–7 days in vitro (Hill et al., 2003; Hur et al., 2004; Ito et al., 1999). Most studies suggest that these short-term cell enrichment protocols enhance vessel formation by paracrine assistance by providing a potent mixture of pro-angiogenic growth factors rather than integration into the vasculature for repair (reviewed in Sen et al., 2011). The 'late outgrowth EPCs' have been rebadged endothelial colony forming cells (ECFCs) and are the EPC type most studied as they display spontaneous vasculogenic properties, integrate long-term into the systemic vasculature of the host animal and remodel into arteries and veins in vivo (Bompais et al., 2004; Gulati et al., 2003; Hur et al., 2004; Ingram et al., 2004; Melero-Martin et al., 2007; Timmermans et al., 2007). ECFCs express the mature EC marker CD144 (VE-cadherin), do not express the progenitor cell marker CD133 and are phenotypically very similar to fully differentiated ECs with the exception of an increased proliferative rate (Hur et al., 2004; Timmermans et al., 2007). ECFCs have entered the clinic with, for example, 2.5×10^6 autologous ECFCs per kilogramme (1.75×10^8 for a 70 kilogramme adult) infused intravenously approximately four weeks after BM aspiration and cell expansion. The same number of cells is again transplanted approximately one week later (NCT01468064, (Lee et al., 2010)). Importantly, the time required to generate the ~2×10^8 of autologous ECFCs is approximately 5 weeks which (i) falls well outside of the effective cell delivery time of 4–10 days post-ischemic event (Dimmeler et al., 2008) and (ii) attracts a high cost associated with long term culture and expansion of adherent cells. Clearly, if we are to provide EPCs as a cell based therapy new strategies to improve their expansion are required and investigation of an allogeneic source is warranted.

In this study we demonstrate the effectiveness of interleukin (IL)-3 to rapidly expand CD133$^+$ non-adherent endothelial forming cells (naEFCs) to a therapeutically relevant number within a clinically beneficial time frame. Recently we and others demonstrated that naEFCs are capable of in vivo vascularisation (Appleby et al., 2012; Janic et al., 2010) and herein we show the expansion of freshly isolated or cryopreserved cells in a serum-free medium to $>2 \times 10^8$ within only 12 days (EXnaEFCs). Furthermore, we demonstrate that these cells harbour low immunogenicity with validated pro-angiogenic capabilities in vitro and in vivo. We also demonstrate improvement of heart function in a rat model of acute myocardial infarction.

Materials and methods

Ethics statement

The collection of primary human umbilical vein endothelial cells (HUVECs) and umbilical cord blood was approved by the Human Research Ethics Committees of the Royal Adelaide Hospital and the Women's and Children's Health Network, Adelaide, South Australia. Informed written consent was obtained from the subjects in accordance with the 'Declaration of Helsinki'. Animal experiments were approved by the Animal Ethics Committee of SA Pathology and conform to the guidelines established by the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes'.

Isolation and culture of CD133$^+$ naEFCs

Umbilical cord blood was collected from healthy, consenting women undergoing elective caesarean sections in cord blood bags (MacroPharma, Mouvaux, France). Blood was diluted 1:1 in sterile phosphate buffered saline (PBS). Mononuclear cells (MNCs) were isolated using Lymphoprep™ (Axis-Shield, Oslo, Norway). Mononuclear cells were washed in human umbilical vein endothelial (HUVE) media [Media 199 (Sigma-Aldrich, St. Louis, MO); containing 20% FCS (Hyclone, Logan, UT), 1.5% sodium bicarbonate, 2% HEPES buffer solution, penicillin streptomycin, sodium pyruvate (Gibco Invitrogen, Gaithersburg, MD) and non-essential amino acids (Sigma-Alrich)]. Cells were blocked with 10 ml of 5% normal mouse serum (Abcam, Cambridge, England) in HUVE media for 10 min, washed in HUVE media and blocked with 100 μl of human FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) prior to an incubation with 100 μl of CD133$^+$ microbeads (Miltenyi Biotec) for 30 min at 4 °C. Cells were washed again in HUVE media and CD133$^+$ cells isolated using the AutoMacsPro (Miltenyi Biotec) as per manufacturer's instructions. CD133$^+$ cells were resuspended at a concentration of $0.5–1 \times 10^6$ cells/ml in CellGro SCGM (CellGenix GmbH, Freiburg, Germany) supplemented with 10 ng/ml Thrombopoietin (TPO, Sigma-Alrich), 40 ng/ml stem cell factor (SCF) and 40 ng/ml Ftl3L (both R&D Systems, Minneapolis, MN), here on in referred to as CellGro medium. In some experiments IL3 (20 ng/ml) and placental growth factor (PlGF, 25 ng/ml, R&D Systems) were added to the media in addition to the other growth factors.

Freezing and thawing of EXnaEFCs

CD133^{+} isolated EXnaEFCs were suspended at 1×10^6 cells/ml at 4 °C in cryopreservation media [90% FCS (Hyclone) and 10% DSMO (Sigma-Alrich)] in a Cyto.S™ vial (Greiner Bio-One GmbH, Frickenhausen, Germany) immediately following isolation and placed in a Nalgene Mr. Frosty™ freezing container (Thermo Fisher Scientific, Waltham, MA) for 24 h at −80 °C before being transferred to liquid nitrogen. To thaw EXnaEFCs, the vial was placed into a 37 °C water bath prior to cold HUVE media (10 ml) being added dropwise. Cells were centrifuged at 300 ×g for 5 min and pelleted cells were resuspended in CellGro medium to 0.5×10^6 cells/ml. Every 2–3 days cells were counted and viability was assessed using a 0.4% trypan blue solution and haemocytometer. Additional media were then added to maintain the cells at 0.5×10^6 cells/ml.

Human umbilical vein endothelial cell (HUVEC) isolation and culture

Primary HUVECs were extracted from human umbilical veins by collagenase digestion and cultured in HUVE media as previously described (Litwin et al., 1997; Wall et al., 1978) and were used after no more than two passages.

Flow cytometric analysis of cell surface protein expression

EXnaEFCs were analysed for cell surface expression of various markers by flow cytometry. Cells were treated with 10 μl Human FcR block (Miltenyi Biotec) diluted in 30 μl of HUVE media. They were then incubated with a panel of mouse anti-human fluorochrome-conjugated antibodies against progenitor markers CD34, CD117, and CD133, vascular markers CD31, CD144, and CD146 and VEGFR2, hematopoietic markers CD14, CD38, and CD45 and IL3RA or appropriate isotype controls (all BD Bioscience, except anti-CD133 is Miltenyi Biotec) for 30 min at 4 °C. Cells were washed in PBS and 7AAD (BD Biosciences) was added prior to cell analysis on an Accuri flow cytometer (BD Biosciences) to determine viability. Data was analysed using FCS Express 4 Flow Cytometry: Research Edition (De Novo Software, Los Angeles, CA).

Up-take of acetylated-low density lipoprotein and *U. europaeus* lectin binding

Cells were incubated at 37 °C for 4 h with 10 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine per-chlorate acetylated-low density lipoprotein (DiI-Ac-LDL; Biomedical Technologies, Stoughton, MA, USA) and 10 μg/ml of FITC bound *U. europaeus* agglutinin 1 lectin (UEA, Sigma-Aldrich). Incorporation of DiI-Ac-LDL and binding of FITC-UEA-1 was assessed using flow cytometry and compared to unlabelled control cells.

Immunoblotting

Cells expanded for 10 days were starved of growth factors for 5 h prior to stimulation without or with IL3 (50 ng/ml, in house) for 10 min prior to being lysed in PLCLB lysis buffer (150 mM NaCl, 5% glycerol, 1% Triton X-100, 1.5 M MgCl2, 50 mM HEPES, pH 7.5) supplemented with 1 mM sodium orthovanadate, 2 mM phenylmethylsulphonyl fluo-ride (PMSF) and protein electrophoresed in SDS-PAGE Criterion gels (5–20% gradient, Bio-Rad, Gladesville, NSW, Aust.). Protein expression was detected using the following antibodies; anti-phosphorylated ERK1/2 (Cell Signaling, Danvers, MA, USA) and anti-β actin (Sigma-Aldrich) probed the membrane overnight at 4 °C followed by secondary antibody incubation at room temperature (RT) for 1 h prior to visualisation by ECL (GE Health Science, Piscataway, NJ, USA) and a luminescent image analyser (LAS4000, Fujifilm, Stamford, CT, USA).

Assessment of immunogenicity of EXnaEFCs

Donor matched EXnaEFCs and HUVECs were isolated (as above) from a single cord and cultured for one week. As a positive control donor matched mature dendritic cells (DCs) were produced as previously described (Rojas-Canales et al., 2012) with mature DCs (mDCs) confirmed by flow cytometry utilising antibodies against HLA-DR, CD11c, CD14, CD80, CD83, CD86 (all BD Biosciences) and CD209 (Miltenyi Biotech).

