Encapsulation of macrophages enhances their retention and angiogenic potential

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Cell therapies to treat critical limb ischaemia have demonstrated only modest results in clinical trials, and this has been partly attributed to poor cell retention following their delivery directly into the ischaemic limb. The aim of this study was to determine whether alginate encapsulation of therapeutic pro-angio/arteriogenic macrophages enhances their retention and ultimately improves limb perfusion. A reproducible GMP-compliant method for generating 300 µm alginate capsules was developed to encapsulate pro-angio/arteriogenic macrophages. Longitudinal analysis revealed no detrimental effect of encapsulation on cell number or viability in vitro, and macrophages retained their pro-angio/arteriogenic phenotype. Intramuscular delivery of encapsulated macrophages into the murine ischaemic hindlimb demonstrated increased cell retention compared with injection of naked cells (P = 0.0001), and that this was associated with enhanced angiogenesis (P = 0.02) and arteriogenesis (P = 0.03), and an overall improvement in limb perfusion (P = 0.0001). Alginate encapsulation of pro-angio/arteriogenic macrophages enhances cell retention and subsequent limb reperfusion in vivo. Encapsulation may therefore represent a means of improving the efficacy of cell-based therapies currently under investigation for the treatment of limb ischaemia.

npj Regenerative Medicine (2019) 4:6 ; https://doi.org/10.1038/s41536-019-0068-5

INTRODUCTION

Critical limb ischaemia (CLI) is a severe manifestation of peripheral arterial disease (PAD) that is characterised by pain and gangrene.1 The limb salvage rate for patients with CLI remains poor,9 with a significant proportion of patients not amenable to standard treatments, including surgical bypass and angioplasty. This has been the driver for the development of angiogenic cell-based therapies aimed at limb salvage in these no option patients. Clinical trials of cell therapy to date have only shown a modest benefit with disappointing results attributed to the lack of potency of cells injected, including a functional impairment of autologous cells harvested from patients with multiple co-morbidities.3–10

The poor retention of cells after injection into the target site is also thought to limit their potential for effecting robust collateralisation. Cells injected directly into the calf muscle are susceptible to clearance by immune cells or apoptosis triggered by the hypoxic, pro-inflammatory environment.11 Mononuclear cells injected intramuscularly in the ischaemic hindlimb have a short-lived survival, which is not improved with repeated injection.12 There are currently no studies to assess retention of cells injected into the ischaemic limb in man, but clinical studies of therapeutic cell injection into the heart reveal a similar precipitous loss, with only ~12% of cells retained after 1 h.13

The use of implantable biomaterials, containing therapeutic cells, to enhance cell-based therapies is gaining traction in a number of cell therapy areas, including the use of bone marrow-derived mesenchymal stem cells for revascularisation of infarcted myocardium and ischaemic hindlimbs.14,15 Encapsulation in polymeric matrices, including alginate, can be used to deliver therapeutic cells as it not only enhances cellular retention and survival16,17 but also provides a semi-permeable membrane for diffusion of nutrients, stimulants and waste products.18

Alginate is an unbranched algae-derived polysaccharide, which gels upon contact with divalent cations.19 Its biocompatibility, paired with ease of use makes it an attractive option for the development of cell therapy. We have previously identified a subset of human monocyte/macrophages that promote limb revascularisation in mice20 and carried out a first in man study involving delivery of this subset in patients with limb ischaemia (unpublished data). Here, we use murine pro-angio/arteriogenic macrophages to optimise and standardise a good manufacturing practice (GMP)-compliant encapsulation strategy, and to study the effect of this procedure on their viability and capacity to enhance revascularisation of the ischaemic limb, in readiness for clinical trials in patients with limb ischaemia.

RESULTS

Optimisation of alginate capsule generation

A number of encapsulation parameters were optimised to allow reproducible generation of capsules of a consistent shape and diameter, prior to generating cell-seeded capsules for subsequent
in vitro and in vivo experiments. Capsule diameter was affected by increasing the flow rate of alginate solution through the cell encapsulator, but varying the concentration of sodium alginate had little effect (Fig. 1a). Increasing the voltage applied to the alginate suspension decreased the capsule diameter (Fig. 1b). A flow rate of 12 ml/min, with 1.0% sodium alginate and 6.8 kV reliably produced capsules of 300 µm diameter and a round shape (Fig. 1a, c).

