Endothelialization of arterial vascular grafts by circulating monocytes

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Recently our group demonstrated that acellular tissue engineered vessels (A-TEVs) comprised of small intestinal submucosa (SIS) immobilized with heparin and vascular endothelial growth factor (VEGF) could be implanted into the arterial system of a pre-clinical ovine animal model, where they endothelialized within one month and remained patent. Here we report that immobilized VEGF captures blood circulating monocytes (MC) with high specificity under a range of shear stresses. Adherent MC differentiate into a mixed endothelial (EC) and macrophage (Mφ) phenotype and further develop into mature EC that align in the direction of flow and produce nitric oxide under high shear stress. In-vivo, newly recruited cells on the vascular lumen express MC markers and at later times they co-express MC and EC-specific proteins and maintain graft patency. This novel finding indicates that the highly prevalent circulating MC contribute directly to the endothelialization of acellular vascular grafts under the right chemical and biomechanical cues.
Acellular vascular grafts continue to show great promise in various animal models as well as human clinical trials. Decellularized tissue engineered constructs have been utilized with increasing frequency and demonstrated improved patency and regeneration potential in pre-clinical studies\textsuperscript{12-13} and clinical trials\textsuperscript{14,15}. In addition to decellularized grafts, non-biological grafts composed of various polymeric biomaterials have also been used to engineer cell-free vascular grafts\textsuperscript{16-24}. All of these acellular materials must promote endothelialization of the lumen to achieve patency and promote development of the vascular wall through extensive, long-term remodeling. However, despite extensive experience with engineering vascular grafts, the mechanism of endothelialization, remains unknown.

Rapid endothelialization has been reported in small animal models as well as in pigs and dogs, which occurred mostly via migration of endothelial cells from the anastomotic sites. However, trans-anastomotic endothelialization is very limited in ovine or humans\textsuperscript{25,26} and therefore, the mechanism of endothelialization of acellular vascular grafts remains unclear. Several studies employed immobilized peptides or growth factors to the luminal surface to promote endothelialization. Growth factors such as stromal derived factor (SDF1\textalpha) was used to home circulating stem cells to the graft lumen. However, these studies showed incomplete endothelialization, especially in the center of the grafts\textsuperscript{27,28}. In our lab we developed an acellular vascular graft that was based on small intestinal submucosa (SIS) with immobilized heparin and VEGF on the graft lumen to capture VEGF receptor expressing cells from the blood. When implanted into the abdominal aorta of a mouse model, the 1-mm diameter VEGF grafts were fully endothelialized within 1 month, consisted of pro-regenerative-anti-inflammatory cells and exhibited distinct vascular remodeling toward the native state\textsuperscript{29}. Furthermore, when implanted into the carotid arteries of a clinically relevant ovine animal model, such small diameter (4.5 mm), 5-cm-long grafts exhibited high patency rates, fully endothelialized within 1 month, and developed a functional and contractile medial layer by 3 months post-implantation\textsuperscript{30-32}.

Given the success of VEGF-based vascular grafts and the lack of trans-anastomotic migration in the ovine animal model, we sought to determine the mechanism of rapid endothelialization of otherwise a-cellular grafts occur given the clear role in inflammation plays in the mouse model. Herein we identify a novel means of endothelialization via the capture and subsequent differentiation of circulating monocytes (MC) on the VEGF coated lumen. We show that VEGF captures MC, which significantly outnumber endothelial progenitor cells (EPCs) in the blood, and differentiate into functional EC that produces nitric oxide and affords patency to neo-arteries.

**Results**

**Cells of mixed EC and M2 macrophage phenotype populate vascular grafts.** At 1-week post-implantation, the lumen of VEGF functionalized SIS grafts contained cells that were devoid of endothelial cell (EC) markers, such as CD144 and eNOS. In addition, by 1 month the lumen was completely populated with cells and there was no gradient of cell density between the anastomotic sites and the middle of the graft. These observations prompted us to hypothesize that the lumen might be endothelialized with cells from circulating blood.

To this end, the phenotype of luminal cells was assessed by immunocytochemistry. Interestingly, at 1-week post-implantation graft lumens were comprised of CD14\textsuperscript{+} and CD163\textsuperscript{+} cells, but lacked EC markers CD144 or eNOS (Fig. 1a, b). Surprisingly, at 1 and 3 months post-implantation, luminal cells of explanted grafts co-expressed the endothelial specific marker CD144 and the M2-macrophage specific marker CD163 (Fig. 1a). Similarly, they also co-expressed the EC-specific marker, eNOS and the monocyte/macrophage specific marker, CD14 (Fig. 1b).