Donor matched HUVECs, EXnaEFCs, mDCs and unmatched bone marrow-derived mesenchymal stem cells (MSC, Millipore) were labelled with mouse anti-human antibodies against MHC class I (clone FMC16), MHC class II (FMC52) or the isotype control (X63), all gifts from H Zola (formerly Flinders Medical Centre, Adelaide, South Australia). Following incubation (30 min, 4 °C), cells were washed in medium and labelled with goat anti-mouse Dylight650 secondary antibody (1/100 dilution in medium, Abcam) for 30 min at 4 °C. Cells were washed, labelled with 7AAD, washed again and then analysed by FACS.

In selected experiments EXnaEFCs, HUVECs and MSCs were cultured with 10 ng/ml of human TNF or IFNγ (R&D Systems) for 48 h prior to antibody labelling and FACS analysis.

In vitro Matrigel™ tube formation assay

In vitro tube formation of HUVECs and EXnaEFCs was assessed. HUVECs were stained with 10 μg/ml DiI-Ac-LDL for 4 h, washed once and incubated overnight under cell culture conditions. EXnaEFCs were stained with 10 μM Calcein-AM (eBioscience, San Diego, CA) for 20 min prior to use. Matrigel™ (10 μl, BD Biosciences) was pipetted into the wells of an angiogenesis μ-slide (ibidi GmbH, Martinsried, Germany) and set for 30 min at 37 °C prior to labelled HUVECs (1×10^4 or 2×10^4 cells per well) without or with EXnaEFCs (1×10^4 cells per well). Tube formation was monitored regularly and seven overlapping phase contrast images were captured (inverted IX70 microscope 4×/0.13NA objective, an S15 F view camera and Analysis Life Sciences software (Olympus, Tokyo Japan)) after 6 h of tube formation. Overlapping images were 'stitched' together using Adobe Photoshop (Adobe Systems, San Jose, CA) and the number of tubes and branches was quantified manually on blinded images using ImageJ 1.47 (National Institute of Health, Bethesda, MD). Fluorescent images were captured on IX71 microscope (Olympus) with 10×/0.4NA objective and

a Hamamatsu Orca-ER camera using the Analysis Life Sciences software.

In vivo Matrigel™ plug tube formation assay

CD133+ EXnaEFCs were expanded for 6–8 days prior to 5×10^5 EXnaEFCs being mixed with 500 μl of high concentration Matrigel™ (BD Biosciences) containing 2 μg/ml basic fibroblast growth factor (R&D Systems) and 50 units/ml heparin (Sigma-Aldrich) and injected subcutaneously into one flank of a 6–8 week old female NOD/SCID mouse. The opposite flank received a control high concentration Matrigel™ plug containing no cells. Mice were humanely killed by cervical dislocation on day 14 post-injection and plugs were removed, washed in PBS, fixed in 4% paraformaldehyde (VWR International, Radnor, PA) overnight, embedded in paraffin, cut into 8 μM sections on a microtome (RM 2235 Leica, Solms, Germany) and placed onto slides (Polysine®, Menzel-Gläser, Thermo Scientific). The sections were investigated for cells expressing CD31 (1:1000, goat anti-mouse/human CD31, Santa Cruz, Dallas, TX) or human mitochondria (MTC02, Abcam) using biotinylated rabbit anti-goat secondary (Abcam) diluted 1:500 with PBS + 3% normal human serum (NHS, Life Technologies, Carlsbad, CA) or biotinylated goat anti-mouse secondary (Vector Labs) used at 1/250 in PBS/3% NHS. Sections were treated with Vectastain elite ABC regent (Vector Labs, Burlingame, CA) for DAB staining as per manufacturer's instructions, followed by haematoxylin and eosin staining. Negative controls included isotype matched irrelevant antibodies as well as secondary antibody alone. Images of slides were collected using a Hamamatsu Nanozoomer slide scanner with CD31+ vessels counted manually and section areas were quantified using the NDPview software (Hamamatsu Photonics, Hamamatsu, Japan).

Acute myocardial infarction in CBH-Rnu rats

CBH-Rnu male rats were anaesthetised using isoflurane (1% in 3–4 l O_2/min, Bomac Laboratories, Sydney, Australia) and mechanically ventilated (tidal volume 3.6 ml, ventilatory rate 60 breaths/min) (Inspira ASV Ventilator (Harvard Apparatus, Massachusetts, USA)). Once ventilated, rats underwent a permanent surgical ligation of the left anterior descending coronary artery to induce acute myocardial infarction (AMI). Rats then received either 100 μl PBS or 1×10^6 EXnaEFCs (expanded for 6–8 days) in 100 μl PBS by transepicardial injection directly below the ligation site before the wound was closed in 3 layers. Rats were allowed to recover on 100% oxygen and received Carprofen (5 mg/kg) and Norocillin (10 mg/kg, both Norbrook Laboratories, Melbourne, Australia) for pain and bacterial management. Control rats did not undergo surgery. Following a cardiac magnetic resonance image (MRI) at 7 days post-AMI, blood was collected from tail veins for serum creatinine detection by a colorimetric test at SA Pathology (Adelaide, Australia) before humane killing. Hearts were collected for histology and RNA isolation.

Cardiac magnetic resonance imaging (MRI)

Cardiac MRI was performed on rats using a 1.5 T MR system (Magnetom Sonata, Siemens, Germany). Cardiac MRI was performed on rats prior to AMI and one week post-AMI.

Animals were placed supine recumbent in the isocentre of the magnet with a human carotid radiofrequency coil placed over the thorax. Anaesthesia was maintained by isoflurane. Two electrocardiography (ECG) electrodes were attached to the thorax thereby generating a vector ECG. Accordingly, all MRI images were free breathing, ECG-gated, acquisitions. Transverse and coronal localiser images were acquired followed by short axis pilot images, from which a true short axis stack was prescribed. TrueFISP (Fast Imaging with Steady-State Precession) cine images (gated to alternate R waves) were acquired. The stack comprised three contiguous left ventricular slices (each 3 mm thick, with no intersection gap) providing almost complete coverage of the left ventricle. The image matrix was 384 × 384, field of view 185 mm, repetition time 14.72 ms, echo time 1.55 ms, flip angle 90°, and 20 heart phases were acquired. The average scan time per animal was 10 min.

MRI image analysis

Left ventricular volumes and derived ejection fraction (EF) were measured off-line from cine images using commercially available software (QMASS v7.2, Medis, Netherlands). Papillary muscles were excluded from calculations. The end-diastolic (ED) and end-systolic (ES) cine frames were identified for each slice and the endocardial and epicardial borders were manually traced. The end-diastolic (EDV) and end-systolic volumes (ESV) were then calculated using the true disk summation technique (sum of cavity volumes across all continuous slices), as previously described (Teo et al., 2008).

PCR and immunohistochemistry of heart tissue collected from AMI rats

Quantification of mRNA levels was carried out using qPCR. Primers designed for rat genes using Primer Blast (National Institute of Health) and purchased from GeneWorks (Adelaide, Australia). Primers were designed to span an intron/exon border to avoid genomic DNA amplification (Table 1). qPCR amplification was performed using QuantiTect™ SYBR Green master mix (Qiagen) on a Rotor-Gene thermocycler (Corbett Research, Mortlake, Australia) with reaction parameters: 15 min at 95 °C, then cycling of 10 s 95 °C, 20 s 55 °C and 30 s 72 °C; for 45 cycles followed by a melt phase. Data obtained was analysed using Rotor-Gene Analysis Software version 6 (Corbett Research). Relative gene expression levels were calculated by normalising to the rat house-keeping genes β-actin, CycA, HPRT and YWAHZ using geNorm software (Vandesompele et al., 2002).

Rat hearts were harvested, fixed in 4% (v/v) buffered formalin at 4 °C overnight and then embedded in OCT compound and frozen at −80 °C. Human placenta was used as a positive control. Tissues sectioned to 5-μm-thick were stained with anti-human IL3Rβ (CSF2RB: clone 9F5, 3.3 μl/ml (Sun et al., 1996)) or an isotype matched negative control and biotinylated goat anti-mouse IgG (2 μl/ml, BD Biosciences) employing VectorStain ABC kit as the detection system (Vector Laboratories, Burlingame, CA, USA). Counterstaining was performed using Mayer's haematoxylin (Dako Agilent Technologies, Glostrup, Denmark). Analysis was

Table 1   Rat primers used in PCR analysis of gene expression in AMI cardiac tissue.

Rat gene	Forward and reverse prime sequence
Tnf	F-5′*AAAGCATGATCCGAGATGTG3′*
	R-5′*AGCAGGAATGAGAAGAGGCT3′*
Sdf1	F-5′*CCGATTCTTTGAGAGCCATGT3′*
	R-5′*CAATGCACACTTGTCTGTTGTT3′*
Cx3cl1	F-5′*GCAGCTTTCCGCTCTGAATA3′*
	R-5′*CTGTTCTGAGCTTCCACCTATC3′*
Cd14	F-5′*GGAAAGAAACTGAAGCCTTTCTC3′*
	R-5′*AGCAACAAGCCGAGCATAA3′*
Ccl2	F-5′*GTCTCAGCCAGATGCAGTTAAT3′*
	R-5′*CTGCTGGTGATTCTCTTGTAGTT3′*
Mmp9	F-5′*CAACCTCACGGACACACA3′*,
	R-5′*CTGCTTCTCTCCCATCATCTG3′*
β-actin	F-5′*CTTCCTTCCTGGGTATGGAATC3′*,
	R-5′*CTGTGTTGGCATAGAGGTCTT3′*
CycA	F-5′*GGCTATAAGGGTTCCTCCTTTC3′*
	R-5′*TTGCCACCAGTGCCATTA3′*
HPRT	F-5′*GACCTCTCGAAGTGTTGGATAC3′*
	R-5′*TCAAATCCCTGAAGTGCTCAT3′*
YWAHZ	F-5′*GACATCTGCAACGACGTACT3′*,
	R-5′*CGGTAGTAGTCACCCTTCATTT3′*

performed using a BX45 microscope, a XC10 camera and Analysis Life Sciences software (Olympus).

