

Host non-inflammatory neutrophils mediate the engraftment of bioengineered vascular networks

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Notwithstanding the remarkable progress in vascular network engineering, implanted bioengineered microvessels mostly fail to form anastomoses with the host vasculature. Here we demonstrate that implants containing assembled human vascular networks (A-grafts) fail to engraft owing to their inability to engage non-inflammatory host neutrophils upon implantation into mice. By contrast, unassembled vascular cells (U-grafts) readily engage alternatively polarized neutrophils, which in turn serve as indispensable mediators of vascular assembly and anastomosis. The depletion of host neutrophils abrogated vascularization in U-grafts, whereas an adoptive transfer of neutrophils fully restored vascularization in myeloid-depleted mice. Neutrophil engagement was regulated by secreted factors and was progressively silenced as the vasculature matured. Exogenous addition of factors from U-grafts re-engaged neutrophils and enhanced revascularization in A-grafts, a process that was recapitulated by blocking Notch signalling. Our data suggest that the pro-vascularization potential of neutrophils can be harnessed to improve the engraftment of bioengineered tissues.

Issue engineering holds great promise in regenerative medicine as a solution for the increasing demand for donor organs and tissues¹. In this context, bioengineering functional vascular networks is central^{2,3}. The last decade has seen remarkable progress in the collective effort to bioengineer such networks by self-assembly of vascular cells within suitable biomaterials^{4–9}. However, efforts remain mostly empirical owing in large part to the inability of bioengineered microvessels to connect to the host circulatory system upon implantation. This challenge is similar to that of primary tissues during surgical grafting^{10–14}. Indeed, clinical experience with primary tissues has repeatedly shown that inadequate revascularization remains a common outcome, leading to various degrees of graft resorption and failure^{15,16}. Therefore, the search for new approaches to advance graft revascularization continues to be a pressing priority in regenerative medicine.

Previous studies have shown that adult neovascularization and angiogenesis rely on myeloid cells^{17,18}. Efforts have been primarily focused on the effects of monocytes and macrophages on tumour angiogenesis^{19–21}. Nevertheless, studies have also established a prominent role for neutrophils in tumour angiogenesis^{22–24}. In grafting models, studies have collectively demonstrated that transplanted hypoxic tissues recruit proangiogenic matrix metalloproteinase (MMP)-9-delivering neutrophils in a vascular endothelial growth factor (VEGF)-dependent manner and that inhibiting neutrophil recruitment impairs ingrowth of host vessels into the grafts^{25,26}. In bioengineered grafts, however, the contribution of neutrophils remains largely unknown.

In this study, we sought to determine the extent to which host myeloid cells, in general, and neutrophils, in particular, govern the engraftment of bioengineered vascular networks. To this end, we examined microvasculatures at two contrasting states of maturation.

On the one hand, we bioengineered grafts containing fully assembled vascular networks embedded in 3-dimensional hydrogel constructs (referred to as assembled grafts or A-grafts). These vascular networks mimicked the indolent state of mature microvessels and as such mostly failed to spontaneously connect with the host circulation upon implantation. On the other hand, we bioengineered grafts that simply contained an unassembled suspension of vascular cells embedded in a hydrogel (referred to as unassembled grafts or U-grafts), a configuration that we have previously shown to effectively produce perfused networks of microvessels following implantation^{27,28}. By comparing these two distinct graft models, we found that the inefficient engraftment of A-grafts was due to the inherent inability of their mature microvasculature to engage non-inflammatory host neutrophils, which in U-grafts served as indispensable mediators of vascularization.

Results

Inefficient engraftment of bioengineered human microvessels. To evaluate the engraftment of bioengineered microvessels, we generated grafts containing fully assembled vascular networks embedded in 3-dimensional constructs (A-grafts). In addition, we prepared grafts that simply contained an unassembled suspension of the same vascular cells (U-grafts) (Fig. 1a). In both cases, grafts were prepared *in vitro* by combining human endothelial colony-forming cells (ECFCs) with mesenchymal stem cells (MSCs) (4×10^5 cells; 1:1 ratio) in gelatin-based hydrogels (GelMA) that were previously shown to be compatible with vascular morphogenesis^{5,28}. U-grafts were suspensions of the cells in the hydrogel on day 0, whereas A-grafts were formed by allowing ECFCs to self-assemble into mature networks over seven days *in vitro* (Fig. 1a). We studied the engraftment of both types of grafts following subcutaneous implantation into nude

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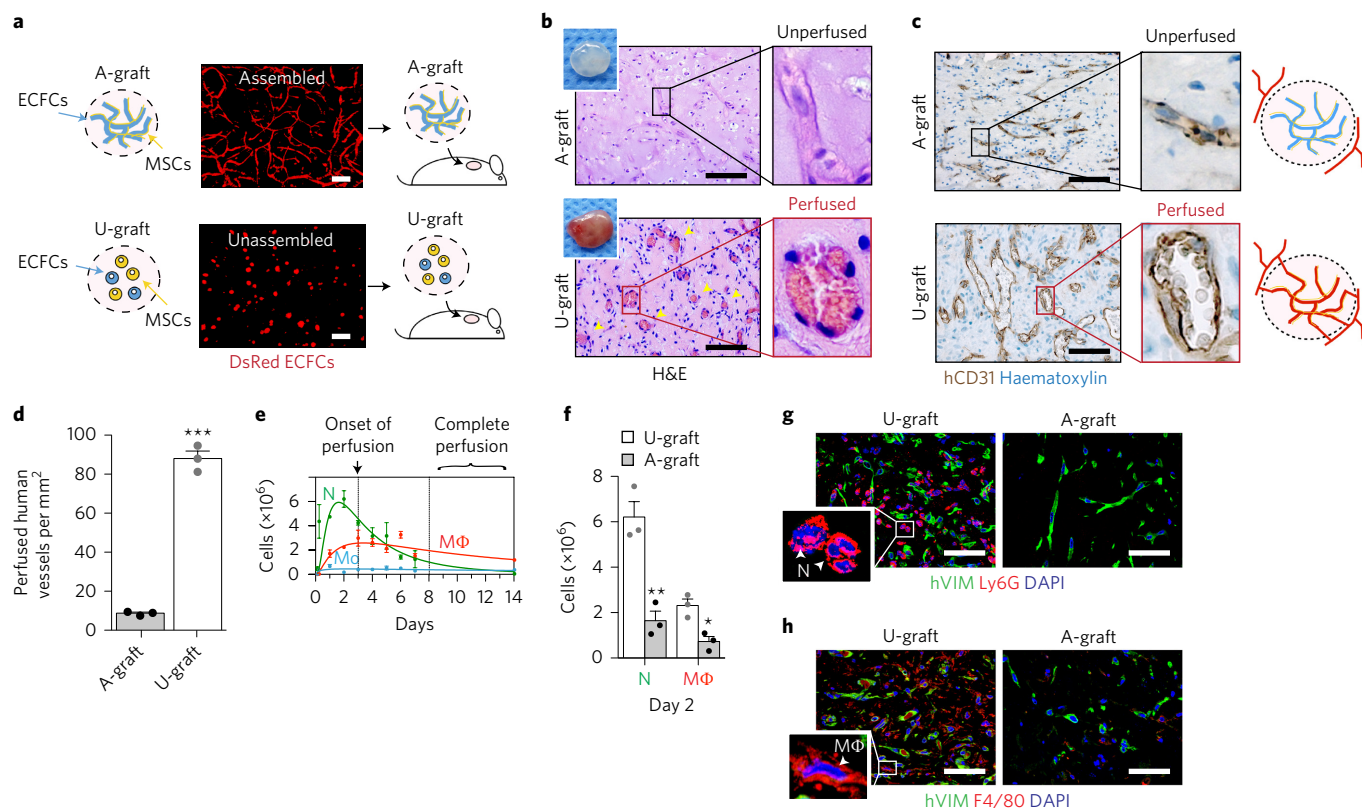


Figure 1 | Engraftment of bioengineered microvascular networks. **a**, Schematic of microvascular graft models. Grafts were prepared *in vitro* by combining human ECFCs with MSCs in hydrogels and then surgically implanted into nude mice. Assembled vascular grafts (A-grafts) were created over seven days *in vitro*, whereas unassembled grafts (U-grafts) contained a suspension of cells. **b**, Haematoxylin and eosin (H&E) staining of A-grafts and U-grafts explanted after seven days *in vivo*. Insets are macroscopic views of the indicated explants. Perfused vessels were identified as luminal structures containing red blood cells (RBCs) (arrowheads). **c**, Immunohistochemical staining of hCD31+ cells on day 7. hCD31+ vascular structures were identified as either perfused (lumens containing RBCs) or unperfused (cord structures). **d**, Density of perfused human blood vessels on day 7. Data are mean $\pm$ s.d.; $n = 3$ mice per group (indicated by individual dots). *** $P < 0.001$ between A-grafts and U-grafts. **e**, Time-course cytometric quantification of myeloid cell subpopulations in U-grafts. M Φ , macrophages; Mo, monocytes; N, neutrophils. **f**, Cytometric quantification of neutrophils and macrophages in U-grafts and A-grafts on day 2 after implantation. Data are mean $\pm$ s.d.; $n = 3$ grafts per group. ** $P < 0.01$, * $P < 0.05$ between A-grafts and U-grafts. **g,h**, Immunofluorescence staining of Ly6G+ neutrophils (**g**, red) and F4/80+ macrophages (**h**, red) in U-grafts and A-grafts on day 2 after implantation. Human cells visualized as hVIM+ cells (green). Nuclei are stained with DAPI. Scale bars, 100 μ m (**b,c**) and 50 μ m (**a,g,h**).

mice. After seven days *in vivo*, U-grafts contained an extensive network of perfused microvessels (Fig. 1b). Their lumens expressed human (h)CD31, confirming the human nature of the endothelium (Fig. 1c), and contained mouse erythrocytes, indicating connection to the host circulatory system (Fig. 1b,c and Supplementary Fig. 1). By contrast, the number of perfused human vessels in A-grafts was very low (Fig. 1d). Instead, the ECFCs remained organized as cellular cords, but these were rarely perfused (Fig. 1b,c). This lack of anastomosis formation by pre-assembled vessels in A-grafts recapitulated the indolent state observed in the microvasculature of transplanted primary tissues (both mouse and human) (Supplementary Figs 2, 3). Thus, this A-graft/U-graft model was deemed suitable for the study of the mechanisms regulating engraftment of a bioengineered vascular network in the absence of tissue parenchyma.