**VEGF captures monocytes from whole blood under flow.** These results prompted us to hypothesize that the lumen of VEGF decorated grafts might be endothelialized with monocytes from circulating blood. To address this hypothesis, we employed a microfluidic device to examine whether cells from blood could be captured by immobilized VEGF. The device consisted of a single channel (length: 1 cm; width: 400 μm; height: 200 μm) that was kept in place by vacuum (Fig. 2a) and coated with a layer of chitosan (positively charged) that was used as adhesive to immobilize heparin (negatively charged) as shown previously\textsuperscript{33}. VEGF was then immobilized by binding to heparin via its heparin binding domain. The surface concentration of VEGF increased with increasing the VEGF concentration in solution until saturation was reached at ~1000 ng/cm\textsuperscript{2} (Fig. 2b). Furthermore, the chitosan/heparin/VEGF (denoted as CHV) surfaces supported proliferation of human umbilical vein endothelial cells (HUVEC) in a VEGF surface concentration dependent manner (Fig. 2c), demonstrating that immobilized VEGF on CHV surface is biologically active.

Next, freshly drawn human blood was passed over the VEGF-containing channel surface under different flow rates corresponding to shear stress ranging from 1 to 15 dyne/cm\textsuperscript{2} (Fig. 2d) and captured cells were fixed and assessed via immunocytochemistry. As indicated in Fig. 2e (representative images at shear stress of 1 dyne/cm\textsuperscript{2}) and quantified in Fig. 2f, all captured cells expressed the monocyte (MC) marker CD144, independent of the level of shear stress (e.g. 100% of 234 ± 16 cells at 15 dyne/cm\textsuperscript{2}; n = 10 independent runs per shear stress were tested). In comparison <1% of captured cells expressed the EC marker CD144 (only one out of 234 ± 16 cells were positive; n = 5 independent runs at 1 dyne/cm\textsuperscript{2} or 15 dyne/cm\textsuperscript{2}; no CD144 cells were present in any run at shear stress of 5 or 10 dyne/cm\textsuperscript{2}). Furthermore, between 1 and 10 dyne/cm\textsuperscript{2}, all captured cells expressed CD31, a shared marker between MC and EC lineages. Interestingly, 64.3 ± 7.1% of captured cells expressed the anti-inflammatory macrophage marker CD163. These results suggested that VEGF may be capturing blood MC, which are known to express the VEGF receptor 1 (VEGFR1).

To verify any potential MC capture by surface immobilized VEGF, CD14\textsuperscript{+}/CD16\textsuperscript{+} MC were isolated from peripheral human blood by negative selection and run over the microfluidic channel (0.5 × 10\textsuperscript{6} cells/mL) under the same shear conditions. Indeed, MC were captured by surface bound VEGF with capture efficiency similar to that of human EC, ovine EC, and a murine macrophage cell line (Fig. 2g; n = 10 independent runs at 1 dyne/cm\textsuperscript{2}). In contrast, little or no capture was observed when using murine and human fibroblasts or human mesenchymal stem cells.

**Immobilized VEGF captures MC from peripheral blood mononuclear cells.** Next, we examined the phenotype of VEGF captured MC using multi-color flow cytometry. As a control we used FN-coated surface that has been previously used to culture murine and human blood by negative selection and run over the microfluidic channel (0.5 × 10\textsuperscript{6} cells/mL) under the same shear conditions. Indeed, MC were captured by surface bound VEGF with capture efficiency similar to that of human EC, ovine EC, and a murine macrophage cell line (Fig. 2g; n = 10 independent runs at 1 dyne/cm\textsuperscript{2}). In contrast, little or no capture was observed when using murine and human fibroblasts or human mesenchymal stem cells.
and non-classical MC (CD14+/CD16+, FN: 28.5 ± 2.1%; VEGF: 26 ± 3.4%). As expected, VEGF-captured MC were highly enriched in VEGFR1 expressing cells (99 ± 1.2% were VEGFR1+), as were the FN bound cells, albeit to a lesser extent (73 ± 5.6%). However, no VEGFR2+ cells were found on either the VEGF or FN coated surface beyond background noise (~2%). Histograms for each antibody and corresponding IgG controls are shown in Fig. 3d–g. IgG gating is depicted in Supplementary Fig. 1.