Hematopoietic colony formation assay

UCB isolated CD133⁺ EXnaEFCs expanded without and with IL3 for 7 days were plated in methylcellulose with a 10× cell solution (200 μl, IMDM with 2% FCS) mixed with 2 ml of MethoCult (Stem Cell Technologies) containing erythropoietin (EPO; 3 U/ml), granulocyte macrophage colony-stimulating factor (GM-CSF; 20 ng/ml), stem cell factor (SCF; 50 ng/ml) and interleukin 3 (IL-3, 10 ng/ml). The cell/MethoCult mix (0.37 ml) was added to 24 well plate wells at 1670 cells/well. Assays were incubated at 37 °C, 5% CO_2 and haematopoietic colonies were scored after 14 days.

Statistical analysis

Results are generally expressed as mean ± standard error of the mean (SEM). An unpaired or paired Student *T*-test, 1- or 2-way ANOVA for multiple comparisons was performed to determine statistical significance between groups with p values < 0.05 considered significant.

Results

Interleukin-3 greatly expands CD133⁺ non-adherent endothelial forming cells

CD133⁺ cells were isolated from umbilical cord blood *via* magnetic sorting and cultured in CellGro SCGM media supplemented with TPO, Flt3L and SCF (complete CellGro media). Following 6−7 days of culture naEFCs expanded

14.6 ± 4.7 fold and by 12−13 days this rose further to 98.6 ± 35.4 fold. To clearly distinguish these expanded cells from our previously published 'four day enriched naEFCs' we have named the expanded population 'EXnaEFCs'. Because IL3 and placental growth factor (PlGF) have been shown to have pro-angiogenic properties (Autiero et al., 2003; Li et al., 2003; Li et al., 2006), they were also tested here. We used 20 ng/ml and 25 ng/ml, respectively, as concentrations that saturate cell surface receptors. As shown in Fig. 1A, the inclusion of IL3 into the medium significantly enhanced EXnaEFC expansion both at days 6−7 (Fig. 1Ai) and 12−13 (Fig. 1Aii) when compared to donor matched cells grown in the 'complete CellGro' medium alone. Notably, by days 12−13 of culture the mean fold increase of EXnaEFCs in medium containing IL3 was ~ 350-fold.

Addition of PlGF did not increase the expansion rate of EXnaEFCs at either days 6−7 or 12−13 of culture. Furthermore, co-culture of PlGF and IL3 did not increase the expansion rate beyond that seen with IL3 alone. These data suggest that the addition of PlGF to the EXnaEFC expansion media had no effect but identified a specific effect of IL3 (Fig. 1A). Furthermore, cell viability remained high (~ 90%) across all groups within 6−7 days of cell culture (Fig. 1Aiii) and fell slightly thereafter at days 12−13 but did not differ significantly between groups (Fig. 1Aiv).

To determine if CD133⁺ EXnaEFCs could be successfully expanded post-freeze/thaw, EXnaEFCs were expanded in CellGro medium either directly after isolation from cord blood or following freezing, liquid nitrogen storage and thawing. Over the course of 14 days the expansion rates (Fig. 1Bi) and viability (Fig. 1Bii) of both fresh and frozen expanded cells were similar.

Expansion of EXnaEFCs maintains its pro-vascular profile in the presence of IL3

We next used flow cytometric analysis to check for possible variations in EXnaEFC surface profile during expansion. As shown in Fig. 2, after nine days of cell expansion (without or with IL3) the EXnaEFCs retained expression of the progenitor cell markers CD34, CD117 and CD133. Further phenotyping revealed that VEGFR2 and CD31 were expressed by the EXnaEFCs, while CD144 and CD146 (both mature EC markers) were not detectable (Fig. 2). The CD133⁺ EXnaEFCs revealed a relatively low but detectable level of CD45 and IL3RA at the cell surface but not CD38 or CD14. These cells were also capable of taking up acetylated-low density lipoprotein (Ac-LDL) and binding *U. europaeus* lectin (UEA-1) (Fig. 2), both features of ECs (Timmermans et al., 2009).

Addition of IL3 did not alter the cell surface expression of any progenitor cell, vascular cell or leukocyte markers (Fig. 2). Similarly, expression of the IL3RA, uptake of Ac-LDL and binding of UEA-1 lectin were all unchanged by the presence of IL3. This suggests that IL3-induced increase in EXnaEFC numbers is likely due to an expansion of a homogenous population rather than selective skewing of a sub-population. Investigation into the cellular mechanisms underpinning this proliferation suggests that the MAPK pathway is involved with a significant increase in activated (ie phosphorylated) ERK1/2 identified in EXnaEFCs with temporary cell starvation of growth factors (5 h) prior to
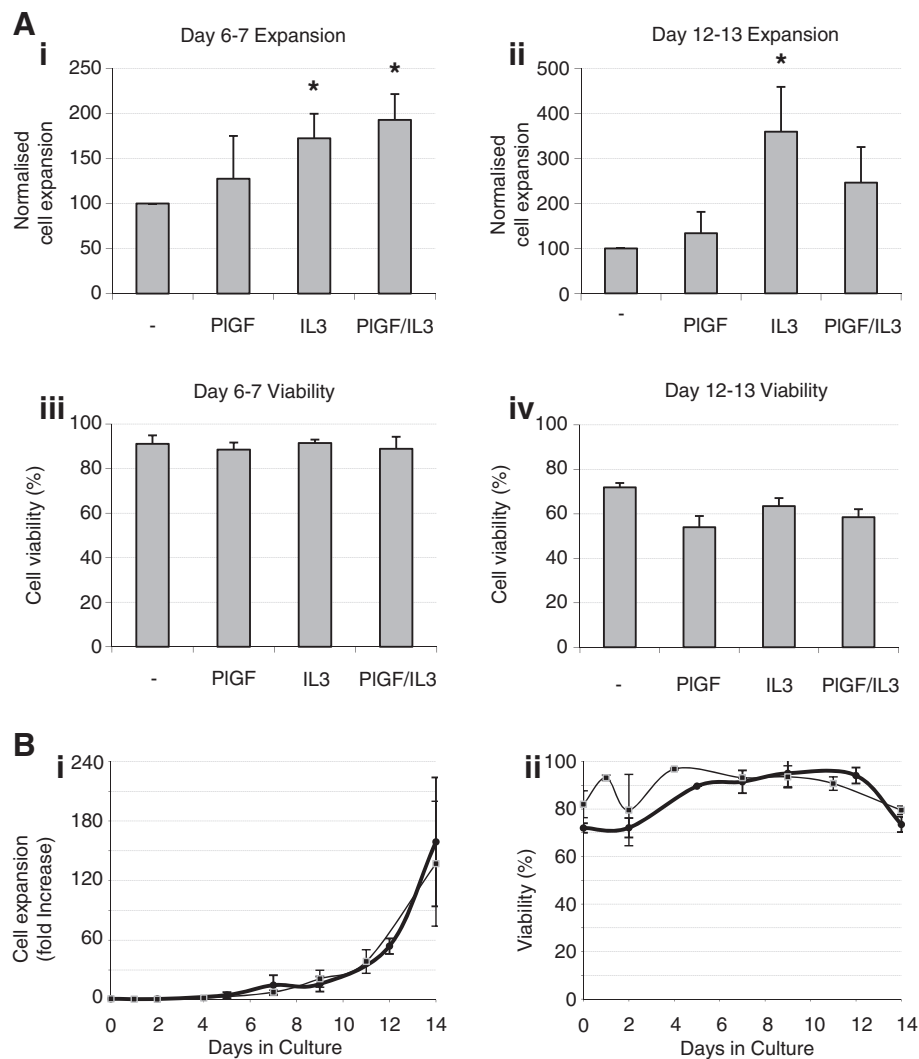
Figure 1 CD133^{+} naEFCs expansion is enhanced by IL3 and is not affected by freeze/thaw. CD133^{+} naEFCs isolated from a single donor were split and cultured under four different conditions; media alone (−), media + PlGF, media + IL3 and media + PlGF + IL3. (A) Cell proliferation (i and ii) and cell viability (iii and iv) in different growth conditions were assessed after 6−7 days of expansion (i and iii) and 12−13 days of expansion (ii and iv). Data is shown as mean ± SEM, with proliferation data expressed as the fold change from the starting number of CD133^{+} naEFCs normalised to media alone expansion, , n = 5−8, *p < 0.05 *versus* media alone. (B) To determine the ability of CD133^{+} naEFCs to be expanded after being frozen and thawed naEFCs were isolated from cord blood based on CD133 expression and expanded in media (thick black line) or frozen down in 90% FCS/10% DSMO and placed into liquid nitrogen for at least one week before thawing and expanding (thin black line). Cell expansion (i) and viability (ii) were recorded for 14 days, graphs show mean ± SEM, n = 3.