Host neutrophils are indispensable for vascularization. Previously, we have shown that host myeloid cells participate in the vascularization of U-grafts²⁹. In addition, we have shown that the hydrogel material used in our grafts is not immunoisolating and that host myeloid cells, including neutrophils, can freely invade our constructs²⁸. However, the mechanisms of action and the specific nature of these myeloid subpopulations have yet to be elucidated. Moreover, the role of myeloid cells in the engraftment of A-grafts is unknown. Here we identify a dynamic presence of three distinct host myeloid

subpopulations in U-grafts: (1) lymphocyte antigen 6 complex, locus G (Ly6G)⁺F4/80^{high} macrophages; (2) Ly6G⁺F4/80^{dim} monocytes; and (3) Ly6G⁺ neutrophils (Fig. 1e and Supplementary Fig. 4). Neutrophil levels peaked before the onset of perfusion (around 6×10^6 cells per graft on day 2) and then progressively faded as U-grafts became vascularized, whereas macrophages peaked around day 3 (approximately 3×10^6 cells per graft) and then remained moderately constant (Fig. 1e). Of note, there was a spatially uniform lack of both Ly6G⁺ neutrophils and macrophages throughout the A-grafts, which was in clear contrast to the abundant presence found in U-grafts (Fig. 1f–h).

To examine the role of myeloid cells in U-grafts, we devised two separate strategies to deplete either circulating monocytes (anti-F4/80 treatment) or neutrophils (anti-Ly6G) in recipient mice (Fig. 2a,b and Supplementary Fig. 4). Depletion of circulating monocytes had no significant effect on vascularization; U-grafts implanted into anti-F4/80-treated mice displayed a microvascular density similar to the control group (140.7 ± 60.6 (mean $\pm$ s.d.) vessels per mm² versus 195.6 ± 44.1 vessels per mm² in IgG-treated mice) (Fig. 2c,d). By contrast, depletion of neutrophils was detrimental to vascularization; U-grafts implanted into anti-Ly6G-treated mice had a reduced presence of perfused vessels on day 7 (Fig. 2c) and microvascular density was significantly lower (50.2 ± 16.5 vessels per mm²) than in the control group (Fig. 2d).

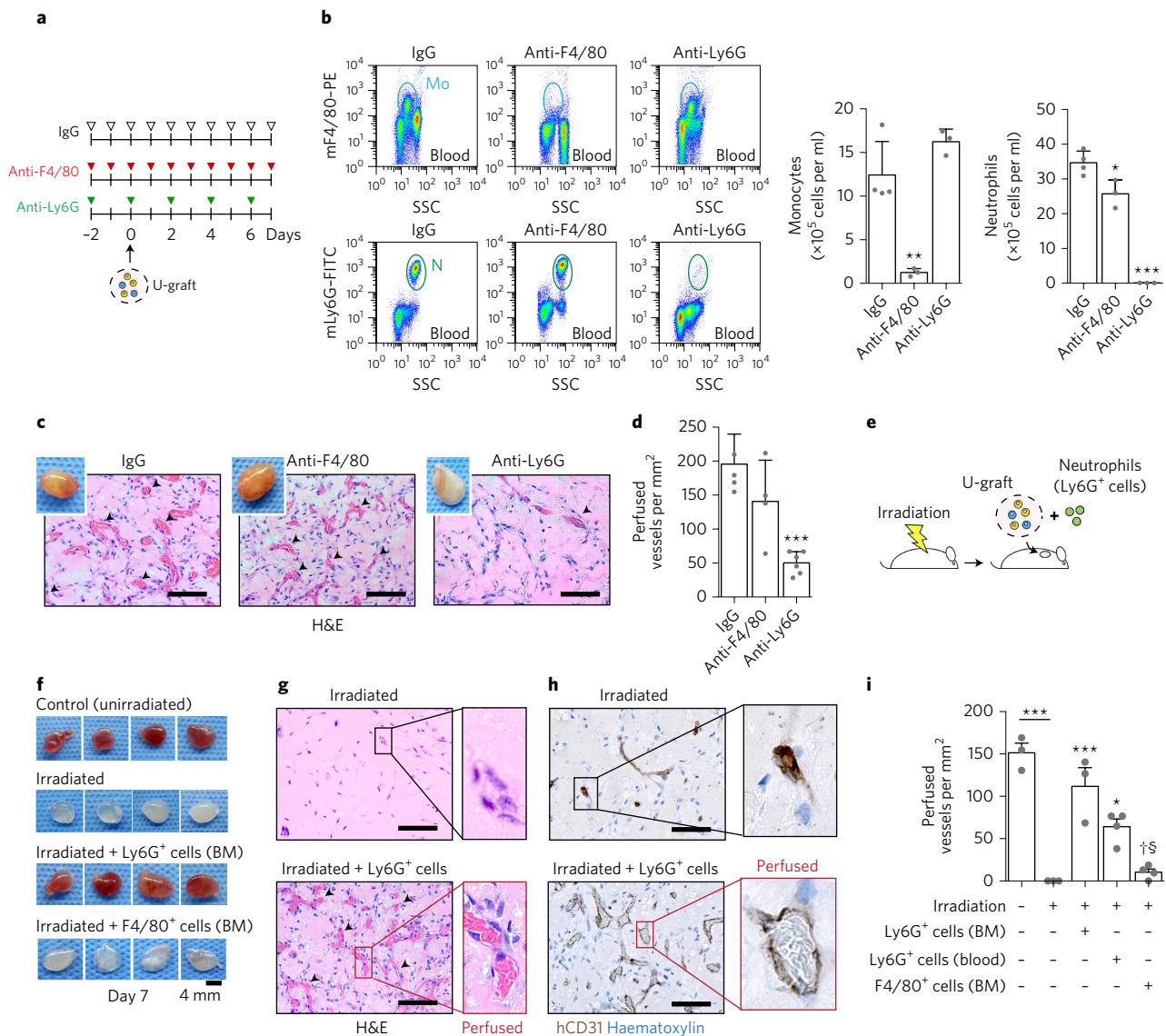


Figure 2 | Host neutrophils are indispensable for graft vascularization. **a**, Schematic with myeloid depletion strategies. Circulating monocytes depleted with anti-F4/80 antibodies (anti-F4/80). Neutrophils depleted with anti-Ly6G antibodies (anti-Ly6G). U-grafts were implanted on day 0. Control (IgG antibodies) and treatments initiated on day -2 and maintained until day 7. **b**, Flow cytometric analysis of blood samples two days after depletion treatment. Gates for circulating monocytes (Mo) and neutrophils (N) are shown. Data are mean $\pm$ s.d.; $n = 3-4$ (as indicated by individual dots). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with the IgG group. SSC, side scatter. **c**, Haematoxylin and eosin staining of U-grafts explanted on day 7. Insets are macroscopic views of the explants. Perfused vessels were identified as luminal structures containing RBCs (arrowheads). **d**, Density of perfused blood vessels on day 7. Data are mean $\pm$ s.d.; $n = 4-6$ mice per group. *** $P < 0.001$ compared with the IgG group. **e**, Schematic with the neutrophil adoptive transfer strategy. U-grafts were implanted into irradiated mice and neutrophils were simultaneously transferred from unirradiated donors. **f**, Macroscopic views of U-grafts explanted on day 7 from (from top to bottom): unirradiated mice, irradiated mice, irradiated mice combined with the transfer of bone-marrow-derived (BM) Ly6G⁺ cells and irradiate mice combined with the transfer of bone-marrow-derived F4/80⁺ cells. **g, h**, Haematoxylin and eosin (**g**) and immunohistochemical (hCD31⁺ cells) (**h**) staining of U-grafts explanted on day 7 from irradiated mice with and without bone-marrow-derived Ly6G⁺ cell transfer. Perfused vessels are marked by arrowheads. **i**, Microvessel density on day 7. Data are mean $\pm$ s.d.; $n = 3-4$ mice per group. *** $P < 0.001$, * $P < 0.05$ compared with the irradiated group (no transfer). † $P < 0.001$ compared to the irradiated group with bone-marrow-derived Ly6G⁺ cell transfer. § $P < 0.05$ compared with the irradiated group with blood-Ly6G⁺ cell transfer. Scale bars, 100 μ m (**c, g, h**).

To confirm the pivotal role of neutrophils, we devised an adoptive transfer approach with irradiated mice (radiation effectively depleted more than 90% of the circulating myeloid cells for over a week, but it was not lethal; Supplementary Fig. 5). In brief, U-grafts were implanted into irradiated mice with or without a transfer of neutrophils from unirradiated donor mice (Fig. 2e). Transferred neutrophils were an enriched population of mouse (m) CD11b⁺mLy6G⁺ cells (around 95%; Supplementary Fig. 6) obtained using a neutrophil-selection kit. U-grafts implanted into irradiated

mice completely failed to vascularize; however, an adoptive transfer of bone-marrow-derived mLy6G⁺ cells fully rescued vascularization, enabling the formation of extensive networks of perfused blood vessels (Fig. 2f–i). Transfer of blood-derived mLy6G⁺ cells also rescued vascularization, although to a lesser extent (Fig. 2i). Of note, the transfer of bone-marrow-derived F4/80⁺ cells (that is, monocytes and/or macrophages) failed to rescue vascularization (Fig. 2f, i and Supplementary Fig. 7), which underscored the distinctive capability of neutrophils. Collectively, these results confirmed