Inducing spreading and proliferation of surface captured MC. Next, we examined whether MC that were attached on VEGF or FN could be differentiated into functional EC similar to what was

Fig. 1 Monocytes are incorporated as the endothelium of acellular vascular grafts. Immunostaining of explanted VEGF-based A-TEVs at the indicated time points of 1 week, 1 month, and 3 months compared to native carotid artery. a Co-staining for the macrophage marker CD163 (red) and the endothelial marker CD144 (green). b Co-staining for the monocyte marker CD14 (red) and the functional endothelial marker eNOS (green). Note that at 1-week lumens are devoid of EC markers but express MC markers, CD14 and CD163. At 1 and 3 months lumens comprise of cells co-staining for MC (CD14, CD163) and EC markers (CD144, phosphorylated (active) eNOS). White letter “L” indicates the lumen. Scale bars 50 µm. White arrows indicate autofluorescent inner elastic lamina.
observed on the vascular graft lumens in vivo. To this end, we developed a protocol based on two important findings (Fig. 4a). First, we found that the ROCK inhibitor, Y-27632 induced rapid adherence and spreading on both iVEGF and FN (Fig. 4b, c). Quantification of cell area indicates that Y-27632 has a dramatic effect on spreading, with an average cell area of 1126.2 ± 43.8 μm² (mean ± SD) and 1006.4 ± 13.7 μm² on FN and iVEGF when cultured with Y-27632 (50 nM for 3 days) as compared to 443.9 ± 33.4 μm² and 331.0 ± 22.1 μm² on FN and iVEGF when cultured without Y-27632 (Fig. 4c).

Also, it is well documented that primary MC are non-dividing cells when cultured in vitro. Interestingly, we discovered that Wnt activation using the GSK3β antagonist, CHIR-99021 (CHIR), induced proliferation of adherent MC, as shown by immunostaining for Ki67 (Fig. 4d). Specifically, treatment with CHIR for 2 days induced Ki67 expression in 44.4 ± 11.6% (p < 0.05, n = 3) of cells on FN and 50.2 ± 11.6% (p < 0.05, n = 3) of cells on VEGF, as compared to only 6.3 ± 4.5% of Ki67

Monocyte differentiation to endothelial cells. Based on these observations, we developed an optimized differentiation protocol as outlined above in Fig. 4a. Briefly, monocytes were cultured on either FN or iVEGF in a modified endothelial basal media (EBM; Lonza) with all supplements (EGM2 bullet kit) with Y-27632 (10 μM), MCSF (10 ng/mL), soluble VEGF (100 ng/mL), and 10% autologous activated platelet rich plasma (PRP). On day 3, Y-27632 was removed and CHIR was added for 2 days. Thereafter, the medium was replaced with the same basal medium but without Y-27632 or CHIR until day 14. While initially cultured monocytes displayed a spindle like morphology, they gradually...
Fig. 3 Characterization of PBMNs from whole blood. Multi-color flow cytometry assessment of PBMNCs a isolated directly from whole blood; or b after 1 h capture on FN surface; or c after 1 h capture on iVEGF. Cells were only gated by FSC/SSC (green gate), no sub-gates were used in order to show the entire population of cells adhered to the surfaces. Flow cytometry histograms demonstrating positive signal shifts (d–g). HUVEC served as a positive control for VEGFR2.
changed into a cobblestone morphology as cells proliferated and formed tight colonies typical of EC (Fig. 4f, g).

Over the 14-day differentiation, we assessed multiple genes involved in EC differentiation and function as well as genes associated with pro-inflammatory (M1) and anti-inflammatory (M2) macrophage activation states (Fig. 5). Interestingly, the differentiation process resulted in a genotype comprising both classic endothelial as well as macrophage genes, especially M2-associated genes. While some monocyte genes were quickly downregulated such as CD14 (Fig. 5a) and CX3CR1 (Supplementary Fig. 2A), the non-classical monocyte marker CD16 was upregulated nearly 100-fold during initial differentiation and decreased to pre-differentiation levels by day 14 (Fig. 5b). A similar trend was observed for M1 associated genes such as IL6, IL12, iNOS, and TNF-α (Fig. 5c–e, Supplementary Fig. 2a). Conversely, some M2 associated genes were dramatically upregulated during differentiation and remained elevated above pre-differentiation levels such as CD163, a gene that is completely lacking in EC such as HUVEC and human carotid endothelial cells (HCAEC) (Fig. 5f). Other M2-associated genes followed a similar trend including EGR2, IL10, ARG1, and FN (Fig. 4g, h, Supplementary Fig. 2a).