In order to standardise experimental conditions, we encapsulated immortalised murine bone marrow-derived macrophages engineered to express the Tie2 receptor (Tie2-iBMMs, see Methods) to provide a uniform population of angiogenic cells for these proof-of-concept studies aimed at developing a standardised GMP-compliant encapsulation method and deciphering the effect of encapsulation on cells. Uniformly seeded capsules were produced when Tie2-iBMMs were seeded into the alginate solution at a concentration of 1 × 10^7 cells/ml, with capsules containing approximately 200 cells each (Fig. 1c).

The effect of encapsulation on Tie2-iBMM viability and phenotype was assessed longitudinally, in vitro, for cell viability and phenotype in order to ascertain whether encapsulation was detrimental to cell health (Fig. 2, Table 1). Microscopic analysis of the capsules demonstrated maintenance of capsule integrity and retention of the cells within the capsules up to day 21 postencapsulation (Fig. 2a–d). There was no significant loss of cells from the capsules in vitro up to day 21 (Fig. 2e, day 0: 196 ± 2.5 vs. day 21: 188 ± 1.4 cells/capsule). Analysis of cell viability by annexin V and propidium iodide (PI) staining (Fig. 2f, g, Table 1), across 7 days in vitro, demonstrated that the majority of cells remain viable.

We assessed the phenotype of macrophages following long-term in vitro culture of Tie2-iBMMs in alginate capsules. After 21 days, the expression of Tie2 and the mouse macrophage marker, F4/80, on naked Tie2-iBMMs (nTie2-iBMMs) remained constant and was comparable to that of eTie2-iBMMs (Fig. 2h, Supplementary Data, Table S1).

| Sodium Alginate (% (w/v)) | Flow Rate (ml/min) | Capsule Size (µm) |
|---------------------------|--------------------|-------------------|
| 0.8                       | 2.0                | 143.04±2.36       |
|                           | 4.0                | 155.91±5.69       |
|                           | 6.0                | 208.89±4.12       |
|                           | 12.0               | 300.47±5.82       |
| 1.0                       | 2.0                | 143.25±4.38       |
|                           | 4.0                | 154.46±3.67       |
|                           | 6.0                | 203.71±3.68       |
|                           | 12.0               | 300.86±2.56       |

**Fig. 1** Optimisation of capsule generation. **a** The diameter of alginate capsules generated under a range of alginate concentrations (0.8% and 1.0%), and encapsulator flow rates (2–12 ml/min) was measured microscopically (n = 10/group). **b** Variation of the encapsulator voltage settings influenced the diameter of capsules generated (n = 5/group, error bars = s.d.). **c** Consistently round 300 µm diameter capsules were generated using 1.0% alginate with a flow rate of 12 ml/min and were used to encapsulate Tie2-iBMMs at a cell density of 1 × 10^7 cells/ml. Scale bar = 100 µm.
Fig. 2  The effect of prolonged encapsulation on Tie2-iBMMs. a–d Alginate capsules seeded with Tie2-iBMMs at days: a 1, b 7, c 14 and d 21 postencapsulation. e Tie2-iBMM retention within the capsules was quantified up to day 21 following encapsulation (n = 5/group, P = n/s by one-way ANOVA). f Flow cytometric analysis of cell viability using annexin V/PI staining (Q1 = cell debris; Q2 = dead cells; Q3 = apoptosing cells; Q4 = live cells). g Quantification of annexin V/PI staining of encapsulated Tie2-iBMMs at days 1, 2, 3 and 7 post-encapsulation (n = 4/group, P = n/s by two-way ANOVA and Bonferroni post-test). h Measurement of cell phenotype by flow cytometry in nTie2-iBMMs (green) and eTie2-iBMMs (grey) up to 21 days in vitro (n = 5/group, P = n/s P < 0.001 by two-way ANOVA and P = n/s Bonferroni post-test). Error bars = s.d. Scale bar = 100 µm.
macrophage markers CD80 and CD86 did not differ between nTie2-iBMMs and eTie2-iBMMs at any time point (Fig. 2h, Supplementary Data, Table S1). Expression of Mannose receptor C-type 1 (MRC1), an ‘M2’ macrophage marker, was not significantly different between nTie2-iBMMs and eTie2-iBMMs at any time point (Fig. 2h, Supplementary Data, Table S1).

