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IMPLANTED BIOMATERIALS

Neutrophil-mediated vascularization

The recruitment of neutrophils is indispensable for the formation of functional blood vessels in bioengineered grafts.

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Clinical applications of tissue engineering are currently limited to thin or avascular *in vitro*-engineered tissues, such as skin and cartilage[1]. This is because one of the main factors limiting engraftment is the inability to provide sufficient blood supply to the engineered graft during the first days after implantation, which leads to nutrient deficiency, hypoxia and, ultimately, tissue necrosis. In response to graft hypoxia, and guided by released pro-angiogenic signals, host blood vessels can grow spontaneously into the tissue following transplantation. However, it takes several weeks for a complete vascular network to form in an implant of several millimetres[2], which is too long for the grafted cells to survive. Strategies exist to circumvent this problem, such as *in vitro* pre-vascularization of the graft to accelerate graft connection to the host vasculature and to limit the period of graft hypoxia. Yet despite important progress in this regard, the inability to form anastomoses — connections between existing blood vessels — remains an important complication that often leads to graft failure. As described in *Nature Biomedical Engineering*, Juan Melero-Martin and colleagues now show that non-inflammatory host neutrophils are indispensable mediators of the vascularization of implanted tissue grafts[3].

Melero-Martin and co-authors compared bioengineered grafts containing unassembled (U-grafts) or assembled (A-grafts) vascular networks. They found that A-grafts, which contained a fully developed and mature microvascular network, completely failed to spontaneously connect to the host circulation after transplantation, whereas the U-grafts, which contained an unassembled suspension of vascular cells, effectively produced perfused vascular networks seven days following implantation. In parallel, dynamic and sequential recruitment of Ly6G$^+$ neutrophils and F4/80$^+$ macrophages occurred for the U-grafts but not for the A-grafts[3]. By using selective-cell-depletion strategies, the authors demonstrated that the removal of neutrophils impaired the vascularization and perfusion of the U-grafts (Fig. 1). These results mirror the need for neutrophils in the revascularization of syngeneically transplanted islets in striated muscle[4].

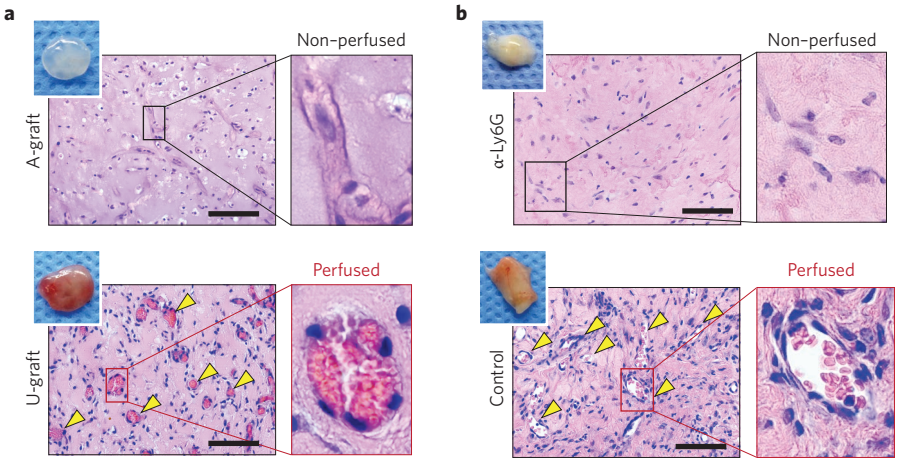

Figure 1 | Neutrophils mediate blood-vessel assembly and perfusion. **a**, Haematoxylin and eosin stainings of A-grafts (top) and U-grafts (bottom) implanted in mice and explanted after seven days. Only U-grafts show vessels containing red blood cells (yellow arrowheads). **b**, The formation and perfusion of vessels in U-grafts are inhibited when neutrophils are depleted by antibody injection (α-Ly6G), but not when isotype control antibodies are used. Right insets: close-up views of individual vessels. Top insets: photographs of the explanted grafts. Scale bars, 100 μm. Figure adapted from ref. [3], Macmillan Publishers Ltd.