Several transcription factors that are well known to be crucial to EC differentiation during development were upregulated during differentiation. Interestingly, the pattern of upregulation loosely followed the accepted temporal sequence of EC transcription factors during development. ETV2 and GATA2 are transcription factors that appear early in EC development and as shown reach maximum upregulation on day 5 (Fig. 5i, j). Following the paradigm, SOX17 reached maximum upregulation after ETV2 and GATA2, on day 7 and remained elevated similar to typical EC levels at later times (Fig. 5k). Another important EC transcription factor, ERG was upregulated early during differentiation and then decreased but remained at levels similar to HUVEC (Fig. 5l). Interestingly, the transcription factor HEY1, a venous EC differentiation marker that is expressed in HUVEC but not HCAEC, was significantly downregulated during MC differentiation.
differentiation (Fig. 5m). Conversely, its arterial counterpart expressed in HCAEC but not in HUVEC, HEY2, was upregulated early on but later decreased on FN. Interestingly, HEY2 was significantly upregulated by nearly 100-fold, and remained at high levels on iVEGF (Fig. 5n).

Next, we assessed markers of mature and functional EC. Of particular interest is KDR/VEGFR2, which was not expressed in MC as demonstrated already using flow cytometry (Fig. 3b, c). As indicated in Fig. 5o, KDR was dramatically upregulated during differentiation, reaching EC levels by day 14. FLT1/VEGFR1 expression was upregulated early on iVEGF but then returned to similar levels as FN before increasing to EC and MC levels (Fig. 5p). VE-Cadherin/CD144 expression was upregulated significantly and remained at high levels through day 14 (Fig. 5q). Similarly, vWF,
Conversely, the venous EC marker HEY1 and surprisingly, and similar to the shear related transcription factors iVEGF conferring much greater expression of upregulated during differentiation on both FN and iVEGF, with levels, which are approximately 5-fold higher than MC (Fig. 5t). arterial HCAEC and above the venous HUVECs (Fig. 5u).

MC/Mφ and EC both express PECAM1/CD31, therefore, it is not surprising that expression of CD31 was fairly stable at MC levels with a final upregulation towards EC levels, which are approximately 5-fold higher than MC (Fig. 5t). Surprisingly, and similar to the shear related transcription factors HEY1 and HEY2, the arterial EC marker EphB2 was dramatically upregulated during differentiation on both FN and iVEGF, with iVEGF conferring much greater expression of EphB2, similar to the arterial HCAEC and above the venous HUVECs (Fig. 5v). Conversely, the venous EC marker EphB4 was slightly upregulated during MC to EC differentiation but remained low compared to the expression levels observed in HUVECs or HCAECs (Fig. 5v). Other EC markers were also upregulated during differentiation such as TIE1, TIE2, NRP1, and NRP2 as indicated in Fig. 5w, x and the heat map of Supplementary Fig. 2a, b. Similar results were obtained when cells were cultured on SIS substrate functionalized with heparin and VEGF (SHV) (Supplementary Fig. 2C).

Monocytes differentiate to an EC and M2 macrophage mixed phenotype. Next, we investigated the expression of endothelial proteins KDR, eNOS, CD31, and CD144 by immunocytochemistry (Fig. 6; Supplementary Fig. 3: secondary antibody assay controls and Supplementary Fig. 4: endothelial cell assay controls). Interestingly, the monocyte markers CD14 and CD16 were still present by day 14, albeit expression of CD16 was low (Fig. 6a, g). Interestingly, cells formed VE-cadherin (CD144) junctions, while maintaining expression of the M2 macrophage marker CD163 either on FN or iVEGF (Fig. 6b, h). Similarly, cell junctions contained CD31, while cells continued to express CD14 (Fig. 6d, j). Initially MC lacked expression of VEGFR2, however, VEGFR2 was highly expressed by day 14 of differentiation (Fig. 6c, i). Finally, MC-derived EC expressed the phosphorylated form of eNOS, indicating acquisition of EC function but lacked expression of EphB2 or EphB4 at the protein level (Fig. 6e, f, k, l).