The effect of alginate encapsulation on Tie2-iBMM function

Quantification of human umbilical vein endothelial cell (HUVEC) tubule formation induced by culturing in the presence of either empty capsules (Fig. 3a), vascular endothelial growth factor (Vegfa, Fig. 3b), nTie2-iBMMs (Fig. 3c) or eTie2-iBMMs (Fig. 3d) demonstrated increased EC tubule area in eTie2-iBMM co-cultures compared with empty capsules (Fig. 3e, P = 0.002), and this was comparable to that induced by the Vegfa positive control (P > 0.1) and nTie2-iBMMs (P > 0.9).

In order to determine whether encapsulation of Tie2-iBMMs affected the secretion of factors that may promote or inhibit angiogenesis, we compared the conditioned media produced by nTie2-iBMMs and eTie2-iBMMs left in culture up to 21 days. We found that although there was a reduction in biofluorescence in both treatment groups over 28 days (Fig. 4a), eTie2-iBMMs were significantly better retained at days 7, 14 and 21 than nTie2-iBMMs (Fig. 4b, P < 0.0001).

Encapsulated Tie2-iBMMs induced greater reperfusion of ischaemic hind limbs than treatment with nTie2-iBMMs (P < 0.01). Mice injected with eTie2-iBMMs or nTie2-iBMMs demonstrated greater revascularisation of the ischaemic limb over 21 days compared with animals treated with empty alginate capsules (Fig. 5a, b, P < 0.0001 and P < 0.05, respectively). Histological analysis revealed an increase in the number of arterioles, (Fig. 5c, f, P = 0.03), and a trend to increased arteriole diameter (Fig. 5d, f, P = 0.057) of α-smooth muscle actin (α-SMA-) arterioles; as well as increased angiogenesis (capillary: fibre ratio, Fig. 5e, f, P = 0.023) in ischaemic muscle specimens of mice treated with eTie2-iBMMs compared with nTie2-iBMMs. Mice treated with empty alginate capsules had significantly less angiogenesis and arteriogenesis compared with those treated with eTie2-iBMMs (Fig. 5c–e, P = 0.01). Alginate capsules persisted in the hindlimb after 21 days (Fig. 5g), and still contained cells at this time (Fig. 5h).

There was no significant difference in the number of CD45+ cells in hindlimbs injected with eTie2-iBMMs compared with nTie2-iBMMs (Fig. 6a). Deep phenotyping of the CD45+ population showed no significant difference in the proportion of neutrophils (CD11b+Ly6G+), or monocytes and macrophages (CD11b+Ly6G and CD11b+Ly6Chi F4/80+ cells, Fig. 6b–d, Supplementary Data, Table S3). Treatment with eTie2-iBMMs was associated with a significantly reduced proportion of the endogenous CD11b+Ly6G- monocytes expressing Ly6C (Ly6Chi) (Fig. 6e, Supplementary Data, Table S3) compared with nTie2-iBMMs and empty capsule-treated mice (P < 0.05). Histological analysis of muscle specimens revealed no significant difference in the number of cells expressing the apoptosis marker activated caspase-3 between treatment groups (Fig. 6f, g), or any difference in muscle damage between treatment groups (Fig. 6f, h).

**DISCUSSION**

To date, cell-based therapies for the treatment of CLI have demonstrated limited efficacy in clinical trials. A possible contributing factor to these modest results is poor cell retention following direct injection of cells into the ischaemic limb. This suggests a need for an alternative delivery system, such as encapsulation of therapeutic cells within a biocompatible material prior to implantation that promotes cell retention to ensure a better outcome.