IL3 stimulation for 10 min (Fig. 2B). The activation of ERK1/2 was observed in cells expanded both without and with IL3.

EXnaEFCs exhibit low immunogenicity

As immunological rejection of cells represents a major hurdle for transplantation, the immunogenicity of CD133-isolated and EXnaEFCs is of key importance for their use in allogeneic cell therapy. Herein we examined MHC class I and class II expression on EXnaEFCs and compared them to donor-matched HUVECs and mature dendritic cells (mDCs), as well as unmatched mesenchymal stem cells (MSCs). When data is expressed as a

fold change in mean fluorescence intensity (MFI) compared to the isotype control, EXnaEFCs, HUVECs and MSCs all had very similar levels of MHC class I expression, while the positive control, mDCs, had significantly higher levels (Fig. 3A). However, when the MFI fold change of each HUVEC and mDC line is normalised to its donor-matched EXnaEFCs, HUVECs express less and mDCs express more MHC class I compared to EXnaEFCs (Fig. 3C). For MHC class II, data expressed as a fold change in MFI compared to the isotype control (Fig. 3B) shows EXnaEFCs expressing higher levels of class II than both HUVECs and MSCs, but significantly less than the mDCs. Similarly, when the MFI fold change of HUVECs and mDCs is normalised to its donor-matched EXnaEFCs, HUVECs express less, while mDCs express more, MHC class II compared to EXnaEFCs (Fig. 3D).
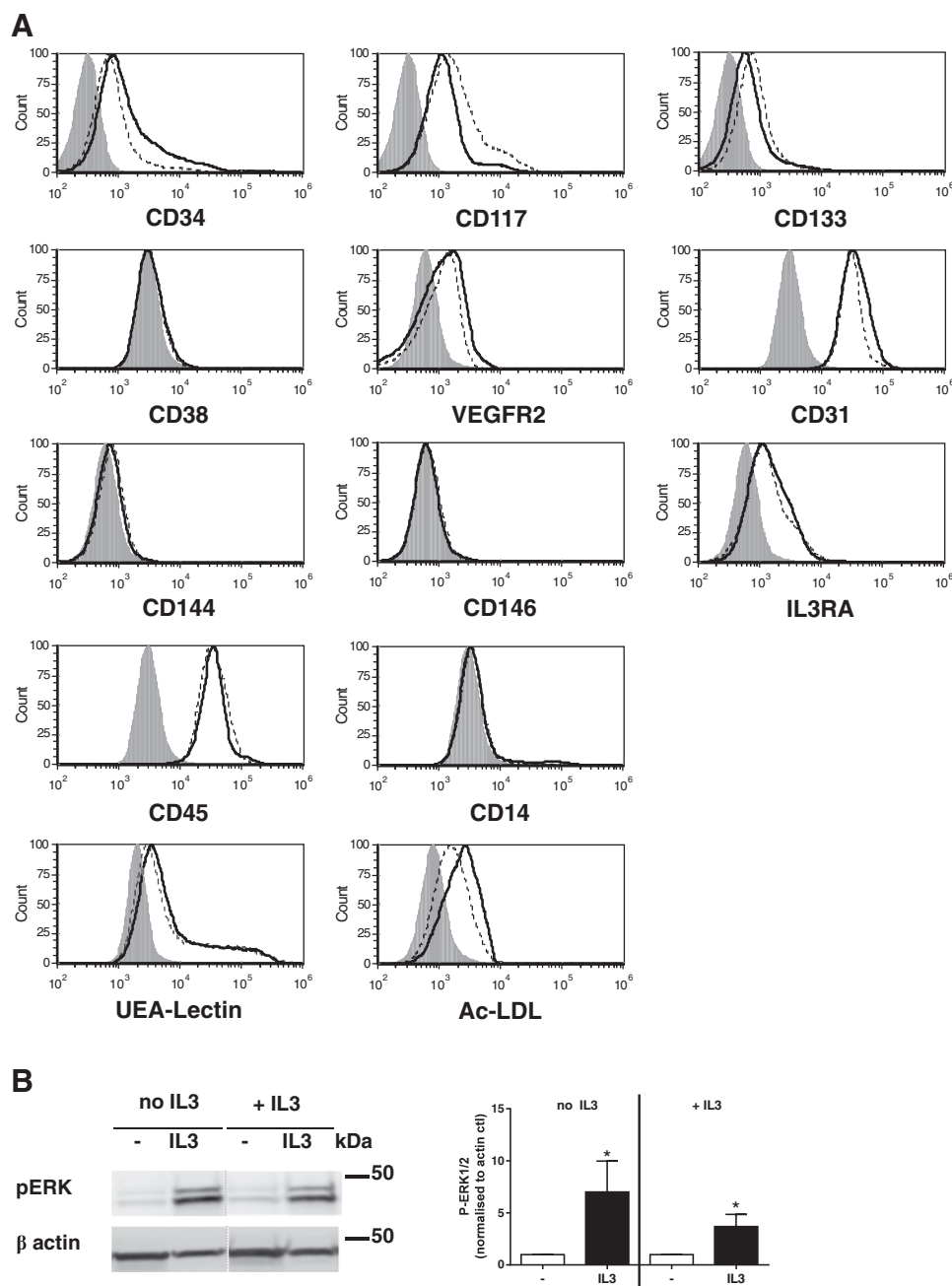
Figure 2 IL3 supports a vascular profile for expanded CD133+ naEFCs and activates ERK1/2. (A), CD133+ isolated naEFCs were expanded in CellGro media with IL3 (dashed grey line) or without IL3 (solid black line). Solid grey peak represents negative control. Cells were labelled with fluorescently tagged antibodies targeting hempoietic progenitor, endothelial, leukocyte and monocyte cell marker. The ability of cells to take up Dil-Ac-LDL and FITC-labelled UEA-1 lectin were also assessed by flow cytometry. Representative donor cell line on day 9 of expansion, n = 4. (B), EXnaEFCs expanded without or with IL3 for 10 days prior to growth factor starvation (5 h) then without or with IL3 stimulation for 10 min. Cell lysates blotted for phosphorylated ERK1/2 or β actin loading control (left panel). Quantified data expressed as mean band intensity ± sem, n = 4, *p < 0.05 *versus* untreated (right panel).

As *de novo* expression of MHC I and MHC II occurs in response to TNF and IFNγ, respectively (Collins et al., 1986; Pober et al., 1983), we next examined induction of MHC molecules on EXnaEFCs, HUVECs and MSCs in response to these pro-inflammatory cytokines. Each cell type cultured with cytokine for 48 h was normalised to untreated controls.

As shown in Fig. 3E, MHC class I was significantly increased in EXnaEFCs exposed to TNF and there was a trend towards an increase in response to IFNγ. HUVECs also increased MHC class I in response to TNF and IFNγ. To note, the extent of the increase appeared less in EXnaEFCs when compared to HUVECs. Cytokines did not alter the expression of MHC class I
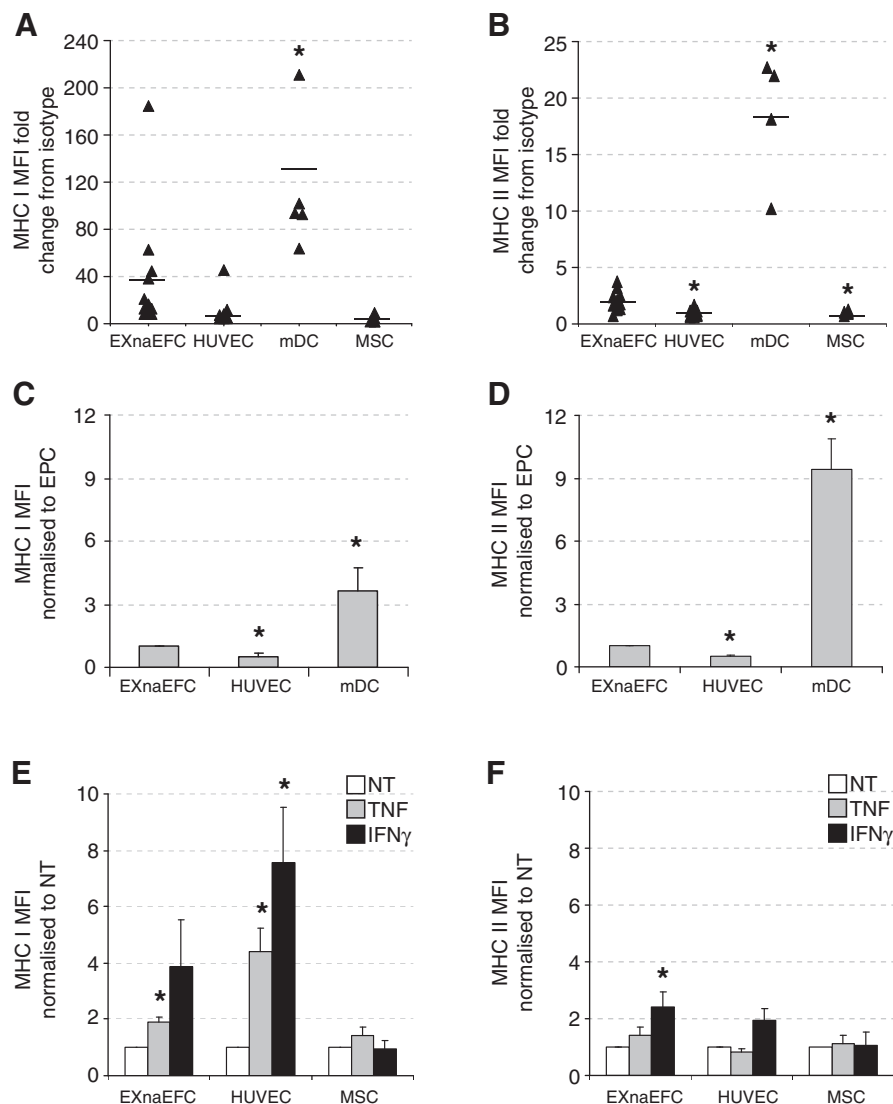
Figure 3 EXnaEFCs exhibit low immunogenicity. Donor matched EXnaEFCs, HUVECs and mDCs were isolated from umbilical cords. After 7 days in culture cells were labelled with antibodies against MHC class I (A,C,E), MHC class II (B,D,F) or isotype control. (A,B) The fold change in mean fluorescence intensity (MFI) compared to the isotype control was calculated for each cell type from each donor, in addition non-donor matched MSCs were used as a comparator cell. Each triangle represents one data point, the line represents the mean. (C,D) The expression of MHC on each HUVEC and mDC was normalised to the donor matched EXnaEFCs, n = 4–9. (E,F) Donor matched EXnaEFCs and HUVECs and unmatched MSCs were cultured with 10 ng/ml of TNF (grey bars) or IFNγ (black bars) for 48 h prior to antibody labelling, the change in MHC expression was normalised to the respective cell type grown in the absence of cytokine (no treatment (NT), white bars), n = 3–6. Bar histograms show mean ± SEM. *p < 0.05 *versus* naEFC (A–D) or NT cells (E–F).