MC-derived EC develop EC function. To assess the function of MC-derived EC, we employed acetylated LDL (acLDL) uptake and neo-vessel (tube) formation in vitro. After 14 days of differentiation, cells were assessed for acLDL uptake and the percentage of acLDL+ cells quantified as shown in Fig. 7a. Of note, only 5 ± 3.3% (n = 3 independent biological assessments) of MC could uptake acLDL. In comparison, when MC were activated to a traditional macrophage phenotype, the percentage of cells that could uptake acLDL increased to 67 ± 9.3%. When MC were differentiated towards EC either on FN or iVEGF using our defined protocol, 92 ± 5.6% and 88 ± 3.9% of cells could uptake acLDL, similar to HUVEC and HCAEC (85.3 ± 7.2% and 93.6 ± 8.4%, p < 0.0001 as compared to initial MC). Although both macrophages and EC uptake acLDL, only EC are known to organize into neo-vessels/tubes on matrigel. Interestingly, MC-derived EC on FN or iVEGF formed tubes within 24 h of adhesion to matrigel. In addition, cells within the neo-vessels expressed VE-cadherin/CD144 and CD31 that were localized at the cell junctions. Surprisingly, these cells also expressed CD16 as indicated in Fig. 7b, c.

Shear stress augments MC-EC differentiation and EC function. Shear stress is present at all times after implantation in vivo and is well known to induce EC differentiation into a mature phenotype and alignment along the direction of flow. To examine the effect of shear stress on MC to EC differentiation, MC cells were differentiated for 9 days under static conditions; then subjected to
gress of EphB2 - but not EphB4 - at the cellular junctions, other hand, when cells were exposed to low shear (1 dyne/cm²) for

had subjected to shear stress; and HCAEC subjected to shear stress.

examined the global transcriptome of individual cells in the three

Furthermore, using single cell RNA-sequencing (scRNA-Seq) we

acquisition of MC-EC phenotype, similar to bona

and showed that the initial population of MC clustered separately from HCAEC, while the resulting MCEC overlapped with HCAEC and not MC (Fig. 9a). Co-expression analysis reveals that only the resulting MCEC contain cells co-expressing KDR and PROM1/CD133, whereas our starting MC population contained only one cell expressing KDR and a separate cell expressing PROM1 (Fig. 9b), suggesting that our initial MC population did not contain any EPC/ECFCs, which are known to co-express KDR/VEGFR2 and PROM1/CD133. Interestingly, heatmaps constructed using EC and MC associated genes revealed that the resulting MCEC cells expressed predominantly EC genes but also some MC genes (Fig. 9c), in agreement with the RT-PCR data (Fig. 5). Violin plots of individual gene expression in each population further support this point (Supplementary Fig. 6).

MCEC are derived exclusively from MC and not contaminating EC. To provide further evidence of the differentiation potential of MC into EC and exclude the possibility that MCEC originated from rare circulating EPC/EC in the initial MC population, we employed a MC-specific promoter to select the initial MC population before the onset of differentiation. Specifically, we used a lentiviral vector (pCD68-ZsG-Puro) encoding for ZsGreen and Puromycin phosphotransferase under the control of the MC specific promoter, CD6834,35 (see schematic in Fig. 10a). After transduction of MC with pCD68-ZsG-Puro and puromycin selection, all cells were ZsGreen+ (Fig. 10b), indicating active CD68-Pr. As a control, HCAEC cells were also transduced with the same vector but all cells died upon puromycin selection, suggesting that CD68-Pr was not active in EC as expected.

Additionally, HCAEC were transduced with a dual promoter lentiviral vector (pCD68-LVDVP) encoding for ZsGreen under the CD68-Pr and DsRed2 under the human (h)PGK promoter (Fig. 10a). This vector was developed in our laboratory and contains insulator and terminator sequences that diminish promoter interference36,37. Transduced HCAEC with pCD68- LVDVP expressed DsRed but not ZsGreen (Fig. 10c), in agreement with the lack of CD68-Pr activity seen with puromycin selection.

MCEC are distinct from MC and cluster with mature EC. Furthermore, using single cell RNA-sequencing (scRNA-Seq) we examined the global transcriptome of individual cells in the three cell populations: starting MC pooled from three donors after 1 h adherence to FN; MC-derived EC (from the same three donors) subjected to shear stress; and HCAEC subjected to shear stress.

gradually increasing shear stress by ramping from low to high shear stress (1 to 10 dyn/cm²) over 2 days; and further cultured under high shear stress for 3 days (for a total of 14 days of differentiation). As shear stress is known to induce NO production, we quantified NO₂ content via Griess reagent. Indeed, shear induced NO₂ production significantly, as compared to static conditions when cultured on FN and especially on iVEGF at levels similar to HUVEC and HCAEC (Fig. 8a).