This study investigates the effect of alginate encapsulation on the phenotype and function of a pro-angiogenic/murine macrophage line (Tie2-iBMMs), in revascularising the ischaemic limb. We describe a GMP-compliant methodology for the consistent generation of uniform alginate capsules containing these cells that does not adversely affect their viability, phenotype and function in vitro. Encapsulation enhanced Tie2-iBMM

### Table 1. Assessment of encapsulated Tie2-iBMM viability in culture

| Day | Live       | Dead       | Apoptosing |
|-----|------------|------------|------------|
| 1   | 89.00 ± 0.1| 2.43 ± 0.5 | 2.52 ± 1.1 |
| 2   | 84.03 ± 2.9| 4.28 ± 8.4 | 1.90 ± 0.3 |
| 3   | 84.03 ± 1.8| 2.78 ± 0.5 | 3.57 ± 0.3 |
| 7   | 90.85 ± 1.1| 1.90 ± 0.1 | 3.57 ± 0.4 |
| 21  | n/s        | n/s        | n/s        |

*P value*
Fig. 3 The effect of alginate encapsulation on the pro-angiogenic function of Tie2-iBMMs. a–d Representative light microscope images of HUVEC/fibroblast angiogenesis assays for HUVECs co-cultured with empty alginate capsules a, 100 ng/ml VEGF b, naked Tie2-iBMMs c or alginate encapsulated Tie2-iBMMs d. e HUVEC tubule area was compared (n = 6–12/group, *P = 0.05, **P = 0.01, ***P = 0.0001 by Kruskal Wallis test, error bars = s.d.). f Quantification of PlGF-2, VEGF, MMP9, IL-10 and IL-1β secreted by nTie2-iBMMs (green) and eTie2-iBMMs (grey) following in vitro culture for up to 21 days (n = 5/group, **P = 0.01, ***P < 0.001 by two-way ANOVA and Bonferroni post-test, error bars = s.e.m.). g, h Quantification of Vegfa164 g and MCP-1 h secreted by stimulated Tie2-iBMMs using ELISA (n = 6/group, **P = 0.01 by Mann Whitney test, error bars = s.d.)
retention following implantation into the ischaemic hindlimb and this was associated with significantly greater angio/arteriogenesis and overall limb revascularisation compared with non-encapsulated Tie2-iBMMS.

Tie2-expressing macrophages are thought to facilitate revascularisation either through a paracrine action or via direct contact with ECs and, therefore, their utility as therapeutic cells necessitates their delivery in close proximity to an ischaemic region to maximise their revascularisation potential. Maintenance of their retention at the site of delivery is thought to be another important factor in achieving optimal therapeutic benefit, with significant cell loss from the site of implantation noted when directly injected into both the ischaemic heart and limb. Cell encapsulation maintains retention and has proved efficacious in different clinical settings, including pancreatic islet cell and hepatocyte transplantation for the treatment of diabetes and liver failure. The data presented demonstrates that Tie2-expressing macrophage secretion of pro-angio/arteriogenic cytokines is preserved or even enhanced following encapsulation. PlGF-2, VEGF and MMP9 have proven potential for promoting ischaemic tissue repair through induction of angiogenesis, progenitor cell recruitment and improved integration of injected cellular biomaterials and, therefore, the greater degree of limb reperfusion in eTie2-iBMM-treated animals could be attributed to the improved retention of these cells in the ischaemic region, facilitating the action of these growth factors. In addition to providing a physical barrier for preventing cell loss through wash out by the vascular and lymphatic systems, alginate encapsulation of cells has also been shown to inhibit migration of cells out of the capsule into the surrounding host tissues.

An advantage of encapsulating cells, in addition to improving retention, is their immuneprivileged status within the capsule. Although immunogenicity is not a consideration when using autologous cells for therapeutic purposes, murine studies suggest that co-morbidities associated with CLI can adversely affect the angio/arteriogenic potential of monocyte/macrophages. Allogeneic macrophages from healthy individuals, that may have more potent angio/arteriogenic properties for promoting limb salvage, could be used in combination with encapsulation technologies, to enhance the efficacy of cell-based strategies.