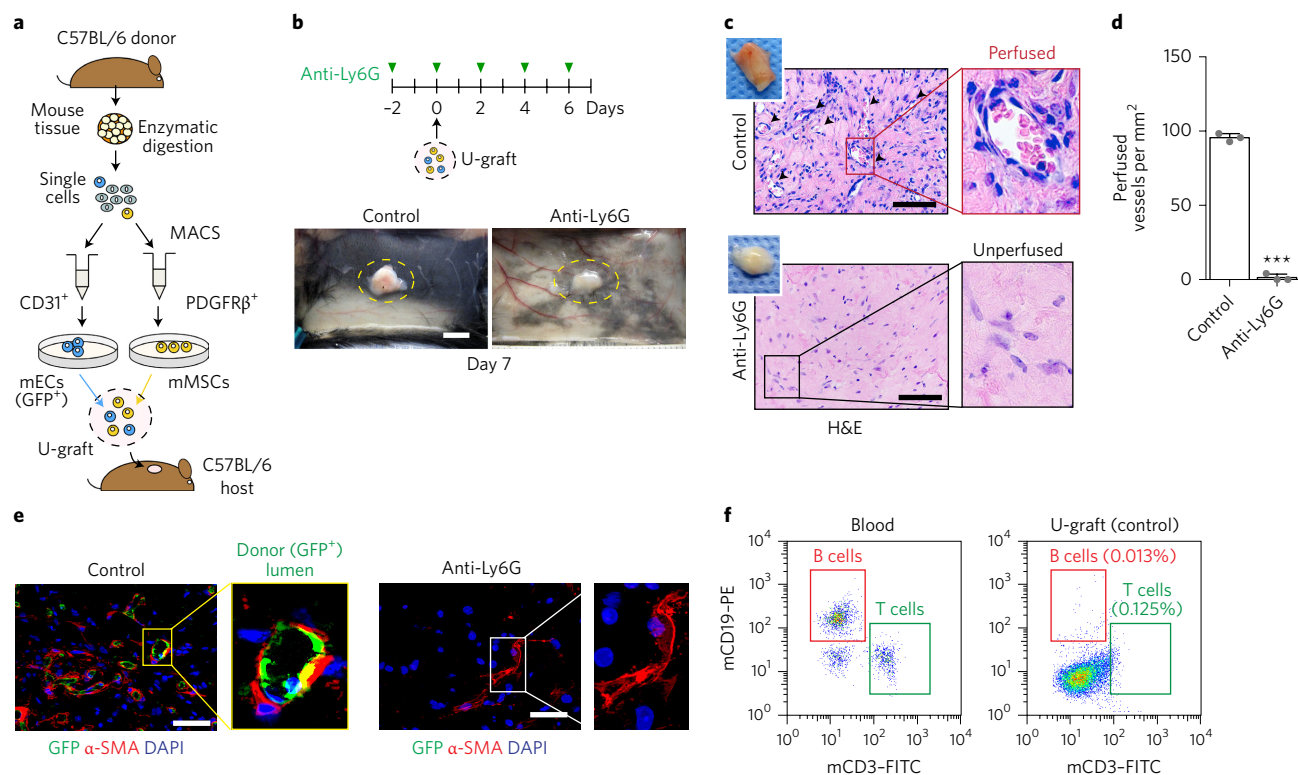


Figure 3 | Host neutrophils are indispensable for graft vascularization in a syngeneic mouse C57BL/6 model. **a**, Schematic of the syngeneic grafting model in C57BL/6 mice. Subcutaneous tissues from C57BL/6 mice were enzymatically digested into single-cell suspensions. MACS was performed to purify mECs (CD31⁺) and mMSCs (PDGFRβ⁺). mECs were lentivirally transduced to express GFP. GFP-mECs and mMSCs were then implanted as U-grafts into host C57BL/6 mice. **b**, Neutrophils were depleted with anti-Ly6G antibodies in C57BL/6 mice. U-grafts containing GFP-mECs and mMSCs were implanted on day 0. Control (IgG antibody) and anti-Ly6G antibody treatments were initiated on day -2 and maintained until day 7. Bottom: macroscopic views of the subcutaneous grafts on day 7 (dotted circles). **c**, Haematoxylin and eosin staining of U-grafts explanted on day 7. Insets are macroscopic views of the explants. Perfused vessels were identified as luminal structures containing RBCs (arrowheads). **d**, Density of perfused blood vessels on day 7. Data are mean ± s.d.; n = 3 mice per group. ***P < 0.001 compared with control. **e**, Vessels lined by donor mECs were identified by GFP and perivascular coverage by α-SMA⁺ staining. Nuclei are stained with DAPI. **f**, Flow cytometric analysis of mouse U-grafts implanted in C57BL/6 hosts. Cytometric analyses included blood samples and cells retrieved from explanted U-grafts on day 2. B cells were identified as mCD19⁺ cells and T cells as mCD3⁺. Scale bars, 4 mm (**b**) and 100 μm (**c,e**).

that host mLy6G⁺ neutrophils are indispensable for vascularization in implanted U-grafts.

Vascularization of U-grafts in immunocompetent mice. To validate the dependency of graft vascularization on host neutrophils in immunocompetent hosts, we generated unassembled U-grafts using mouse autologous endothelial cells (ECs) and MSCs isolated from C57BL/6 mice (cells referred to as mECs and mMSCs) (Fig. 3a). These mouse cells were isolated from excised subcutaneous tissues that were enzymatically digested and cells were purified by using magnetic beads with antibodies against mouse CD31 (for mECs) and platelet-derived growth factor receptor-β (PDGFRβ) (for mMSCs) using magnetic-activated cell sorting (MACS). Purified mECs and mMSCs were expanded in culture and their phenotype and purity were confirmed as we have previously described³⁰. mECs were transduced to express green fluorescent protein (GFP) (referred to as GFP-mECs) and combined with mMSCs to generate U-grafts, which were then subcutaneously implanted into immunocompetent C57BL/6 mice. Implants were collected on day 7 and histological (haematoxylin and eosin) examination revealed that extensive networks of blood microvessels had formed and that these contained mouse erythrocytes, thereby confirming perfusion (Fig. 3b,c). Microvessels were lined by the implanted GFP-mECs as confirmed by GFP immunostaining (Fig. 3e). In addition, GFP⁺ microvessels were mostly covered

by α-smooth muscle actin (α-SMA)-expressing perivascular cells, indicating stability (Fig. 3e). Taken together, these data demonstrate that U-grafts containing mouse cells (mECs and mMSCs) can form microvascular networks in fully immunocompetent hosts (C57BL/6 mice).

Next, we examined whether neutrophils were also indispensable for vascularization in immunocompetent hosts (Fig. 3b). As in the xenograft model, depletion of neutrophils was detrimental for vascularization in syngeneic grafts; mouse U-grafts that were implanted into anti-Ly6G-treated C57BL/6 mice completely lacked perfused vessels (neither donor nor host) on day 7 (Fig. 3c,e) and microvascular density was negligible and significantly lower (1.4 ± 1.3 vessels per mm²) than in the control group (95.6 ± 4 vessels per mm²) (Fig. 3d).

In addition, we examined the presence of lymphocytes in mouse U-grafts implanted into immunocompetent C57BL/6 hosts. To this end, grafts were explanted on day 2, enzymatically digested, and the retrieved cells were analysed by flow cytometry. We examined the presence of mCD45⁺mCD19⁺ B cells and mCD45⁺mCD3⁺ T cells and found that the presence of both B and T cells in the grafts was minimal (less than 0.2%) and significantly lower than the myeloid contribution (mCD11b⁺ cells were around 56.7% on day 2 in syngeneic mouse U-grafts implanted into C57BL/6 mice) (Fig. 3f). Indeed, U-grafts implanted into anti-Ly6G-treated mice failed to vascularize despite normal levels of circulating lymphocytes.