Neutrophils are quickly recruited from circulation to injured tissues, as they respond instantly to chemotactic cues of infection or inflammation or of the existence of damaged cells[5]. In Melero-Martin and colleagues' study, subcutaneous plugs containing conditioned medium from cultured U-grafts potently recruited neutrophils, whereas medium from A-grafts did not. This demonstrates that the unassembled endothelial cells in the U-grafts secrete factors that are chemotactic for neutrophils, and is in agreement with recent observations using *in vitro* cultures of aortic rings, where added neutrophils traffic to the angiogenic sprouts[6] (Fig. 2a). The grafts were then analysed and compared with respect to chemoattractants and vessel-maturity markers. The messenger-RNA levels of the vessel-maturity markers (downstream of Notch signalling) were found to be higher in the A-grafts than in the U-grafts, which was opposite to the expression of neutrophil chemoattractants (chemokine ligands CXCL1 and CXCL8 and cytokine interleukin 6), which were lower in A-grafts than U-grafts. Moreover, the inhibition of Notch signalling 24 hours before implantation increased the expression of neutrophil chemoattractants by the A-grafts, leading to an enhanced recruitment of neutrophils and to the formation of anastomoses and functional vascular plexa.

Primarily described in the field of cancer, neutrophils are also important actors of the angiogenic processes in ischaemic diseases as well as in physiological situations such as the menstrual cycle and wound healing[7]. Similar to the distinction made between the pro-inflammatory M1 and the alternatively activated M2 macrophage subpopulations, neutrophils are categorized into the N1 population, with canonical pro-inflammatory function, or into the N2 population, with an alternatively activated phenotype[8]. As for the macrophages, numerous discrete neutrophil subpopulations with varied functions have been described[9], and outline a significantly more complex picture. For example, a pro-angiogenic subpopulation

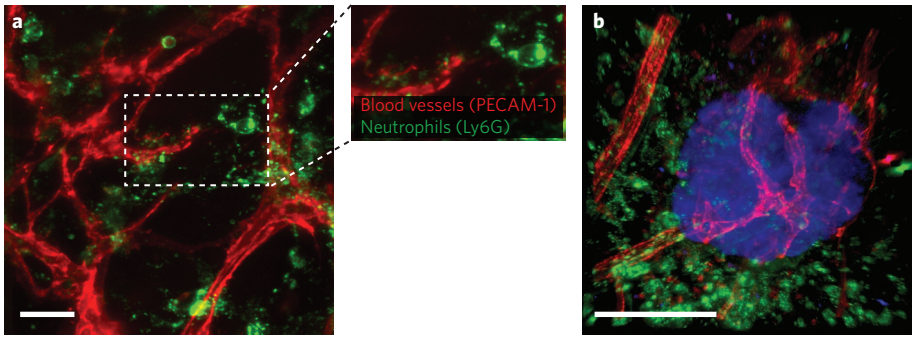

Figure 2 | Neutrophils recruited to sprouting vascular endothelium *in vitro* and *in vivo*. **a**, Using the *in vitro* aortic ring assay of cultured rings of mouse aorta, neutrophils added to the culture are seen to traffic to and interact with the angiogenic sprouts (inset). Endothelium (red) is visualized by platelet endothelial cell adhesion molecule 1 (PECAM-1) monoclonal antibodies and neutrophils (green) are stained by Ly6G monoclonal antibodies. Scale bar, 20 μm. **b**, Syngeneic transplantation of isolated islets of Langerhans into the striated muscle of mice reveals rapid recruitment of neutrophils to the engraftment site, where they coincide with the formation of the islet vascular network. The pancreatic islet is stained by CellTracker (blue), endothelium (red) is visualized by PECAM-1 monoclonal antibodies, and neutrophils (green) are stained by Ly6G monoclonal antibodies. Scale bar, 50 μm. Images courtesy of G. Christoffersson and J. Lomei, Department of Medical Cell Biology, Uppsala University.