The present study highlights the promise offered, through the use of a GMP-compliant biomaterial encapsulation process, to enhance the efficacy of cell therapies for treating limb ischaemia. We employed the murine macrophage iBMM cell line in our experiments to ensure replicability and fair comparison in our proof of concept study. Further studies, using human macrophages in place of the mouse cell line tested here, would be required to allow the translation of this work into clinical trials. Here, we show not only an improvement in the method for delivering cells, but also the potential for a whole new cell product for therapeutic use when human macrophages are encapsulated under GMP conditions. Sodium alginate is a well-established material for the purposes of cell encapsulation, although there now exists an expansive range of biomaterials that have been engineered to specifically promote the reparative function of cells.
Biomaterial-based cell therapies may be further enhanced through engineering to allow for the temporal release of pro-angio/arteriogenic factors that may increase the potency of encapsulated cells. Growth factor-containing hydrogel cores within alginate microcapsules are postulated to improve cell survival, with MSC-VEGF co-encapsulation demonstrating promise in the treatment of myocardial infarction. Co-encapsulation of different cells may also enhance therapeutic cell function and survival. It is possible therefore, that although the present study highlights the benefit of cellular encapsulation in promoting retention of therapeutic cells and their activity in revascularising the ischaemic limb, there could be scope for further improvements to enhance their efficacy through the development of co-encapsulation modalities.

Fig. 5 The effect of cell encapsulation on revascularisation of the murine ischaemic hindlimb. a Laser Doppler images of mice treated with direct injection of naked or encapsulated Tie2-iBMMs, or acellular alginate capsules, measured over 21 days ($n = 11–15/group$). b Perfusion index of murine hindlimbs following induction of ischaemia up to day 21 (ischaemic limb flux/contralateral limb flux, $P < 0.05$ by two-way ANOVA *$P = 0.05$ **$P = 0.01$ ***$P = 0.0001$ by Bonferroni post-test, error bars = s.e.m.). c, d Mean α-SMA+ arteriole number per field of view and diameter in the adductor muscle of mice following HLI surgery at day 21 ($n = 6–9/group$, *$P = 0.05$ **$P = 0.01$ by Kruskal Wallis test, error bars = s.d.). e Capillary fibre ratio of gastrocnemius muscle samples harvested from mice at day 21 (CD31+ capillaries:laminin+ muscle fibres, $n = 6–9/group$, *$P = 0.05$ **$P = 0.01$ by Kruskal Wallis test, error bars = s.d.). f Representative fluorescence microscopy images of arteriole staining for α-SMA (red) and laminin (green) and capillary/fibre staining for CD31 (red) and laminin (green). g Murine hindlimbs at day 21 treated with direct injection of naked or encapsulated cells (white arrow). h H&E analysis of capsules harvested from HLI mice at day 21. Scale bar = 100 µm

Published in partnership with the Australian Regenerative Medicine Institute npj Regenerative Medicine (2019) 6
In summary, these studies provide an optimised methodology for the generation of alginate capsules containing pro-angiogenic arteriogenic macrophages, and show that encapsulation in this biopolymer is not detrimental to cell viability, phenotype or function. These data show that encapsulation both enhances macrophage retention and their pro-angiogenic arteriogenic potential in the ischaemic murine hindlimb, which leads to greater limb perfusion, compared with naked cells. This work may have important implications for cell-based therapies currently being trialled for treatment of CLI.
Fig. 6 The effect of eTie2-iBMM treatment of the ischaemic hindlimb on inflammation, apoptosis and muscle damage. a CD45⁺ cells (white arrow) within the adductor muscle of nTie2-iBMM, eTie2-iBMM or empty capsule-treated mice. CD45⁺ cells were quantified as a total proportion of DAPI-stained cells (n = 5/group, P = n/s by Kruskal Wallis test, error bars = s.e.m.). b–d Quantification of b neutrophil, c monocyte and d macrophage content of ischaemic hindlimb muscle after 7 days following delivery of nTie2-iBMMs (grey), eTie2-iBMMs (purple) or empty alginate capsules (white). Data are represented as a proportion of CD45⁺ cells (n = 5/group, P = n/s by Kruskal Wallis test, error bars = s.d.). e Analysis of proportion of Ly6CHigh (purple) and Ly6Clow (grey) monocytes isolated from ischaemic muscle (n = 5/group, P = 0.05 by Kruskal Wallis test). f Representative fluorescence microscopy images of cells (blue, DAPI) expressing activated caspase-3 (red, white arrow) in adductor muscle specimens from nTie2-iBMM, eTie2-iBMM and empty alginate capsule-treated mice; and H&E stained microscopy images of adductor muscle from nTie2-iBMM, eTie2-iBMM and empty alginate capsule-treated mice. Quantification of g cells expressing activated caspase-3 (n = 4/group, P = n/s by Kruskal Wallis test, error bars = s.e.m.) and h muscle damage/repair (n = 5/group, P = n/s by Kruskal Wallis test, error bars = s.e.m.) in ischaemic adductor muscle from mice treated with nTie2-iBMMs, eTie2-iBMMs and empty alginate capsules. Scale bars = 100 µm.
METHODS