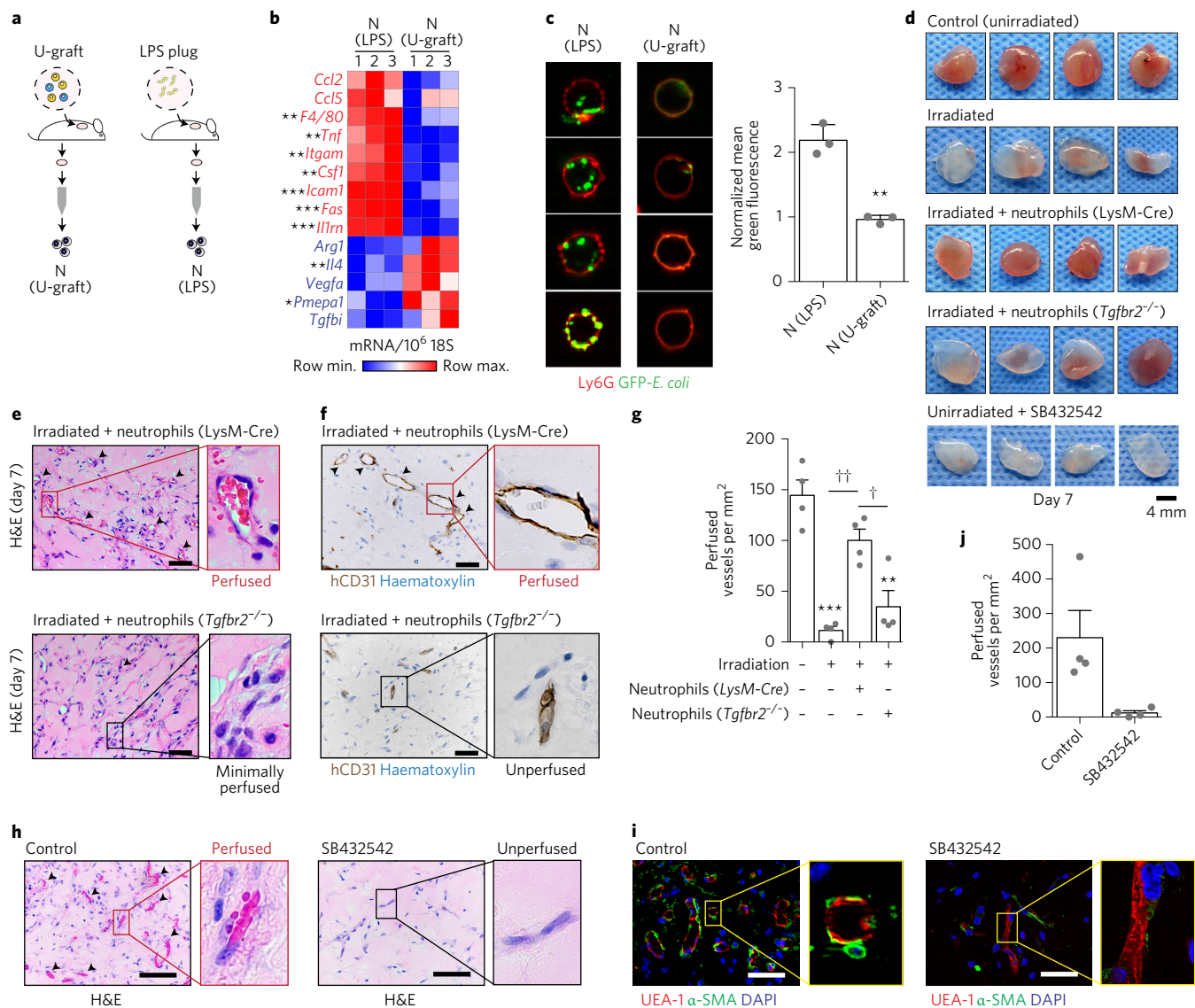


Figure 4 | Alternatively polarized neutrophils mediate U-graft vascularization. **a**, Schematic of flow cytometric sorting of neutrophils (N) isolated from either explanted (day 1) U-grafts or from LPS-containing plugs. **b**, Heat map of gene expression levels (by qRT-PCR) in neutrophils from U-grafts or LPS plugs. Primers were mouse-specific (Supplementary Table 2). mRNA data are row-relative and normalized to ribosomal 18S rRNA. $n = 3$ mice per group. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ between U-graft and LPS groups. **c**, Representative Ly6G⁺ (red) neutrophils from U-grafts and LPS plugs after exposure to GFP-*E. coli* (green). Phagocytosis of GFP-*E. coli* was visualized under a fluorescence microscope and quantified by flow cytometry. Data are the normalized mean green fluorescence $\pm$ s.d. measured in gated Ly6G⁺ neutrophils; $n = 3$ mice per group. ** $P < 0.01$ between U-graft and LPS groups. **d**, Macroscopic images of U-grafts explanted on day 7 from unirradiated mice, irradiated mice, irradiated mice with a transfer of bone-marrow-derived *LysM-Cre* neutrophils, irradiated mice with a transfer of bone-marrow-derived *Tgfb2*^{-/-} neutrophils, and unirradiated mice + TGF β receptor inhibitor (SB432542). **e**, Haematoxylin and eosin and **f**, immunohistochemical (hCD31⁺ cells) staining of U-grafts explanted on day 7 from irradiated mice with transfer of either bone-marrow-derived *LysM-Cre* or bone-marrow-derived *Tgfb2*^{-/-} neutrophils. Perfused vessels are marked by arrowheads. **g**, Microvessel density on day 7. Data are mean $\pm$ s.d.; $n = 4$ mice per group. *** $P < 0.001$, ** $P < 0.01$ compared with the unirradiated control group. [†] $P < 0.05$ and ^{††} $P < 0.01$ between indicated groups. **h–j**, U-grafts were implanted in mice treated with SB432542. Mice receiving saline injections served as controls. **h**, Haematoxylin and eosin staining of explanted U-grafts on day 7. **i**, Immunofluorescence staining showed the presence of human vessels (UEA-1⁺ lumen) and perivascular coverage (α -SMA⁺ cells) in the control group; and unperfused human endothelial cord structures in SB432542-treated grafts. **j**, Microvessel density quantified on day 7. Data are mean $\pm$ s.d.; $n = 4$ mice per group. * $P < 0.05$. Scale bars, 100 μ m (e,f,h,i).

In summary, we demonstrated that (1) syngeneic mouse U-grafts did vascularize in immunocompetent host; (2) neutrophil participation was critical for the vascularization of syngeneic U-grafts; and (3) lymphocyte presence in the grafts was minimal.

Alternatively polarized neutrophils mediate vascularization. Recent evidence has suggested that neutrophils can acquire an alternative non-inflammatory phenotype (referred to as N2) that mediates tissue

remodelling³¹. Here, we compared neutrophils retrieved from explanted U-grafts to inflammatory neutrophils isolated from lipopolysaccharide (LPS)-containing plugs (Fig. 4a). Neutrophils in U-grafts did not resemble LPS-activated neutrophils in several respects, including: (1) lower mRNA expression of canonical pro-inflammatory genes (namely, *Ccl2*, *Ccl5*, *F4/80* (also known as *Adgre1*), *Tnf*, *Itgam*, *Csf1*, *Icam1*, *Fas* and *Il1rn*); (2) higher expression of anti-inflammatory (*Arg1* and *Il4*) and pro-remodelling

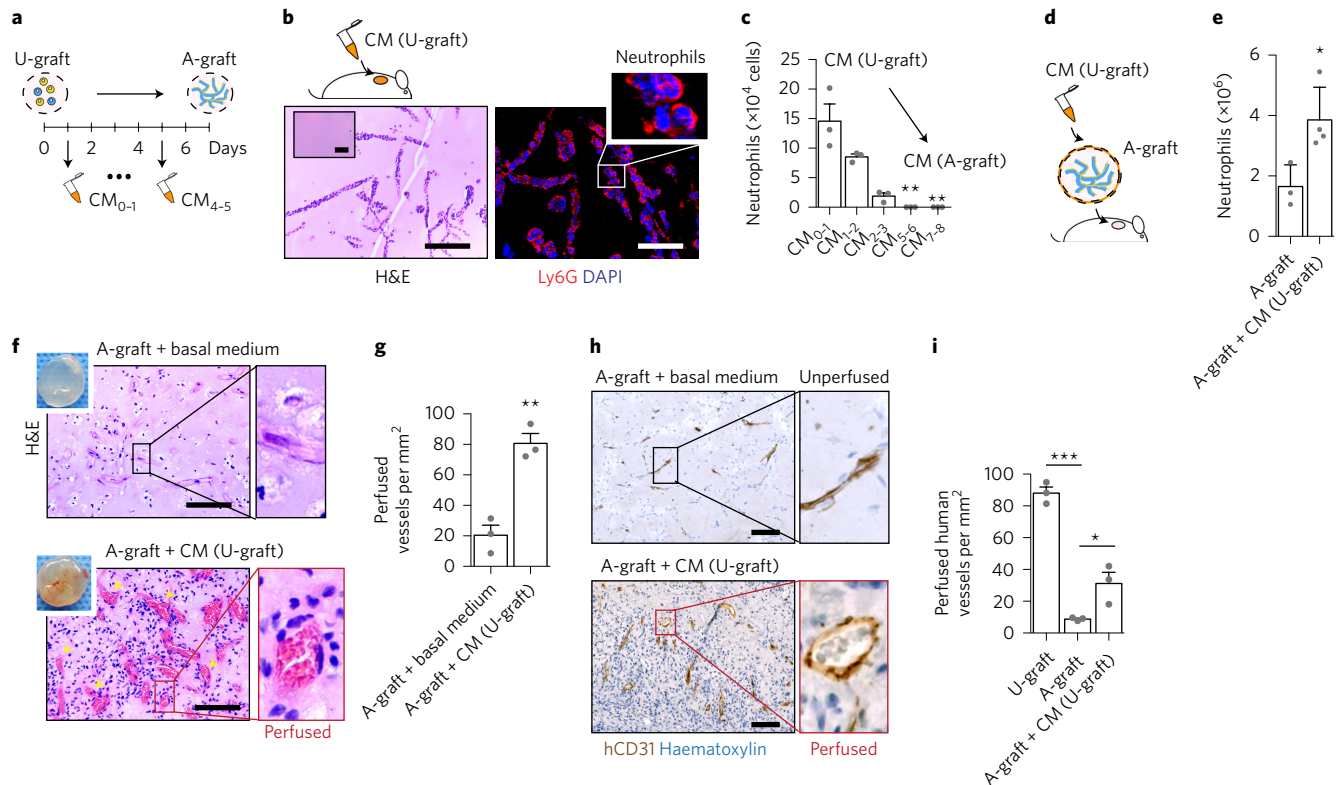


Figure 5 | Neutrophil activity regulated by secreted factors from the graft vasculature. **a**, Schematic of daily samples of conditioned medium collected from U-grafts until they became A-grafts. CM, indicates the day of collection of the conditioned medium. **b**, Haematoxylin and eosin (left) and immunofluorescence (Ly6G, DAPI; right) staining revealing infiltration of neutrophils on day 2 into implanted Matrigel plugs containing conditioned medium from U-grafts. Haematoxylin and eosin inset corresponds to a plug containing control basal medium. **c**, Flow cytometric quantification of neutrophil infiltration on day 2 into plugs containing conditioned medium collected at the indicated times. Data are mean $\pm$ s.d.; $n = 3$ plugs per group. **d**, Schematic of A-grafts infused in conditioned medium from U-grafts and implanted into nude mice. **e**, Flow cytometric quantification of neutrophil infiltration on day 2 into A-grafts that were infused with and without conditioned medium from U-grafts. **f**, Haematoxylin and eosin staining on day 7 of explanted A-grafts that were infused with either conditioned medium from U-graft or basal medium. Insets are macroscopic views of the explants. Perfused vessels are marked by arrowheads. **g**, Microvessel density on day 7. Data are mean $\pm$ s.d.; $n = 3$ mice per group. **h**, Immunohistochemical staining (hCD31⁺ cells) on day 7 in explanted A-grafts that were infused with either conditioned medium from U-graft or basal medium. Perfused human hCD31⁺ vascular structures were identified as lumens containing RBCs. **i**, Density of perfused human microvessels on day 7. Data are mean $\pm$ s.d.; $n = 3$ mice per group. ******* $P < 0.001$, ***** $P < 0.05$ between indicated groups. Scale bars, 100 μ m (**b** (left), **f**, **h**) and 50 μ m (**b** (right)).