Next, we assessed the effect of high shear on a number of key EC genes that are known to be affected by shear stress (Fig. 8b–k). Consistent with the increased NO production, eNOS was upregulated dramatically with shear (Fig. 8b). CD144/VE-cadherin was similarly upregulated by shear, with expression matching EC levels (Fig. 8c), as did key EC mechanosensory proteins such as JAG1, DLL4, and the receptors NOTCH1 and NOTCH4 (Fig. 8d–g). Interestingly, high shear downregulated the venous genes HEY1 and EphB4 and upregulated arterial EC genes HEY2 and EphB2 (Fig. 8h–k).

Following gene expression changes, we assessed protein expression upon exposure to shear. Under high shear stress conditions, the cells aligned parallel to the direction of flow as expected (Fig. 8l–s), with CD144 clearly delineating the cell borders (Fig. 8l, p). Immunostaining showed that both the MC marker CD14 and the M2-MΦ marker CD163 remained consistently expressed on FN and iVEGF (Fig. 8l, m, p, q). Notably, high shear induced expression of EphB2 - but not EphB4 - at the cellular junctions, typical of mature arterial endothelial cells (Fig. 8n, o, r, s). On the other hand, when cells were exposed to low shear (1 dyne/cm²) for 5 days, cell alignment was not as evident, and expression of arterial EC marker EphB2 was not apparent (Supplementary Fig. 5a, c), but the venous EC marker EphB4 was weakly expressed (Supplementary Fig. 5b, d), indicating that shear plays an important role in the acquisition of MC-EC phenotype, similar to bona fide EC.

Uniform Manifold Approximation and Projection (UMAP) dimensionality reduction was applied to the integrated dataset and showed that the initial population of MC clustered separately from HCAEC, while the resulting MCEC overlapped with HCAEC and not MC (Fig. 5b). Violin plots of individual gene expression in each population further support this point (Supplementary Fig. 6).

Fig. 7 MC-derived EC develop endothelial function. a MCEC differentiated on FN or iVEGF uptake acetylated LDL. MC on day 0 are unable to uptake LDL compared to traditionally activated, LPS stimulated MΦ, MCEC on FN or VEGF or HUVEC or HCAEC. (**∗p < 0.0001) statistical significance using one-way ANOVA and Sidak’s test (DF = 12, F = 205.1); n = 3 independent biological replicates; error bars indicate ±SD of the mean. MCEC differentiated on FN (b) or VEGF (c) formed tubular networks when cultured on Matrigel. Representative immunostaining for the monocyte markers CD16 (pink), endothelial marker CD144 (green), and CD31 (red) over n = 5 independent biological replicates.
These results show that CD68-Pr is active in MC but not in EC and therefore, can be used to track MC as they differentiate. Selected MC were subjected to the MCEC differentiation protocol as discussed above. Interestingly, after 14 days of differentiation, the selected cells maintained ZsGreen expression and continued to express the MC marker, CD14. At the same time, these cells also expressed the EC-specific proteins, CD144 and VEGFR2 (Fig. 10d). Notably, upon application of shear stress, expression of both ZsGreen (CD68-Pr activity) and CD14 decreased, while EC markers CD144 and VEGFR2 remained highly expressed in all cells (Fig. 10e), demonstrating that MC, and not a contaminating EC fraction, could differentiate into EC and that shear stress contributed significantly to the MEC phenotype.

Discussion

Various strategies have been employed to capture rare circulating endothelial progenitor cells, EPCs, from the blood to induce endothelialization of implanted materials. Some groups employed antibodies against EPC specific proteins such as VEGFR2, CD34, and CD133, but application in small animal models was met with varying success. In addition, the use of antibodies was shown to reduce functionality of the cell surface protein, especially in the case of VEGFR2. SDF1α, a cytokine that binds to CXCR4 on EPCs, has also been employed but resulted in incomplete endothelialization in the middle of the grafts and neo-intimal hyperplasia. In recent work from our laboratory, we employed VEGF to capture circulating cells that express the VEGF receptor and endothelialize the otherwise acellular grafts. This approach was very successful as shown by high patency rates (92%) and successful remodeling of arterial grafts in both a mouse model and the clinically relevant ovine model. Even though our in vitro studies indicate small differences between iVEGF and FN culture surfaces, likely due to the presence of soluble VEGF in both conditions, in vivo VEGF was essential for maintaining patency and promoting remodeling. In contrast, FN based grafts have been previously shown to fail, likely due to the RGD integrin binding domain present in FN that...
binds a plethora of cell types, including platelets. In the absence of VEGF, grafts failed due to occlusion within hours of implantation in the ovine model, suggesting that heparin alone was not enough to prevent clotting. However, in mouse models, grafts without VEGF did not fail; likely due to the significantly higher flow rate in mouse abdominal aorta compared to the carotid artery of sheep. In addition, heparin only grafts exhibited a high degree of inflammation and lacked a proper endothelium and medial layer. In contrast, VEGF grafts were fully endothelialized within 1 month, consisted of pro-regenerative-anti-inflammatory macrophages and exhibited distinct vascular remodeling towards the native state. Therefore, VEGF promotes capture of cells with patency inducing, pro-regenerative capacity in vivo.