on MSCs (Fig. 3E). As shown in Fig. 3F, TNF failed to increase the expression of MHC II on all cells tested and IFNγ marginally increased MHC II expression on EXnaEFCs only.

EXnaEFCs are pro-angiogenic

A key feature of mature ECs and some progenitor cells, such as ECFCs, is their ability to form tube-like structures on Matrigel™ *in vitro*. We first investigated the ability of EXnaEFCs alone to form tube-like structures on Matrigel™; these cells retained their round appearance and did not form any tube-like structures over a 12 h time course,

irrespective of IL3 exposure (data not shown). To further investigate the pro-angiogenic potential of these cells we cultured 1 × 10⁴ EXnaEFCs with an equivalent number of HUVECs (ie 1 × 10⁴ cells). Control wells contained HUVECs alone either at 1 × 10⁴ or 2 × 10⁴ cells/well. Each donor HUVEC line was performed in triplicate, with tube structures and branching points (branches) counted manually at 6 h and normalised to 2 × 10⁴ HUVECs.

We observed that wells containing 1 × 10⁴ HUVECs formed fewer tubes and branch points than wells containing 2 × 10⁴ HUVECs. Wells containing co-cultures of 1 × 10⁴ HUVECs and 1 × 10⁴ EXnaEFCs (expanded without or with IL3) formed the same number of tubes and branch points as

the wells containing 2×10^4 HUVECs and significantly more tubes and branch points than the wells containing 1×10^4 HUVECs alone (Fig. 4A). This suggests that the EXnaEFCs are pro-angiogeneic *via* their contribution to tube structure formation *in vitro*. Notably, tube structure formation was maintained by EXnaEFCs expanded with IL3 (Fig. 4A).

To investigate the physical association between the EXnaEFCs and HUVECs in the aforementioned experiments we labelled the EXnaEFCs with Calcein-AM labelled and the HUVECs with DiI-Ac-LDL labelled and undertook immunofluorescence microscopy. As shown in Fig. 4B the EXnaEFCs (green) are not randomly scattered throughout the well, but are in close proximity to the HUVEC (red) tubes.

Previously we have shown that the day four enriched naEFCs are unable to form tube structures by themselves *in vitro*, but do form lumenised vessels *in vivo* (Appleby et al.,

2012). Thus, we next tested the ability of EXnaEFCs to contribute to vessel formation *in vivo* by injecting Matrigel™ plugs containing EXnaEFCs (and control plugs containing no cells) into each flank of NOD/SCID mice. Plugs were removed 14 days following insertion, paraffin embedded, sectioned and stained with anti-mouse/human CD31 or MTC02, a human specific mitochondrial antibody. As shown in Figs. 5A and B, the CD31+ EXnaEFCs are clearly visible only within the plug in which they were seeded. Notably, while we were unable to observe EXnaEFCs forming CD31+ vessels containing erythrocytes within these sections, the EXnaEFCs did cluster together within the Matrigel™ and confirmed to be of human origin as they bound MTC02, an antibody targeting the human mitochondria (Fig. 5C). Further investigation of these plugs revealed that the surrounding connective tissue contained CD31+ vascular structures which contained
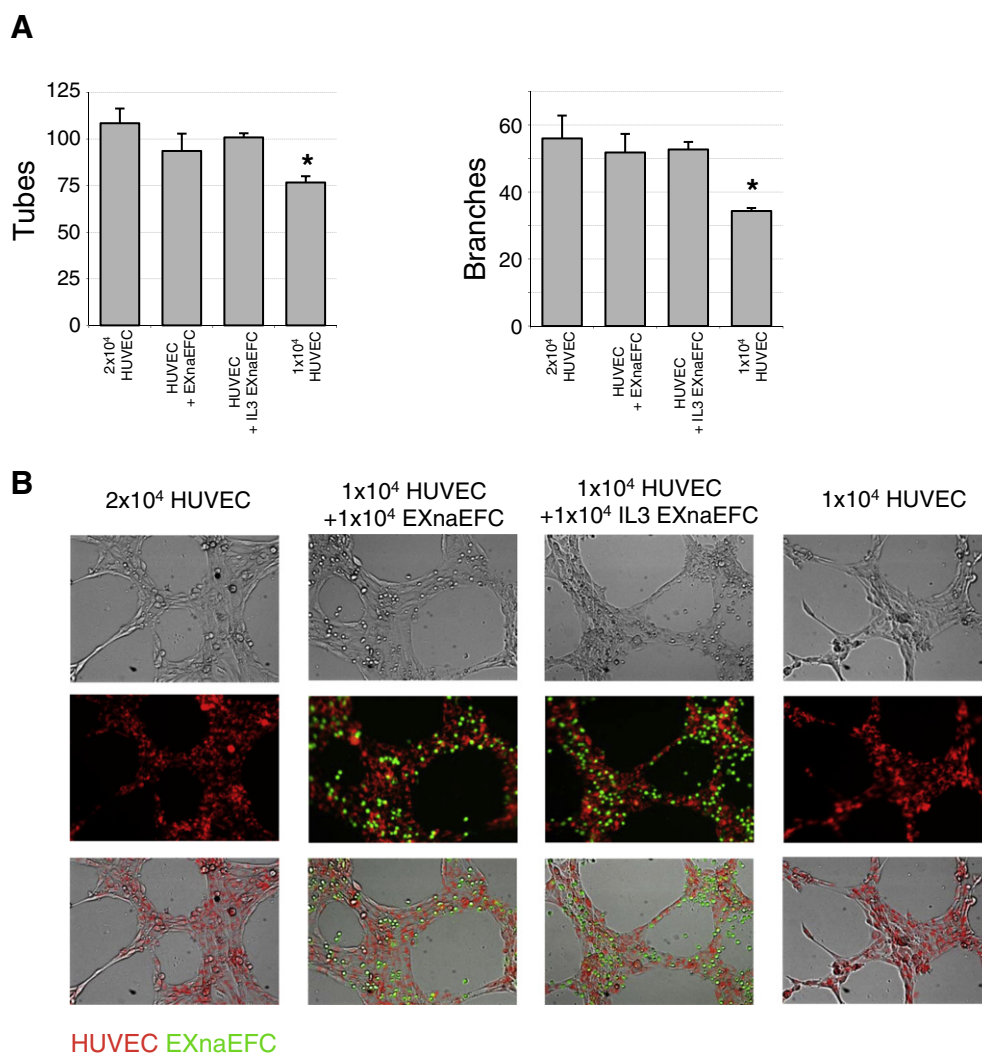

Figure 4 EXnaEFCs contribute to tube formation *in vitro*. To determine the ability of CD133+ EXnaEFCs to contribute to endothelial tube formation, mature HUVECs were cultured *in vitro* on Matrigel™ for 6 h alone or with EXnaEFCs expanded in the presence or absence of IL3. (A) The number of tubes and branches was quantified from 3 experimental replicates from 5 different biological donors. Data expressed as mean ± SEM with *p < 0.05 *versus* 2×10^4 HUVECs alone. (B) Representative images of tube formation after 6 h in culture, with HUVECs labelled with DiI-Ac-LDL (red) and EXnaEFCs with calcein AM (green), bright field, fluorescent and merged images are shown.