(*Vegfa*, *Pmepa1* and *Tgfb1*) genes (Fig. 4b); and (3) lower phagocytic activity (Fig. 4c). Previous studies have shown that N2 polarization is mediated by transforming growth factor- β (TGF β) signalling³¹. To elucidate the potential role of TGF β , we adoptively transferred neutrophils lacking TGF β -receptor 2 (*Tgfb2*^{-/-}; from *LysM-Cre::Tgfb2/loxP* donors) into myeloid-depleted (irradiated) nude mice and examined the ability of U-grafts to vascularize (Fig. 4d–g). *Tgfb2*^{-/-} neutrophils were not able to restore vascularization to the same extent as control neutrophils (34.7 ± 15.9 vessels per mm² versus 100.2 ± 11 vessels per mm² with control *LysM-Cre* neutrophils) (Fig. 4g), indicating that the pro-vascularization potential of neutrophils was dependent on *Tgfb2* expression. To further confirm the need for TGF β signalling, we evaluated the vascularization potential of U-grafts in mice that were treated daily with SB432542, a potent and selective inhibitor of the TGF β type I receptor. We found that treatment with SB432542 impaired the formation of perfused vascular networks. Indeed, the number of perfused vessels on day 7 in U-grafts from SB432542-treated mice was very low (Fig. 4j); although the ECFCs appeared organized as cellular cords, these cords were rarely perfused (Fig. 4h,i).

Together, our results show that neutrophils from the U-graft display a distinctive non-inflammatory phenotype that is consistent with the N2 phenotype; this alternative polarization was dependent

on TGF β R–TGF β signalling, and in turn was essential for the pro-vascularization function of neutrophils. Moreover, blocking TGF β signalling by exogenous treatment with SB432542 completely abrogated U-graft vascularization.

Neutrophils activity is regulated by secreted factors. To gain further insight into how grafts engage host neutrophils, we collected daily samples of conditioned medium from U-grafts over 8 days *in vitro* (that is, until they became A-grafts) (Fig. 5a). Subcutaneous plugs containing conditioned medium from U-grafts robustly recruited neutrophils in nude mice (Fig. 5b)—note that the neutrophil recruitment by conditioned medium from U-grafts was significantly higher than by conditioned medium from ECFCs and from MSCs separately (Supplementary Fig. 8). However, plugs containing conditioned medium from A-grafts had no detectable neutrophils, a decrease that occurred progressively and coincided with the assembly of cells into vascular networks (Fig. 5c). Moreover, human cells retrieved from A-grafts could immediately regain the ability to recruit neutrophils when used in new U-grafts (Supplementary Fig. 8). Thus, neutrophil recruitment was mediated by secreted factors and was directly related to the state of the vascular cells (assembled versus unassembled) within the grafts.

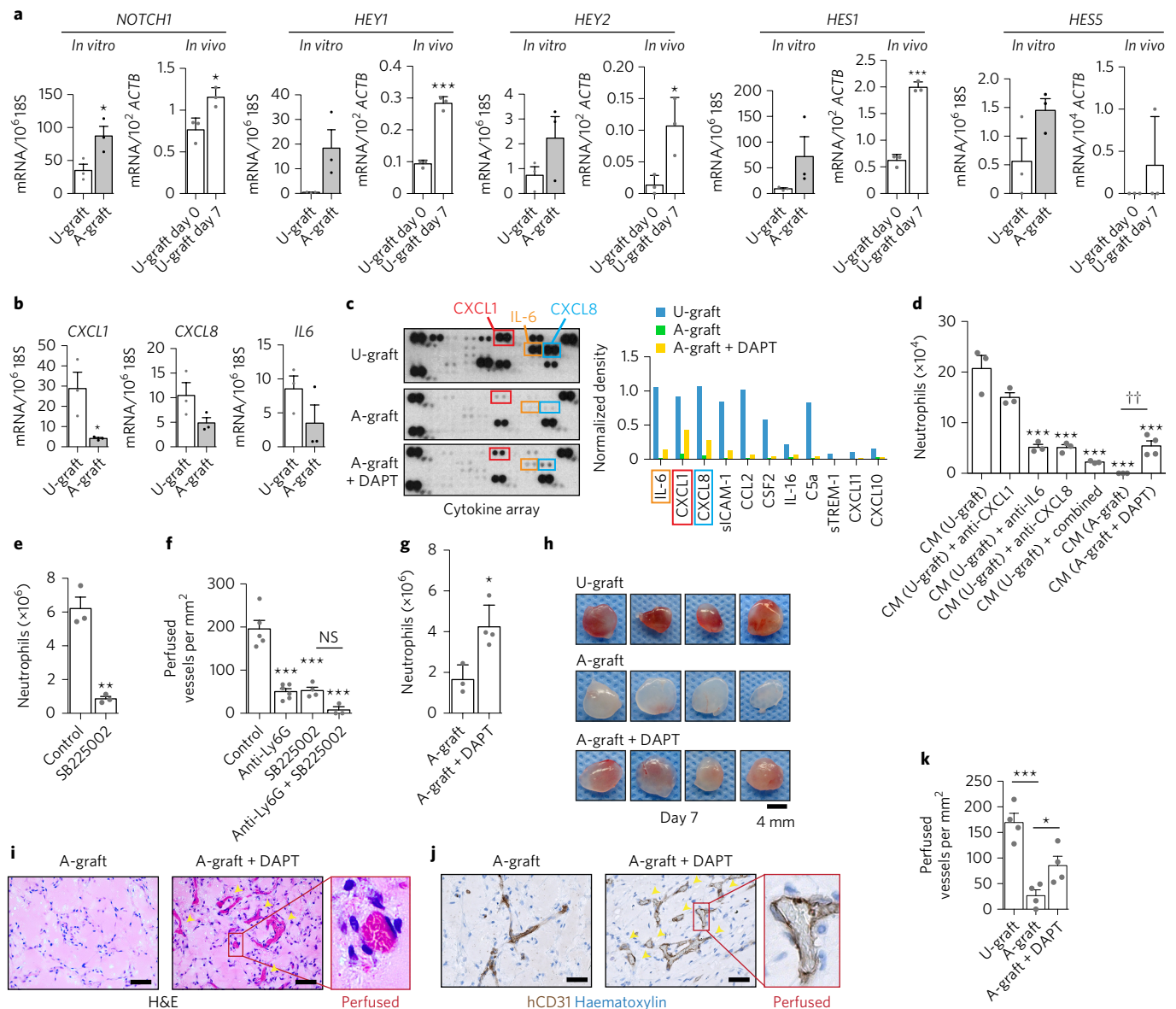


Figure 6 | Notch inhibition promotes A-graft revascularization. **a**, mRNA gene expression (qRT-PCR) of *NOTCH1* and downstream mediators of the Notch signalling pathway (*HEY1*, *HEY2*, *HES1* and *HES5*). *In vitro*, data from ECFCs retrieved from U-grafts and A-grafts and normalized to ribosomal 18S rRNA. *In vivo*, data from total mRNA isolated from U-grafts 30 min (day 0) and seven days after implantation. Data normalized to human β -actin (*ACTB*). Data are mean $\pm$ s.d.; $n = 3$ grafts per group. *** $P < 0.001$, * $P < 0.05$. **b**, Expression of *CXCL1*, *CXCL8* and *IL6* in ECFCs retrieved from U-grafts and A-grafts and normalized to ribosomal 18S rRNA. **c**, Human cytokine array analysis of conditioned medium from U-grafts, A-grafts and A-grafts treated with DAPT (γ -secretase inhibitor; 24 h exposure). Selected cytokines marked with coloured outlines. Quantification of cytokine levels were carried out by densitometry. **d**, Blocking neutrophil recruitment by neutralizing antibodies in subcutaneous plug assays. Neutralizing antibodies against IL6, CXCL8, CXCL1 or a combination of all three antibodies (combined) were added to conditioned medium from U-grafts before implantation. The number of neutrophils recruited into the plugs was measured by flow cytometry on day 2. Data are mean $\pm$ s.d.; $n = 3-4$ mice per group. *** $P < 0.001$ compared with conditioned medium from U-grafts. †† $P < 0.01$ between indicated groups. **e, f**, Effect of the CXCR2 antagonist (SB225002) on neutrophil recruitment and U-graft vascularization. U-grafts implanted in untreated mice served as controls. **e**, Neutrophil recruitment into U-grafts on day 2 measured by flow cytometry. **f**, Density of perfused microvessels on day 7. Data mean $\pm$ s.d.; $n = 3-6$ mice per group. *** $P < 0.001$, ** $P < 0.01$ compared with control. **g**, Neutrophil recruitment on day 2 in A-grafts and A-grafts with DAPT. Data are mean $\pm$ s.d.; $n = 3-4$ mice per group. * $P < 0.05$. **h**, Macroscopic views of U-grafts, A-grafts and A-grafts with DAPT, explanted on day 7. **i**, Haematoxylin and eosin and (**j**) immunohistochemical (hCD31⁺ cells) staining of A-grafts and A-grafts with DAPT explanted on day 7. Perfused vessels identified as luminal structures containing RBCs (arrowheads). **k**, Microvessel density on day 7. Data are mean $\pm$ s.d.; $n = 4$ mice per group. *** $P < 0.001$, * $P < 0.05$ between indicated groups. Scale bars, 50 μ m.

Next, we studied whether exposing A-grafts to conditioned medium from U-grafts could therefore enhance the engraftment of assembled bioengineered vessels (Fig. 5d). First, we found that A-grafts that were simply infused once with conditioned medium from U-grafts at the time of implantation contained significantly more neutrophils on day 2 than the control (Fig. 5e). In addition,

A-grafts infused with conditioned medium from U-grafts contained an extensive network of perfused microvessels on day 7 (Fig. 5f,g). Moreover, the extent to which conditioned medium from U-grafts induced revascularization of A-grafts was significantly higher than that of conditioned medium from ECFCs and MSCs separately (Supplementary Fig. 9). Examination of human-specific (hCD31⁺)

lumens revealed that A-grafts infused with conditioned medium from U-grafts comprised perfused human microvessels that carried mouse erythrocytes, indicating successful connection of pre-assembled vessels to the host circulatory system (Fig. 5h). By contrast, A-grafts infused with basal medium had a significantly lower number of perfused human lumens on day 7 (8.7 ± 0.6 vessels per mm^2 versus 31.2 ± 7.0 vessels per mm^2 in A-grafts infused with conditioned medium from U-grafts) (Fig. 5i). Instead, human ECFCs remained organized as unperfused cellular cords (Fig. 5f,h). Therefore, a major difference between U-grafts and A-grafts is the presence or absence of secreted factors that recruit and activate host neutrophils, which in turn modulate graft vascularization. Notably, exogenous provision of factors from U-grafts could reengage neutrophils and enhance revascularization in A-grafts.