In this study we show that the cells populating the lumen of VEGF-decorated grafts were mostly VEGFR1-expressing MC. Interestingly, VEGFR1 has higher affinity for the VEGF ligand than VEGFR2, which is expressed in EC, and therefore may enable higher selectivity of the VEGF surface towards MC. In addition, MC outnumber EPCs to a great extent, as EPCs represent <0.01%, whereas MC represent over 20% of PBMNC. Indeed, VEGF grafts did not contain any EC on the lumen at 1-week post implantation and instead, the graft lumen was covered in MC-M2 polarized cells (CD14+/CD163+). Binding of MC to immobilized VEGF was also shown in vitro using microfluidic channels with immobilized VEGF that captured MC cells from whole blood under flow. While we cannot exclude the possibility that some EPCs might have been captured on the graft lumen, the vast majority of cells on the graft lumen continued to express MC-Mφ markers at 1 month and even at 3 months post-implantation, further supporting the hypothesis that they originated from MC.

MC are known to play a crucial role in angiogenesis and arteriogenesis. During angiogenesis, Mφ have been found to orchestrate bud development of new vessels and are often found in direct contact with tip cells. Similarly, MC-derived, tumor associated Mφ, are known to induce rapid angiogenesis around growing tumors and have been shown to be present within the

Fig. 9 Single-cell RNA sequencing. Single-cell RNA sequencing of three cell populations, initial MC, MCEC, and HCAEC both subjected to shear stress. a UMAP reduction analysis of unsupervised data of all three cell populations. b Co-expression analysis of KDR and PROM1 expression across all three populations. MC do not express KDR or PROM1. c Heat map representation of MC, M1, M2, and EC genes across all three populations.
growing vessels in contact with tip cells. MCs were also shown to be critical for the success of implanted grafts, as ablating them using genetic or chemical means led to graft failure due to stenosis and lack of proper endothelium. Bone marrow myeloid progenitor cells were shown to induce re-endothelialization after balloon injury in rats. All these studies show that MCs are critical for angiogenesis and endothelialization, but their contribution is thought to be indirect through secretion of growth factors and cytokines that promote endothelial proliferation and migration.

In contrast to these studies, here, we report for the first time, direct incorporation of MC-derived ECs into the endothelium of a neo-artery in a large animal model. At 1-week post-implantation, acellular grafts were coated with cells expressing MC but not EC proteins. At 1 and 3 months post-implantation, the luminal cells retained MC/Mφ markers while also expressing functional EC markers, including expression of eNOS and production of NO, ultimately conferring graft patency. Interestingly, similar results were obtained in our mouse model, whereby the VEGF grafts developed a well-defined endothelial layer comprised of cells expressing both EC and MC/Mφ markers. In contrast to small animals, where ECs can migrate from the anastomotic sites, in humans and large animals such as sheep, EC ingrowth does not occur. Therefore, endothelialization by an abundant cell in the blood such as MC may be critical for the success of arterial grafts and perhaps also venous or cardiac transplants. This novel mechanism may also contribute to the repair of cardiovascular tissues in-vivo following EC disruption by injury or disease.