of neutrophils that expresses vascular endothelial growth factor receptor 1, chemokine receptor CXCR4 and the integrin CD49d (refs [10,11]) are specifically recruited to sites of hypoxia, such as newly transplanted and avascular pancreatic islets, where they initiate the formation of new blood vessels by releasing high quantities of the matrix metalloproteinase MMP9 (Fig. 2b)[11]. This situation is analogous to that of the U-grafts, and it is plausible that the previously described pro-angiogenic neutrophils correspond to those observed to be responsible for vessel formation in the grafts (this would need further investigation). Compared with neutrophils recruited to lipopolysaccharide plugs, neutrophils recruited to U-grafts express higher levels of the anti-inflammatory genes arginase 1 and interleukin 4, as well as the pro-remodelling genes vascular endothelial growth factor A and transforming growth factor beta. Moreover, Melero-Martin and co-authors demonstrated that the positive effect of neutrophils for vessel function is dependent on transforming-growth-factor-beta signalling, although it remains to be demonstrated whether this signalling in neutrophils is crucial for the trafficking to the grafts or for the pro-angiogenic actions.

Macrophages have a profound influence on the promotion of tumour angiogenesis[12], partly through the release of pro-angiogenic factors such as vascular endothelial growth factor, basic fibroblast growth factor or MMP9. In addition, a specific subset of macrophages localize in the perivascular space and help to stabilize vessels[13]. It has also been demonstrated that perivascular macrophages participate in the recruitment of immune cells from circulation, especially neutrophils[14]. And in the zebrafish and mouse embryos, brain vessels form anastomoses by means of macrophages connecting nearby sprouts[15]. In Melero-Martin and colleagues' work, even though macrophages are recruited to the U-grafts before the onset of perfusion, macrophage depletion does not affect the microvascular density. However, it is still possible that the macrophages also contribute to the formation of a mature and functional vascular network.

Melero-Martin and co-authors' study highlights the importance of neutrophil recruitment for proper anastomosis of bioengineered tissues and organs, and thereby opens up perspectives towards improving the engraftment and long-term function of grafts. Importantly, since most transplantation settings involve immunosuppression, the exact nature and means of recruitment of these neutrophils need to be delineated to tailor immunosuppressant therapies accordingly. ❐

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References

1. Novosel, E. C., Kleinhans, C. & Kluger, P. J. *Adv. Drug Delivery Rev.* **63,** 300–311 (2011).
2. Rouwkema, J. *et al. Trends Biotechnol.* **26,** 434–441 (2008).
3. Lin, R.-Z. *et al. Nat. Biomed. Eng.* **1,** 0081 (2017).
4. Christoffersson, G. *et al. Diabetes* **59,** 2569–2578 (2010).
5. Phillipson, M. & Kubes, P. *Nat. Med.* **17,** 1381–1390 (2011).
6. Christoffersson, G. *et al. J. Leukoc. Biol.* (in the press).
7. Fridlender, Z. G. *et al. Cancer Cell* **16,** 183–194 (2009).
8. Sylvestre-Roig, C., Hidalgo, A. & Soehlein, O. *Blood* **127,** 2173–2181 (2016).
9. Seignez, C. & Phillipson, M. *Curr. Opin. Hematol.* **23,** 1–6 (2016).
10. Christoffersson, G. *et al. Blood* **120,** 4653–4662 (2012).
11. Massena, S. *et al. Blood* **126,** 2016–2026 (2015).
12. Murdoch, C. *et al. Nat. Rev. Cancer* **8,** 618–631 (2008).
13. De Palma, M. *et al. Cancer Cell* **8,** 211–226 (2005).
14. Actin, A. *et al. Nat. Immunol.* **36,** 240–249 (2104).
15. Fantin, A. *et al. Blood* **116,** 829–840 (2010).