Inhibition of Notch signalling promotes revascularization.

Microvascular stability is associated with activation of Notch in the endothelium^{32,33}. However, the relation between endothelial Notch signalling and neutrophil activity in the context of grafting remains largely unknown. mRNA expression of *NOTCH1*, as well as several downstream mediators of Notch signalling (*HEY1*, *HEY2*, *HES1* and *HES5*), was upregulated in ECFCs from A-grafts compared to U-grafts (Fig. 6a). Moreover, expression of human-specific genes (*NOTCH1*, *HEY1*, *HEY2* and *HES1*) were all significantly upregulated by the human cells in implanted U-grafts over a seven-day period, confirming the association between vascular maturity and Notch expression *in vivo* (Fig. 6a). Inversely, expression levels of several cytokines with neutrophil chemoattractant activity (namely, *CXCL1*, *CXCL8* and *IL6*) were downregulated in ECFCs from A-grafts (Fig. 6b), which coincided with reduced neutrophil presence. Cytokine analyses of conditioned medium confirmed widespread downregulation of numerous neutrophil chemoattractants in A-grafts compared to U-grafts (Fig. 6c). Of note, exposing A-grafts to the Notch inhibitor DAPT (a γ -secretase inhibitor) for 24 h reactivated the expression of several cytokines (most notably *CXCL1*, *CXCL8*, and *IL6*) (Fig. 6c), suggesting that there is a relationship between Notch activation and the secretion of neutrophil chemoattractants by the vasculature. Moreover, the use of antibodies to specifically block each of the identified cytokines (most notably *IL6* and *CXCL8*) in conditioned medium from U-grafts interfered with the recruitment of neutrophils into subcutaneous plugs (Fig. 6d), whereas plugs containing conditioned medium from DAPT-treated A-grafts recruited significantly more neutrophils than plugs with conditioned medium from untreated A-grafts (Fig. 6d).

To further elucidate the importance of secreted cytokines, we examined the effect of a non-peptide CXCR2 antagonist (SB225002) on the vascularization of U-grafts. CXCR2 is a receptor for both *CXCL8* and *CXCL1*, and previous studies have shown that SB225002 effectively inhibits neutrophil chemotaxis in response to *CXCL8* both *in vitro* and *in vivo*³⁴. Indeed, SB225002 treatment significantly reduced the presence of neutrophils in our implanted U-grafts on day 2 (Fig. 6e). Notably, U-grafts that were implanted into SB225002-treated mice had significantly lower microvessel densities than those implanted into control animals (Fig. 6f). To put this result in context, SB225002-treatment produced a reduction in vascularity that was similar to the reduction produced by depletion of host neutrophils with an anti-Ly6G antibody (Fig. 6f). Moreover, a combined treatment with both an anti-Ly6G antibody and SB225002 further reduced microvessel density in U-grafts (decreased to around 8 vessels per mm^2), although the differences in reduction between the combination and each individual treatment were not statistically significant. Nevertheless, this additional reduction in microvessel density due to the combined treatment might suggest participation of an additional type of host CXCR2⁺ cells that, together with neutrophils, mediate graft vascularization.

Next, we examined whether deactivation of Notch could rescue the inherent lack of revascularization in A-grafts. To this end, we incubated A-grafts with DAPT for 24 h before the implantation into mice. Compared to untreated grafts, DAPT-treated A-grafts had significantly more neutrophils on day 2 (Fig. 6g). In addition, DAPT-treated A-grafts contained more extensive networks of perfused microvessels that were unequivocally of human origin after seven days *in vivo* (Fig. 6h–j), and the difference in microvascular density between DAPT-treated and untreated grafts was significant (Fig. 6k). Notably, human vessels in DAPT-treated A-grafts were surrounded by perivascular α -SMA⁺ cells (Supplementary Fig. 10), indicating that the transient inhibition of Notch signalling did not compromise proper perivascular coverage.

Discussion

In this study, we determined the cellular mechanisms impairing engraftment of bioengineered human vascular networks. We show that the inefficient formation of anastomoses in assembled microvessels (A-grafts) is due to an inherent inability of mature vessels to engage a subset of alternatively polarized (non-inflammatory) host neutrophils at the implantation site. This contrasted with grafts comprising a non-assembled suspension of vascular cells (U-grafts), which rapidly engage neutrophils to mediate proper vascularization. Depletion of host neutrophils significantly abrogated vasculogenesis in U-grafts, whereas an adoptive transfer of neutrophils restored vascularization in myeloid-depleted mice. The dependency on host neutrophils was complete and corroborated in both syngeneic (mouse grafts implanted into mice) and xenograft (human into mouse) models. Neutrophil recruitment and activation were controlled by factors secreted from the implanted vascular cells, a process initially upregulated in unassembled U-grafts, but progressively silenced as the vasculature matured. This mechanism of progressive neutrophil disengagement could explain the inactive nature of assembled microvasculatures—both bioengineered and primary—with regards to their capacity to engraft and connect to host vessels.

Emerging evidence indicate that neutrophils can comprise two distinct subpopulations with different polarized phenotypes: a canonical pro-inflammatory phenotype (referred to as N1) and an alternative anti-inflammatory, pro-remodelling, phenotype (N2)^{31,35,36}, resembling the well-established 'M1–M2' macrophage paradigm. N2 polarization has been substantiated in a variety of tumour models, and it involves TGF β -mediated activation (TGF β inhibition produces a shift to N1, and in turn acquisition of anti-tumour activity), followed by downregulation of pro-inflammatory genes and expression of both anti-inflammatory factors and pro-angiogenic mediators^{31,37,38}. Recently, the contribution of alternatively polarized N2 neutrophils has also been described in non-neoplastic events, including stroke³⁹ and myocardial infarction⁴⁰. However, their participation in tissue grafting has not been described previously. Here, we show that graft neutrophils display a phenotype that is consistent with an alternative N2 polarization state in several respects, including low expression of canonical pro-inflammatory genes, high expression of anti-inflammatory and pro-remodelling genes, low phagocytic activity and TGF β R2-mediated activation. We also found that adoptive transfer of neutrophils from bone marrow restored vascularization in myeloid-depleted mice to a better extent than blood neutrophils, which is consistent with published evidence of immature non-inflammatory neutrophils (equivalent to N2 neutrophils) being more abundant in the bone marrow than in the circulation³⁷.

Although our study focused on the role of neutrophils in graft vascularization, our implants also contained host macrophages and monocytes. Macrophage presence was expected in light of their role as cellular chaperones for vascular anastomosis during angiogenesis⁴¹. Furthermore, macrophage–neutrophil interdependency during angiogenesis is well established^{42–44}. In our studies,

the indispensable role of neutrophils in graft vascularization did not preclude participation of host macrophages, which were also abundant at the onset of perfusion. However, we demonstrated that in the absence of neutrophils, macrophage involvement in vascularization was undoubtedly insufficient. With regard to host monocytes, our results were equally conclusive: depletion of circulating monocytes had no appreciable effect on graft vascularization and adoptive transfer of bone-marrow-derived F4/80⁺ cells could not restore normal vascular activity in myeloid-depleted mice. Our results with the combined treatment with anti-Ly6G (neutrophil depletion) and SB225002 (CXCR2 inhibitor) showed that this combination reduced vascularization in U-grafts more than either of the treatments alone, although the differences were not statistically significant. Nevertheless, the additional reduction in the density of microvessels with the combined treatment might indicate the participation of an additional type of host CXCR2⁺ cells in vascularization, suggesting that neutrophils probably are not solely responsible for the vascularization of the grafts. Further research is warranted to confirm a role and to elucidate the nature of additional host CXCR2⁺ cells that may contribute to the process of graft vascularization.

Prior to our study, the mechanisms regulating engraftment of bioengineered microvessels remained essentially unexplored. Nevertheless, some recent studies did report positive integration between engineered and host vessels. Baranski and colleagues bioengineered vascular networks by the assembly of human ECs (human umbilical vein endothelial cells) and mouse embryonic perivascular cells (10T1/2 cell line), and demonstrated perfusion with host blood upon implantation into the intraperitoneum of nude mice⁴⁵. More recently, Riemenschneider *et al.* constructed patches containing self-assembled microvessels formed by co-entrapment of ECFCs and human fetal brain pericytes in fibrin gel. These patches were sutured onto the epicardial surface of the hearts of athymic rats following permanent ligation of the left anterior descending artery; approximately 25% of the bioengineered vessels were found to be perfused⁴⁶. Therefore, the poor engraftment seen with our bioengineered vessels might seem inconsistent with these previous reports. However, it is important to note that the sources of perivascular cells in the aforementioned studies were from embryonic and fetal tissues, respectively. Considering that xenograft studies have shown that embryonic (but not adult) vasculature can form spontaneous connections with the host⁴⁷, it is conceivable that the sole presence of fetal cells could facilitate the formation of anastomoses. In any case, bioengineering tissues with non-autologous fetal cells poses additional challenges related to immune tolerance and may therefore have reduced translational potential. Further studies are warranted to elucidate whether host neutrophils also mediate vascular anastomoses in bioengineered vessels that use embryonic or fetal cells.

Harnessing the pro-vascularization potential of neutrophils could become the basis for a new strategy to engraft bioengineered tissues. Moreover, this approach could potentially benefit grafting clinical procedures that critically rely on adequate revascularization. Clinical and preclinical studies have repeatedly shown that perfused vessels in adult primary tissue grafts are almost exclusively originated by infiltration of host vessels. However, this process of revascularization poses two main constraints on the grafts: (1) inadequate blood supply to the centre, which leads to necrosis and resorption; and (2) irreversible loss of graft endogenous vasculature. The latter is important because mounting evidence indicates that most tissues have highly specialized endothelium that regulates homeostatic and regenerative processes in a tissue-specific manner^{48–52}. Therefore, loss of endogenous vessels could contribute to long-term malfunctioning of the grafts. Enabling a robust integration of the graft microvasculature could eliminate the abovementioned constraints. In light of our study, new strategies to improve microvascular engraftment could focus on harnessing non-inflammatory

neutrophils by exposing the grafts to specific exogenous vascular factors and/or by blocking Notch signalling, which could reactivate endogenous mechanisms in mature vessels. We anticipate that future studies will address whether engaging host neutrophils would indeed improve revascularization of primary tissue grafts and how this strategy would ultimately affect parenchymal function in the engrafted tissues.