erythrocytes (Fig. 5E) with serial sections confirming CD31 expressing vessels when compared to the negative isotype control (Fig. 5D). Utilising the human-specific mitochondrial antibody (MTC02) we identified that these CD31$^+$ vascular structures were not of human origin (Figs. F–G). Of more interest, we observed that sections of plugs containing the
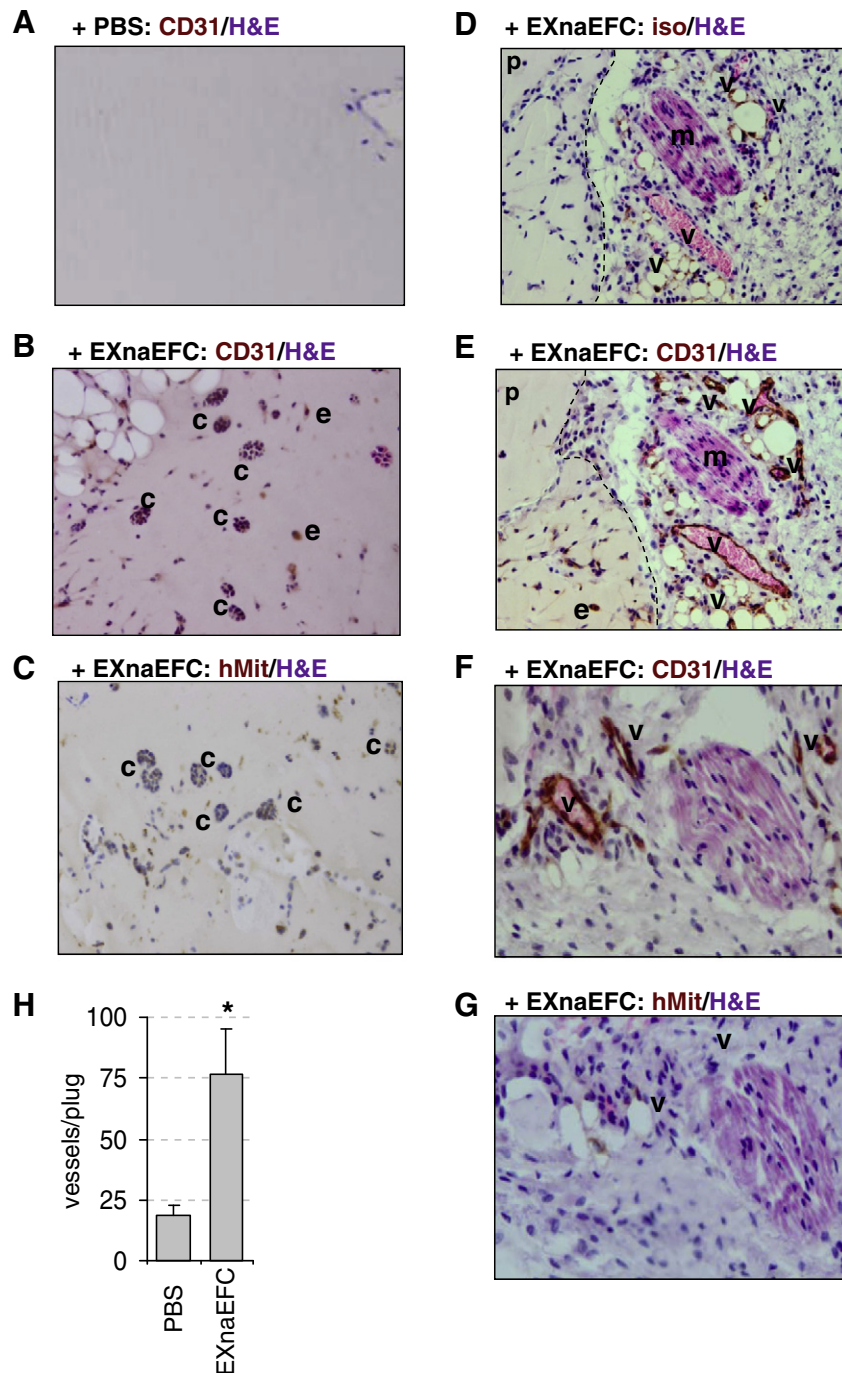

Figure 5   EXnaEFCs are pro-angiogenic *in vivo*. Matrigel™ plugs containing EXnaEFCs or PBS alone were injected into each flank of a NOD/SCID mouse and removed after 13 days. Paraffin embedded plugs were sectioned and stained for mouse/human CD31 or human specific mitochondria (hMit) and counter stained for H&E. (A) Shows PBS containing plugs stained for CD31 and H&E; (B) shows EXnaEFC contained plugs stained for CD31, and (C) shows EXnaEFC contained plugs stained for human mitochondria. (D) The plug–tissue boundary (indicated by the dashed line) with the CD31 (iso) negative control and (E) a serial section of the above stained for CD31$^+$ and showing erythrocyte contained CD31$^+$ vessels, (F) human mitochondria staining of tissue surrounding the plug, (G) serial section of the above with CD31$^+$ staining of vessels in tissue surrounding the plug. c = stain positive cluster, e = single EXnaEFC, m = muscle, p = plug, v = vessel. (H) The number of CD31$^+$ vessels in the tissue surrounding the Matrigel™ plug was quantified per whole plug, mean ± SEM, n = 4, *p < 0.05 *versus* PBS.

EXnaEFCs demonstrated a significant increase in the number of CD31^{+} vasculature structures in the surrounding tissue compared to the controls (Fig. 5H).

Expanded naEFCs ameliorate acute myocardial infarction in rats

To investigate the therapeutic potential of human EXnaEFCs, CBH-Rnu male rats were used in a model of acute myocardial infarction (AMI) as their immunodeficiency provides tolerance to xenografted cells. Briefly, rats underwent a surgical ligation of the left anterior descending coronary artery to induce myocardial infarction prior to receiving either PBS or EXnaEFCs injected transepicardially below the ligation site. Rats were analysed by cardiac MRI pre-surgery and seven days post-surgery to measure changes in the left ventricular mass (LVM) and ejection fraction (EF). Blood was also collected to determine levels of serum creatinine as well as the hearts for gene expression and histology.

We first assessed the presence of transplanted human cells in host myocardium by immunochemistry. As human IL3 was used to expand the cells and this growth factor (and its receptor) has very low homology between human and mouse and thus reagents are not cross-reactive (Hayashida et al., 1990) we used an anti-human IL3Rβc antibody to specifically identify the human EXnaEFCs in the transplanted areas in the rats. Fig. 6A shows in AMI rats treated with PBS alone that neither an isotype control IgG (Fig. 6A, top left panel) nor the anti-IL-3Rβc (Fig. 6A, top middle panel) could detect human cells. In contrast, in AMI rats + EXnaEFCs IL3Rβc expressing cells were identifiable at the site of injection (Fig. 6A, bottom left panel). To note, in the same rat heart, but distal to the EXnaEFC injection site no IL3Rβc expressing cells were detectable (Fig. 6A, bottom middle panel). Human placental tissue was used as a positive control (Fig. 6A, right top and bottom panels). Interestingly, while we did not observe the integration of human EXnaEFCs into the vasculature of the myocardium, the expression pattern of these cells in the rat heart was strikingly similar to that observed in the human placental tissue and suggestive of a pro-angiogenic cell exuding its effects *via* a paracrine function (Fig. 6A, enlarged images).

Fig. 6B shows that compared to the pre-AMI group, rats administered PBS or EXnaEFCs exhibited a significant reduction in ejection fraction. However, the cell therapy group exhibited a 5% improvement of ejection fraction when compared to the PBS alone group. Delivery of EXnaEFCs did not improve LVM (data not shown). Creatine phosphate in the muscle is broken down into creatinine that enters the blood stream. While this is a constant process within the body, high levels of serum creatinine are linked to cardiovascular distress. AMI significantly increased serum creatinine levels in rats treated with PBS. Strikingly however, delivery of EXnaEFCs returned creatinine to similar levels to pre-AMI rats (Fig. 6B).

Expanded naEFCs promote gene expression for repair of AMI

Cardiac tissue was collected and qPCR was utilised to measure endogenous rat mRNA of known AMI affected genes; *Tnf*, *Sdf1*, *Cd14*, *Cx3cl1*, *Ccl2* and *Mmp9*. Expression was normalised to housekeeper genes (*β-actin*, *CycA*, *Hprt* and *Ywahz*) and control rats which had not undergone surgery. As shown in Fig. 7, administration of EXnaEFCs rescued the hearts from AMI-induced (i) suppression of *Tnf*, *Sdf1* and *Cd14* and (ii) increase expression of *Cx3cl1*. To note, the cell therapy group exhibited increased gene expression of *ccl2* and *Mmp9* when compared to both control rats and those treated with PBS immediately post-AMI (Fig. 7).