Previous studies have shown that MCs can express EC genes after in vitro culture but lack VE-cadherin positive adherens junctions and the ability to form tubes, indicating lack of EC functionality. In contrast, we developed a strategy to coax MC to differentiate into functional EC-like cells in vitro. Our strategy involved several steps. First, we utilized the ROCK inhibitor, Y27, which was used in previous studies to promote spreading and prevent death of embryonic stem cells during sub-culture. We also substituted serum with PRP, which has been used previously to enhance the survival of MCs. Most important, activation of the WNT pathway via CHIR was necessary to coax MC towards a functional EC phenotype, exhibiting VE-cadherin junctions and

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**Fig. 10 CD68 promoter driven selection of MC for differentiation.**

- **a** Schematic of lentiviral vectors encoding for ZsGreen and puromycin phosphotransferase under the CD68 promoter (CD68-Pr; pCD68-ZsG-Puro) and Schematic of lentiviral dual promoter (LVDP) encoding for ZsGreen under the CD68-Pr and DsRed under the human (h)PGK promoter (pCD68-LVDP). The two transcriptional units are separated by polyadenylation (SPA, synthetic polyA), terminator (Tactb), and insulator (cHS4) sequences that diminish promoter interference.

- **b** Immunostaining of MC transduced with lentiviral vector shown in (a) and selected with puromycin (ZsGreen+) for the MC marker CD14 (red) prior to differentiation. HCAEC cells were transduced with the lentiviral dual promoter vector shown in b. They express DsRed but not ZsGreen indicating that CD68 is inactive in HCAEC.

- **d, e** Immunostaining for MC marker CD14 and EC markers CD144 and VEGFR2 (red) of puromycin selected MC (ZsGreen+) that were coaxed to differentiate towards EC under (d) static or (e) shear stress conditions. Scale Bar: 20 µm.
fibroin clot (FN; Thermo Fisher) or IVEFG for 1 h in EBWM with no serum or PRP (basal media without supplements). Adherent cells were washed once in warm PBS to remove unbound cells. Adherent cells were further cultured up to 14 days in EGM2 medium, with all supplements except that FBS was replaced by autologous PRP (20% v/v). VEGF (50 ng/ml) and MCSF (1 ng/ml; Thermo Fisher) were added to the EGM2 medium throughout the culture period. On day 0, Y-27632, 10 μM, (Y27; Sigma) was added to the medium and removed on Day 3. On day 3, CHIR-99021, 10 μM, (CHIR, Sigma) was added to the medium and removed on day 5. EGM2 with 20% PRP, 50 ng/ml VEGF, and 1 ng/ml MCSF was replaced every 2–3 days.

Culture under shear conditions. On day 14, differentiated MCs on FN or IVEFG were further cultured in a bio-reactor setup as previously reported. In this procedure circular dishes were modified with attachment of a smaller inverted circular culture dish inside of a larger dish. When this dish is placed on an orbital shaker, the force directs the medium to swirl around the center dish. Shear stress is generated by changing the orbital speed. We adapted this setup with an orbital shaker with a 1.9-cm radius. Shear was determined by the equation:

$$\tau = \rho \eta \pi f$$

where \(\tau\) is the desired shear (dynes/cm²), \(\rho\) is the density of the orbital shaker, \(\eta\) is the viscosity (poise), \(f\) is the density (g/ml), and \(\pi\) is the rotations speed in rounds per min. Using this equation and converting to rotations per minute to 29, 84, 133 rotations per minute equated to 1, 5, and 10 dynes/cm² of shear, respectively. Shear was slowly ramped up from 1 to 10 dynes/cm² over 2 days and
Immunohistochemistry and immunocytochemistry. Explanted A-TEVs and native carotid arteries were cleaned using saline and pressure fixed in 10% Formalin. Samples were then dehydrated in a series of graded ethanol solutions, xylene substitutes and then embedded in paraffin. Tissue sections (10 µm) were deparaffinized and subjected to pressure-activated high temperature antigen retrieval. Paraffin sections were first blocked with 5% (v/v) goat serum in PBS. On day 14 and day 20 (after shear) cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X-100, and blocked in 5% goat serum. Tissue sections and cells were then further incubated with the following primary antibodies: anti-VEGFR1 (1:100, Thermo Fisher Scientific), anti-VEGFR2 (1:100, Thermo Fisher Scientific), anti-smooth muscle alpha actin (1:200, Thermo Fisher), anti-CD144 (1:50, Cell), anti-CD14 (1:200, Abgent), anti-CD38 (1:200, Abcam, Cambridge, MA), anti-CD144 (1:50, Cell), anti-CD144 (1:50, Cell), anti-CD144 (1:50, Cell), and anti-CD144 (1:50, Cell). Cell sections were then washed in PBS three times, then fixed in 4% paraformaldehyde before imaging and quantification.

Quantification of NO production. NO production was measured as a function of NO2 concentration. Media from differentiated cells under static and shear were collected and assessed for nitrite using the Griess colorimetric reagent (Thermo Fisher).
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