Methods

Mice. NU/NU (BALB/c background; Massachusetts General Hospital) and C57BL/6 male mice (Jackson Laboratory) were used for the implantation of human and syngeneic mouse grafts, respectively, unless stated otherwise. Rosa26^{trp} (129S1 background) and Cdh5(Pac)-CreERT2⁺ (C57BL/6 background) transgenic mice^{53,54} were interbred and offspring were given two consecutive intragastric injections of 50 µl tamoxifen (2 mg ml⁻¹ in sunflower-seed oil) on postnatal day (P)1 and P2 to induce Cre-recombinase activity. The resulting Rosa26^{trp};Cdh5(Pac)-CreERT2⁺ mice served as donors and their tissues expressed an eGFP-L10a fusion protein in endothelial cells. For myeloid-specific *Tgfb2* deletion, *LysM-Cre* transgenic mice (Jackson Laboratory, JAX004781) were interbred with conditional *Tgfb2*^{lox/lox} mutant mice (JAX012603; both C57BL/6 background). The offspring were interbred again and genotyped to select homozygous *Tgfb2*^{-/-} (*LysM-Cre*^{+/+}*Tgfb2*^{lox/lox}) alleles. The myeloid-specific deletions of *Tgfb2* were confirmed by PCR. Animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee at Children's Hospital Boston in an AAALAC-approved facility.

Transplantation of primary mouse tissue grafts. Six-week-old Rosa26^{trp};Cdh5(Pac)-CreERT2⁺ transgenic mice served as donors and were perfused with PBS before being euthanized. Myocardium, liver and kidney were collected and trimmed into 3 × 3 × 3 mm³ grafts. Tissue grafts were then surgically implanted into the subcutaneous space on the back of nude mice with 50 µl of collagen–fibrin gel (3 mg ml⁻¹ of bovine collagen, 30 µg ml⁻¹ of human fibronectin, 25 mM HEPES, 10% 10× DMEM, 10% FBS, and 3 mg ml⁻¹ of fibrinogen, pH neutral; mixed with 10 µl of 10 U ml⁻¹ thrombin immediately before implantation). After seven days *in vivo*, fluorescein isothiocyanate (FITC)-conjugated *Griffonia simplicifolia* lectin I (GSL I) isolectin B4 in saline (Iso-B4; 50 µg per 100 µl per mouse) was infused intravenously 10 min before euthanasia. Grafts were explanted and subjected to histological analyses. Anti-FITC and anti-GFP antibodies were used to stain perfused and donor vessels, respectively.

Transplantation of primary human myocardial tissue grafts. Discarded normal human right ventricular myocardial tissues were obtained during clinically indicated procedures in accordance with a protocol approved by the Institutional Review Board. Myocardial tissues were immediately transported to the laboratory in cold sterile saline, trimmed into 3 × 3 × 3 mm³ cubes, and surgically implanted (subcutaneously) into nude mice. 50 µl of collagen–fibrin gel was added around myocardial grafts to improve integration with host tissues. Seven days after implantation, FITC-conjugated *Ulex europaeus* agglutinin-I in saline (UEA-I; 50 µg per 100 µl per mouse) was infused intravenously 10 min before euthanasia. Grafts were explanted and subjected to histological analyses. Perfused human vessels were stained by biotinylated-anti-UEA-I antibody and streptavidin–TexasRed.

Isolation and culture of human MSCs and ECFCs. Human MSCs were isolated from the mononuclear cell fraction of bone marrow aspirates as previously described⁷. MSCs were cultured on uncoated plates using MSC medium: MSCGM (Lonza) supplemented with 10% MSC-qualified FBS (Hyclone), 1× glutamine–penicillin–streptomycin (GPS; Invitrogen). All experiments were carried out with MSCs between passages 6–10. Human ECFCs were isolated from umbilical cord blood samples in accordance with a protocol approved by the Institutional Review Board as previously described²⁷. ECFCs were cultured on 1% gelatin-coated plates using ECFC medium: EGM-2 (except for hydrocortisone; Lonza) supplemented with 20% FBS, 1× GPS. All experiments were carried out with ECFCs between passages 6–8. DsRed-labelled ECFCs were generated by lentiviral infection with a pLVX-DsRed vector (Clontech) and selected with puromycin (2 µg ml⁻¹)⁵.

Fabrication and implantation of bioengineered grafts. Photopolymerizable methacrylated gelatin (GelMA) hydrogel was synthesized as previously described⁵. GelMA prepolymer solution was prepared in PBS at 80 °C (5% w/v final) with photoinitiator Irgacure 2959 (0.5% w/v; CIBA Chemicals), and then allowed to cool down to 37 °C in a water bath. ECFCs and MSCs (4 × 10⁵ total; 1:1 ratio) were suspended in 200 µl of GelMA solution and the mixture was polymerized by exposure to 7.5 mW cm⁻² UV light for 15 s at 37 °C. Polymerized cell-laden constructs are referred to as unassembled grafts or U-grafts. Alternatively, on indicated experiments, U-grafts were constructed with ice-cold phenol-red-free Matrigel (BD Bioscience). Assembled grafts or A-grafts were constructed by culturing U-grafts *in vitro* for seven days using EGM-2 with 5% FBS. A-grafts contained easily observable, fully assembled vascular networks. Both U-grafts and A-grafts were subcutaneously implanted into back of six-week-old male

athymic NU/NU mice for engraftment studies. On specified experiments, DsRed-labelled ECFCs were used to visualize the formation of assembled vascular networks in A-grafts. For Notch-signalling inhibition studies, A-grafts were treated with the γ -secretase inhibitor DAPT (10 μ M in EGM-2 with 5% FBS) for 24 h before implantation; whereas A-grafts cultured for eight days in EGM-2 with 5% FBS served as a control. To evaluate host myeloid cell recruitment, grafts were explanted two days after implantation and subjected to flow cytometric and histological analyses. To assess vascularization, grafts were explanted seven days after implantation and were subjected to histological analysis. On indicated experiments, FITC-conjugated UEA-I (50 μ g per 100 μ l per mouse) was injected intravenously 10 min before euthanasia to label perfused human vessels.

Isolation and culture of mouse MSCs and ECs. Subcutaneous white fat pads were excised from euthanized C57BL/6 mice, minced and digested (1 mg ml⁻¹ collagenase A, 2.5 U ml⁻¹ dispase, 126 μ M calcium chloride and 80 μ M magnesium sulfate in DMEM containing 1% FBS) for 1 h at 37 °C. The stromal vascular fractions were obtained after removal of mature adipocytes by centrifugation (450g for 10 min) and the lysis of erythrocytes with ammonium-chloride solution. The stromal vascular fractions were incubated with a FITC-conjugated anti-mouse-CD45 antibody, followed by anti-FITC magnetic microbeads (Miltenyi Biotec), and passed through magnetic columns (Miltenyi Biotec). The mCD45⁻ cell fraction was then incubated with PE-conjugated anti-mPDGFR β or anti-mCD31 antibodies, followed by anti-PE magnetic microbeads, and passed through magnetic columns. The purified mCD45⁻mPDGFR β ⁺ mouse MSCs (mMSCs) were cultured on uncoated tissue-culture dishes using MSC medium. The purified mCD45⁻mCD31⁺ ECs (mECs) were cultured on fibronectin-coated tissue-culture dishes using ECFC medium. mECs were then transduced with a lentivirus (pLenti-CMV-GFP; Addgene) to express GFP under CMV promoter (referred to as GFP-mECs). All experiments were carried out with mMSCs at passage 3 and GFP-mECs at passage 12.

Syngeneic mouse model of graft vascularization. C57BL/6-derived mMSCs and GFP-mECs were implanted in collagen-fibrin gel (2 $\times$ 10⁶ total; 3:2 ratio; 200 μ l) subcutaneously into host C57BL/6 male mice as U-grafts in a syngeneic mouse model. To evaluate host myeloid cell recruitment, grafts were explanted two days after implantation and subjected to flow cytometric analyses. To assess vascularization, grafts were explanted seven days after implantation and were subjected to histological analysis. Immunofluorescence staining of GFP indicated the grafting of donor GFP-mECs.

Histology and immunohistochemistry. Explanted grafts were fixed overnight in 10% buffered formalin, embedded in paraffin and sectioned (7- μ m thick). Haematoxylin and eosin-stained sections were examined for the presence of erythrocyte-filled blood vessels. For immunostaining, sections were deparaffinized and antigen retrieval was carried out with Tris-EDTA buffer (10 mM Tris-Base, 2 mM EDTA, 0.05% Tween-20, pH 9.0) or citric buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). Sections were then blocked for 30 min in 5–10% blocking serum and incubated with primary antibodies overnight at 4 °C. Horseradish-peroxidase-conjugated mouse secondary antibody (1:200; Vector Laboratories) and 3,3'-diaminobenzidine (DAB) were used for detection of hCD31, followed by haematoxylin counterstaining and Permount mounting. Fluorescent staining was performed using fluorescently conjugated secondary antibodies (1:200) followed by DAPI counterstaining (Vector Laboratories). When indicated, human vimentin (hVIM) staining was used to identify human cells (both ECFCs and MSCs) (please note that the anti-hVIM antibody used is very specific to human vimentin and does not react with mouse cells and tissues)⁷. Primary and secondary antibodies are detailed in Supplementary Table 1.