Multipotency of EXnaEFCs

Asahara recently demonstrated that a single HUCB derived CD133^{+} cell could give rise to cells of either the hematopoietic or endothelial lineage (Masuda et al., 2011). Using a similar methylcellulose hematopoietic colony formation assay we executed a fate analysis of EXnaEFCs expanded without or with IL3. As shown in Table 2, when the EXnaEFCs were cultured with GM-CSF, SCF, IL3 and EPO for 14 days a variety of cell types were detected including early progenitors of both erythrocyte and myeloid lineages (CFU-GEMM), the erythrocyte lineage (BFU-E), the granulocyte/monocyte progenitor lineages CFU-GM, CFU-G and CFU-M. Also shown in Table 2, a comparison of naEFCs expanded without and with IL3 which suggests a similarity between these cells with respect to the number and type of colonies formed.

Discussion

The ability to repair and generate blood vessels is critical to improve outcomes in a number of diseases including cardiovascular disease and diabetes. Endothelial progenitor cells contribute to blood vessel formation and vascular repair in animal models of ischemia (reviewed in (Timmermans et al., 2009; Yoder, 2012)) but human trials have yielded moderate success (Lipinski et al., 2007; Martin-Rendon et al., 2008).

Human EPCs have been isolated by a variety of means that essentially fall into two broad categories, 1) cell culture conditions to enrich for EPC colonies (Hill et al., 2003; Ito et al., 1999; Kalka et al., 2000) and 2) the isolation of cells based on surface marker expression *e.g.* CD34 or CD133 (Appleby et al., 2012; Bompais et al., 2004). These methods warrant refining as they (i) lack the specificity of EPC selection and (ii) are commercially prohibitive based on high cell culture costs and requiring ~5 weeks of culture to reach an ECFC number sufficient for therapeutic application (Gulati et al., 2003; Hur et al., 2004). Herein we have isolated CD133^{+} naEFCs from cord blood and expanded the population ~350-fold in less than two weeks, using serum-free medium. Given that $1-3 \times 10^6$ CD133^{+} naEFCs can be isolated from ~60 ml cord blood, this protocol can produce in excess of 5×10^8 cells within 14 days. Serum-free expansion of CD133^{+} umbilical cord blood cells has previously been achieved using medium supplemented with SCF (100 ng/ml), TPO (20 ng/ml), Flt3L (100 ng/ml), IL3 (20 ng/ml), plus VEGF and IL6 (Masuda et al., 2012). Here, using significantly lower concentrations of SCF (40 ng/ml), TPO (10 ng/ml) and Flt3L (40 ng/ml), together with IL3 (20 ng/ml), we were able to achieve similar expansion in the same time period. Our investigation into cell viability also
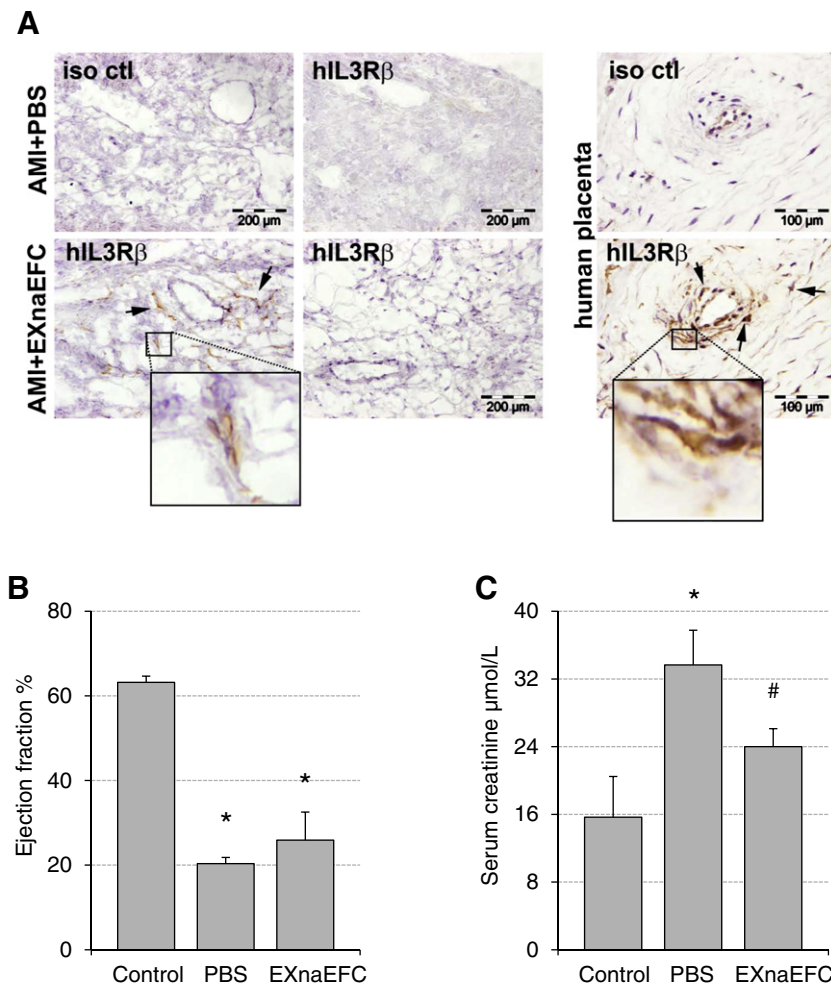
Figure 6 EXnaEFCs are localised near the heart vasculature and reduce cardiac damage in rats following AMI. Rats given surgically-induced AMI received either PBS or 1×10^6 EXnaEFCs *via* injections into the infarct site. (A), Seven days post-surgery, hearts from AMI + PBS and AMI + EXnaEFCs were examined for human cells *via* immunohistochemistry using a human specific anti-IL3Rβ antibody *versus* isotype control (iso ctl). Left and right panels of AMI + EXnaEFC staining represent regions of the heart in close proximity or distal to the site of cell injection, respectively. Human placenta (far right panels) served as controls for human IL3Rβ expression. Images shown represent one of four experiments. Prior to retrieval of the hearts, the ejection fraction was assessed by MRI (B) and peripheral blood was taken to measure serum creatinine (C). Control rats which did not undergo any surgery also had MRI and creatinine data collected. Data is shown as the mean ± SEM, n = 3—6. *p < 0.05 *versus* controls, #p < 0.05 EXnaEFCs *versus* PBS.

suggests that the IL3-induced expansion of the naEFCs was not merely *via* pro-survival effect as the rate of cell viability was similar across all media combinations assessed. We were also able to show that isolated CD133+ EXnaEFCs could be frozen, thawed and expanded equally well as the freshly isolated cells, an advantage for the use of these cells as a potential allogeneic cell therapy.

The expression of CD133 by EXnaEFCs differentiates them significantly from the well characterised ECFCs which are CD133− and suggests that the EXnaEFCs are a more primitive circulating EPC rather than the EC-like ECFCs. EXnaEFCs also express CD117, CD31, and low levels of CD45, but do not express CD14, CD38, CD144 or CD146. The expression of CD45 is a contentious issue in this field but increasing evidence supports that cells expressing this marker are clinically relevant. For example, Asahara's recent development of a clonogenic assay for cells with endothelial potential showed

that these cells clearly expressed CD45 (Masuda et al., 2011). A study by Estes et al. showed a CD34+CD133+CD45lowCD31+CD14− population capable of promoting tumour blood vasculature in mice (Estes et al., 2010). Furthermore, reduced frequency of circulating CD34+VEGFR2+CD45low and CD34+CD133+CD45low cells correlates with peripheral and coronary artery disease, while CD34+VEGFR2+CD45− shows no such correlation (Estes et al., 2010; Schmidt-Lucke et al., 2010). Our data demonstrate that co-culture of EXnaEFCs with HUVECs in Matrigel™ *in vitro* promoted tube-like structure formation. Fluorescence microscopy suggested that expanded naEFCs were in close proximity to and possibly incorporated into HUVEC structures.