Microvessel density. Microvessel density was reported as the average number of erythrocyte-filled vessels (vessels per mm²) in haematoxylin and eosin-stained sections from the middle of the implants as previously described⁷. The entire area of each section was analysed. For specified experiments, human-specific microvessel density was quantified by evaluation of slides immunostained for human-specific CD31 (hCD31). Perfused microvessel density was measured by injection of FITC-labelled lectins (UEA-1 or Iso-B4) before euthanizing graft-bearing mice, followed by staining of lectin-labelled vessels with anti-UEA-1 or anti-FITC antibodies.

Cell retrieval and flow cytometry. Grafts were removed from euthanized mice and cells were retrieved by enzymatic (1 mg ml⁻¹ collagenase and 2.5 U ml⁻¹ dispase) digestion for 1 h at 37 °C. Retrieved cells were then prepared into single-cell suspensions. For indicated experiments, retrieved cells were sorted into hCD31⁺ and hCD31⁻ cells by MACS using magnetic beads (DynaBead) coated with anti-hCD31 antibodies. Retrieved cells were also stained for flow cytometry and analysed using a Guava easyCyte 6HT/2L flow cytometer (Millipore Corporation, Billerica, MA) and FlowJo software (Tree Star Inc., Ashland, OR). Antibody labelling was carried out for 20 min on ice followed by three washes with PBS supplemented with 1% BSA and 0.2 mM EDTA. Cell staining was followed by fixation with 1% paraformaldehyde. To identify the mouse myeloid population, cell suspensions were incubated with

PerCP-conjugated anti-mouse CD45, PE-conjugated anti-mouse F4/80 and FITC-conjugated anti-mouse Ly6G antibodies with concentrations indicated in Supplementary Table 1. Mouse neutrophils were identified as mCD45⁺mLy6G⁺ cells. Mouse monocytes and macrophages were identified as mCD45⁺mLy6G⁺mF4/80^{dim} and mCD45⁺mLy6G⁺mF4/80^{high} cells, respectively. To identify mouse lymphocytes, cell suspensions were incubated with PerCP-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD19 and FITC-conjugated anti-mouse CD3e antibodies. Mouse T cells and B cells were identified as mCD45⁺mCD3⁺ and mCD45⁺mCD19⁺ cells, respectively. For indicated experiments, retrieved cells were sorted into mCD45⁺mLy6G⁺ (neutrophils) and mCD45⁺mLy6G⁻ cells by fluorescence-activated cell sorting (FACS) using a FACSAria II 5-LASER sorter system (BD Bioscience). Sorted cells were analysed immediately for gene expression by qRT-PCR.

Myeloid cell depletion. Antibodies were administered intraperitoneally according to the schedule described in Fig. 2a. The rat anti-mouse Ly-6G (clone 1A8; Bio X Cell) antibody was administered at 200 μ g per mouse two days before U-graft implantation, and then every other day at 100 μ g per mouse. The rat anti-mouse F4/80 (clone CI:A3-1; Bio X Cell) antibody was administered at 400 μ g per mouse two days before U-graft implantation, and then every day at 200 μ g per mouse. Daily injection of rat IgG (200 μ g per mouse) served as a control. Monocyte and neutrophil depletion was confirmed in blood samples by flow cytometry.

Myeloid adoptive transfer model. Circulating myeloid cells were depleted from recipient mice by two 4 Gy gamma irradiation sessions on days -4 and 0. mLy6G⁺ neutrophils or mF4/80⁺ cells (around 10⁷ cells) from unirradiated donor mice were adoptively transferred by injection at the implantation sites in recipient mice and at the same time as the U-graft implantation (day 0). Donor neutrophils were isolated from either bone marrow or blood using a mouse neutrophil isolation kit that enriches for CD11b⁺Ly6G⁺ cells (Miltenyi Biotec; negative selection using a cocktail that contains anti-CD5, -CD45R (also known as Ptprc or B220), -CD49b, -CD117 (also known as Kit or c-kit), -F4/80 and -Ter119 antibodies). Donor mF4/80⁺ cells were isolated from the bone marrow by MACS using magnetic beads (Miltenyi Biotec) coated with anti-mF4/80 antibodies.

Generation of conditioned medium. Samples of conditioned medium were collected daily from U-grafts over eight days *in vitro* (that is, until they became A-grafts). To this end, grafts were cultured in 3-ml tubes with 500 μ l of EBM-2, 5% FBS medium refreshed every 24 h. Collected samples of conditioned medium were filtered (0.2 μ m) and then concentrated tenfold (Amicon Ultra centrifugal filters; 3 kDa cut off; Millipore). For indicated experiments, A-grafts were infused with conditioned medium from U-grafts. To this end, A-grafts were placed on clean Kimwipes (2 $\times$ 2 cm²) and then the concentrated conditioned medium (50 μ l) from U-grafts was slowly added on top of the grafts to allow a replacement of culture medium by capillary force. Infused A-grafts were immediately implanted into recipient mice. A-grafts infused in basal medium (EBM-2, 5% FBS) and conditioned medium from grafts containing only ECFCs or MSC alone served as controls.

Neutrophil recruitment plug assay. Concentrated conditioned medium (50 μ l) was mixed with 200 μ l of Matrigel and injected subcutaneously into NU/NU mice. After two days, plugs were explanted and infiltrated cells were retrieved by enzymatic digestion and subjected to flow cytometric analysis. Concentrated basal medium (EBM-2, 5% FBS) served as a control. For indicated experiments, neutralizing antibodies against IL6, CXCL1 or CXCL8 (5 μ g per antibody per plug) were added to the concentrated conditioned medium 30 min before implantation. Individual neutralizing antibodies and the combination of all three were compared.

CXCR2-antagonist treatment. The CXCR2-antagonist SB225002 (Sigma) was administered at 7.5 mg kg⁻¹ intraperitoneally 30 min before U-graft implantation, and then every day until day 7. Neutrophil recruitment on day 2 and graft vascularization on day 7 were measured as described above. Daily injection of saline served as a control. For selected experiments, SB225002 treatment was combined with rat anti-mouse Ly-6G treatment (clone 1A8; administered as described above) to study the combined effect on U-graft vascularization.

TGF β receptor inhibitor treatment. U-grafts were implanted in mice systemically treated with a TGF β receptor inhibitor (SB432542; 10 mg kg⁻¹; daily intraperitoneal injection). Mice receiving saline injections served as controls. Grafts were collected on day 7 to evaluate vascularization.

LPS plug assay. Lipopolysaccharides (LPS; 10 μ g; from *Escherichia coli* 0111:B4; Sigma) were mixed with 200 μ l Matrigel and this mixture was injected subcutaneously into NU/NU mice. After one day, plugs were explanted, infiltrated cells were retrieved and neutrophils were sorted by FACS for analysis.

Phagocytosis assay. Cells were retrieved from U-grafts or LPS plugs 24 h after implantation and were then incubated with GFP-expressing *E. coli* for 30 min. Cells were washed twice in PBS and free bacteria were removed by centrifugation (300 r.p.m.). Cells were analysed by flow cytometry and immunofluorescence.

Mouse neutrophils were identified as mCD45⁺mLy6G⁺ cells and phagocytosed *E. coli* as GFP⁺ bacteria.

Human cytokine protein array. The presence of selected cytokines was evaluated in samples of conditioned medium with human cytokine protein arrays (R&D Systems) according to the manufacturer's instructions. Antigen–antibody complexes were visualized using LumiGLO substrate (Kirkegaard & Perry Laboratories, Inc.) and chemiluminescent sensitive film (Kodak). Densitometry was performed by image analysis (ImageJ) to estimate the amount of protein present in each sample.

Quantitative qRT-PCR. Quantitative qRT-PCR (qRT-PCR) was carried out in RNA lysates prepared from either explanted grafts, cells in culture or cells retrieved from grafts. Total RNA was isolated with an RNeasy kit (QIAGEN) and cDNA was prepared using reverse transcriptase III (Invitrogen), according to the manufacturer's instructions. Multigene transcriptional profiling, a form of qRT-PCR, was used to determine the number of mRNA copies per cell normalized to ribosomal 18S rRNA abundance, as previously described⁵⁵. Human-specific gene expression in explanted grafts was measured using human-specific primers and normalized to human β -actin (*ACTB*) expression. Real-time PCR primer sequences are shown in Supplementary Table 2. Heat maps were generated using the Gene-E software package from the Broad Institute (www.broadinstitute.org/cancer/software/GENE-E/).

Microscopy. Images were taken using an Axio Observer Z1 inverted microscope (Carl Zeiss) and AxioVision Rel. 4.8 software. Fluorescent images were taken with an ApoTome.2 Optical sectioning system (Carl Zeiss) and 20 $\times$ /0.8 or 40 $\times$ /1.4 oil objective lens. Non-fluorescent images were taken with an AxioCam MRC5 camera using a 40 $\times$ /1.4 oil objective lens.

Statistical analyses. Unless otherwise stated, data were expressed as mean $\pm$ standard deviation (s.d.). For comparisons between two groups, means were compared using unpaired two-tailed Student's *t*-tests. Comparisons between multiple groups were performed by ANOVA followed by Bonferroni's post-test analysis. Samples size, including the number of mice per group, was chosen to ensure adequate power and were based on historical data. No exclusion criteria were applied for all analyses. No specific methods of randomization were applied to group or animal allocation. Investigators were not blinded to group allocation. All statistical analyses were performed using GraphPad Prism version 5 software (GraphPad Software Inc.). *P* < 0.05 was considered statistically significant.

Data availability. The authors declare that all data supporting the findings of this study are available in the paper and its Supplementary Information.

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Author contributions

R.-Z.L. and J.M.M.-M. conceived and designed the project. R.-Z.L., C.N.L., R.M.-L., J.N., P.Z., M.S., M.A.M. and J.M.M.-M. performed the experimental work. All authors discussed and analysed the data and edited the results. W.T.P., B.P. and S.E. provided crucial materials. R.-Z.L. and J.M.M.-M. wrote the manuscript.

Additional information

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Competing interests

The authors declare no competing financial interests.

Erratum: Host non-inflammatory neutrophils mediate the engraftment of bioengineered vascular networks

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In the version of this Article originally published, the *x* axis label 'Days' was missing from Fig. 1e, and in Fig. 2b, the top-right flow cytometry plot was incorrect. These errors have now been corrected.