The infusion of a heterogeneous population of progenitor cells, either from bone marrow or peripheral blood into myocardial infarction patients has been shown to improve left ventricle ejection fraction, end-systolic and diastolic volumes and increase coronary blood flow of the affected
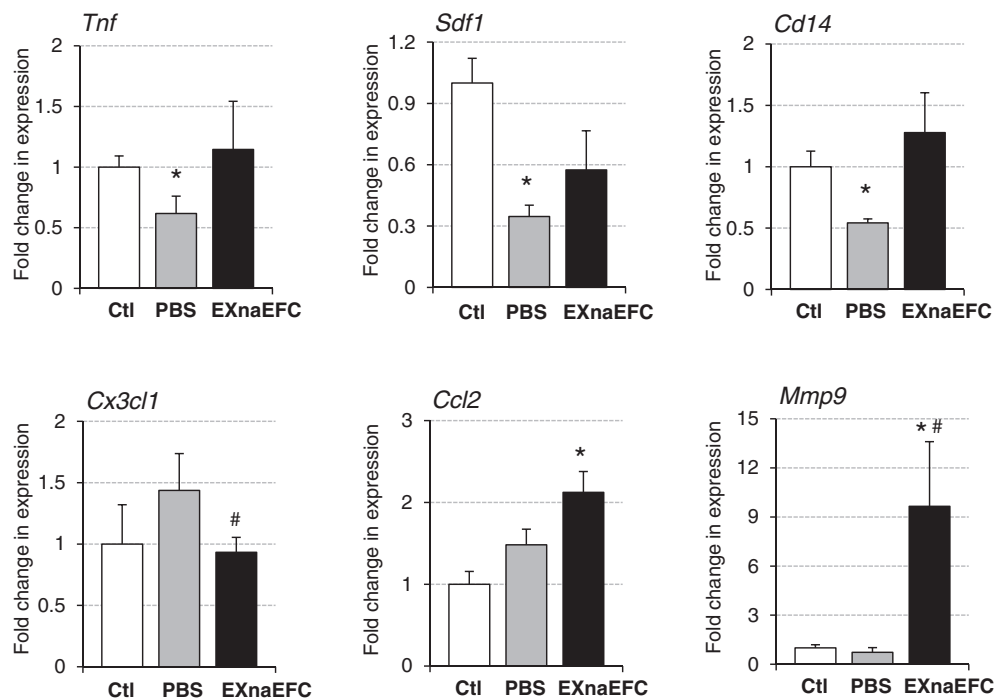
Figure 7 EXnaEFCs control gene expression in cardiac tissue following AMI. Rats given surgically-induced AMI received either PBS (grey bars) or 1×10^6 EXnaEFCs (black bars) *via* injections into the infarct site. Seven days post-surgery cardiac tissue from the infarct site was collected, RNA isolated and rat gene expression was analysed by real time PCR. Control heart tissue was collected from rats which had not undergone any procedure (white bars, Ctl). Gene expression was normalised to housekeepers. Data is shown as the mean ± SEM, fold changed compared to the control rats, n = 3–6, *p < 0.05 *versus* controls, #p < 0.05 EXnaEFCs *versus* PBS.

vessel (Assmus et al., 2002; Jiang et al., 2010; Schachinger et al., 2004; Strauer et al., 2002). As delivery of human EPCs into the infarct site of rats with surgically induced AMI has shown increased cardiac function as measured by ejection fraction (Hong et al., 2013), fraction shortening (Schuh et al., 2008) and left ventricle end systolic and diastolic pressure (Hu et al., 2009) we executed similar experiments to examine the restorative effect of the human EXnaEFCs. Intracardial injection of 1×10^6 EXnaEFCs immediately following AMI demonstrated a decrease in serum creatinine levels when compared to AMI rats administered PBS alone. Notably, the creatinine levels measured in the untreated control CBH/Rnu rats were below published values for immunocompetent rats such as Sprague Dawley which we have previously used in a similar model of AMI (Palm and Lundblad, 2005; Richardson et al., 2013). As measurement of serum creatinine is an indirect determinant of cardiac damage that rises acutely post-myocardial infarction, measurements within the first two to three days would better represent cardiac damage. This will be examined in

future studies. Furthermore, the CBH/Rnu rats in this study were routinely ~80–150 g, half the weight of Sprague Dawley rats which weigh ≥250 g each at the same age (Hong et al., 2013; Schuh et al., 2008). A higher level of AMI-induced mortality was observed in the CBH/Rnu rats and the ejection fraction in our rats was significantly lower than documented elsewhere, both prior to surgery (63% compared to 82%) and post-surgery (20% compared to 39%) (Hong et al., 2013). While we demonstrate a modest 5% increase in EF seven days following administration of EXnaEFCs *versus* PBS controls, it must be noted that a recent meta-analysis from 2625 patients showed that compared to the standard treatment, bone marrow cell transplantation delivered intracoronary exhibited a 3.96% improvement in EF and correlated with a significant decrease in all-cause mortality in these patients (Jeevanantham et al., 2012). Based on these observations, the contribution of EXnaEFCs to alleviate ischemic injury warrants further investigation. Improved cardiac performance in animal models has been shown four-weeks post-EPC transfer (Hong et al.,

Table 2 Hematopoietic properties of EXnaEFCs. Human naEFCs expanded without or with IL3 for 7 days were seeded in MethoCult plus growth factors GM-CSF, IL3, SCF and EPO for 14 days prior to colony counting and staining with May Grunwald/Giemsa to assess cellular morphology. EXnaEFCs formed blast-forming unit-erythroid (BFU-E), colony-forming units (CFU)-GEMM, -GM, -G and -M colonies in methylcellulose. (mean ± sem, n = 3).

	BFU-E	CFU-GEMM	CFU-GM	CFU-G	CFU-M
EXnaEFC no IL3	85 ± 35	11 ± 1	24 ± 11	42 ± 6	9 ± 1
EXnaEFC + IL3	138 ± 43	15 ± 4	10 ± 2	40 ± 10	11 ± 1

2013; Hu et al., 2009; Masuda et al., 2012; Schuh et al., 2008), suggesting a longer time period within our study may well have improved outcomes even further. In addition, our *in vitro* work suggested that the EXnaEFCs expressed less MHC classes I and II when compared to donor matched mature DCs, and were less receptive to TNF and IFNγ for increased surface expression of MHC class I. Our *in vivo* experiments utilised immunocompromised rodents and thus conclusive evidence of 'low immunogenicity' will require immunocompetent animals, longer *in vivo* studies and would also benefit from a comparison to MSCs. This is part of an ongoing study within our laboratory.

To determine a profile of gene activation within the hearts of AMI rats following EXnaEFC administration, we performed a comprehensive PCR analysis of cardiac tissue seven days post-infarct and treatment. We reveal that the rats receiving EXnaEFCs exhibited rescued gene expression of *Tnf*, *Sdf1* and *Cd14*. TNFα has been implicated in stimulating fibroblast proliferation and assisting in heart repair post-AMI (Jacobs et al., 1999). SDF1 supports further EPC mobilisation from the bone marrow (Jin et al., 2006) and CD14 expressing macrophages may aid in the clearance of necrotic tissue (Ertl and Frantz, 2005). Rats administered expanded naEFCs also exhibited levels of *Cx3cl1* closer to those found in control rats. Herein *Ccl2* gene expression was highest in rats receiving EXnaEFCs. Notably, the CCL2 receptor, CCR2, is expressed by EPCs and vascular smooth muscle cells and is associated with angiogenesis (Schober, 2008). *Mmp9*, which was significantly increased in rats administered EXnaEFCs, has been shown to liberate EPCs from the bone marrow (Heissig et al., 2002; Liu and Velazquez, 2008). Together this data suggest that EXnaEFCs trigger the production of a suite of rat-derived pro-angiogenic factors, many of which also mediate the clearance of damaged and necrotic tissue.

While immunocompromised animals, (such as the T lymphocyte deficient CBH/Rnu rats used here), are commonly employed to mimic human AMI (Dick et al., 1997; Masuda et al., 2012; Shultz et al., 1995), studies indicate that immuno-suppression prevents leukocyte recruitment into cardiac tissue post-AMI, reduces the clearance of dead cells and significantly delays recovery time (Kloner et al., 1978). Therefore the suitability of immunocompromised animals in AMI experiments generally is questionable and conclusions must be made prudently. Finally, if we are to consider allogeneic cells for therapeutic application understanding their immunogenicity is important. We show that EXnaEFCs express slightly higher levels of MHC class I and class II when compared to donor-matched ECs. However, when stimulated with the pro-inflammatory cytokines TNF or IFNγ, two cytokines routinely present at a transplant site (Ono et al., 1998), the increase in MHC I expression on EXnaEFCs was modest compared to the ECs. While more reactive than the MSCs, the EXnaEFCs were significantly lower in MHC expression when compared to the mDCs.

Herein we have described the isolation, serum-free expansion and characterisation of a non-adherent population of human endothelial progenitor cells (EXnaEFCs). These cells express progenitor cell and EC markers and display pro-angiogenic properties that can assist EC tube formation *in vitro* and *in vivo*. Interleukin-3 significantly enhanced EXnaEFC expansion and these cells demonstrated a clinically relevant improvement of heart function seven days post-AMI with reduced serum creatinine levels and an increase in pro-angiogenic genes in the rat heart. The mechanisms underpinning the effect of IL3 on EXnaEFCs are yet to be fully elucidated, however data to support its use come from it enhancing CD34+ and proangiogenic cell survival (D'Atri et al., 2011; Zeoli et al., 2008) as well as EC migration and tube formation *in vitro* (Dentelli et al., 1999). Our work contributes to this by demonstrating an increase in ERK activation in response to IL3. With studies showing that high-dose intervention of stem cells within two weeks is most effective in AMI (Dimmeler et al., 2008; Richardson et al., 2013), our rapid expansion of EXnaEFCs to clinically relevant numbers within a therapeutically advantageous window supports the further investigation of IL3 in cell therapy.

Importantly, in our study, the EXnaEFCs did not form capillary-like tubes on their own *in vivo* and therefore do not merit the 'endothelial' status (Hirschi et al., 2008; Yoder, 2012). A broader pro-angiogenic phenotype is noted instead. Taken together, these expanded human cells differ significantly from those we originally characterised and phenotyped as naEFCs with CD133+ isolation from cord blood and enrichment for four days in defined media (Appleby et al., 2012). These results show IL3 as a potent growth factor for human CD133+ cell expansion with clear pro-angiogenic properties, *in vitro* and *in vivo*.

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Acknowledgments

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