Cell culture
Murine bone marrow-derived macrophages were immortalised using a lentiviral vector containing the SV40 large T antigen coding sequence to form iBMMs.\(^4\) Vesicular stomatitis virus-pseudotyped, third generation lentiviruses were produced by transduction of 293T cells. Tie2 expression was subsequently induced via a second lentiviral transduction.\(^3\) Tie2-iBMMs were cultured in complete medium (IMDM (Gibco, UK), 20% foetal calf serum containing 1% (v/v) antibiotic/antimycotic and 50 ng/ml macrophage colony stimulating factor (M-CSF, Peprotech, UK) under standard conditions (37 °C, 21% O\(_2\), 5% CO\(_2\)).

Digitisation of alginate capsules
SLG20 alginate (1.5% (w/v), Pronova Biomedical) was prepared in 0.9% (w/v) NaCl, and cells resuspended in alginate at a concentration of 1.0 × 10\(^7\) cells/ml. Capsules were generated using a GMP-compliant BUCHI B-935 Pro encapsulator, set at a flow rate of 12.0 ml/min, with the cell solution passing through a 120 μm nozzle vibrating at 1800 Hz, and a 6.8 kV electric field, into a polymerisation solution (1.2% (w/v) CaCl\(_2\), 0.9% (w/v) NaCl, Tween-20). Capsules were subsequently washed in 0.9% (w/v) NaCl. Capsule diameter and cell number/capsule was determined by counting the number of cells within ten capsules from three separate experiments under a brightfield microscope. The GMP-grade encapsulation system generated sterile capsules that contained the murine macrophage cell line in order to minimise the possibility of infection and hence any inflammation that might confound our revascularisation results in our animal hindlimb ischaemia (HLI) model.

Preparation of single-cell suspensions from ischaemic muscle for flow cytometry
Adductor muscle samples were harvested from treated animals 7 days after the procedure. Briefly, cells were isolated from dissected tissue following 30 min incubation in a tissue digestion buffer (0.5% bovine serum albumin, 1 mg/ml collagenase, 1 mM EDTA, 500 units/ml hyaluronidase and 100 units/ml DNase I in dPBS (Sigma)). Filtered tissue digests were subject to red blood cell lysis (BD Bioscience) and washed prior to staining and analysis using flow cytometry.

Histological analysis
Unilateral hindlimb ischaemia was surgically induced in 8-week-old C57BL/6 male mice (n = 15/group) by ligation of the femoral artery proximal and distal to the profunda femoris and excision of the intervening segment. nTie2-iBMMs were either directly injected into the adductor muscle of mice undergoing hindlimb ischaemia surgery, or encapsulated in alginate prior to implantation in operated mice. Each mouse received 1 × 10\(^6\) Tie2-iBMMs. Radiance efficiency was quantified using an IVIS Spectrum In Vivo imaging system (Perkin Elmer) at days 0, 3, 7, 14, 21 and 28 using a 60 s exposure time to assess changes in fluorescence intensity using Living Imaging v4.5 software.

Murine model of HLI
Murine bone marrow-derived macrophages were immortalised using a lentiviral vector containing the SV40 large T antigen coding sequence to form iBMMs.\(^4\) Vesicular stomatitis virus-pseudotyped, third generation lentiviruses were produced by transduction of 293T cells. Tie2 expression was subsequently induced via a second lentiviral transduction.\(^3\) Tie2-iBMMs were cultured in complete medium (IMDM (Gibco, UK), 20% foetal calf serum containing 1% (v/v) antibiotic/antimycotic and 50 ng/ml macrophage colony stimulating factor (M-CSF, Peprotech, UK) under standard conditions (37 °C, 21% O\(_2\), 5% CO\(_2\)).

In vitro angiogenesis assay
The angiogenic potential of eTie2-iBMMs was assessed using a previously described HUVEC/fibroblast co-culture assay,\(^4\) and compared with HUVEC tube formation induced by empty alginate capsules. Media containing 100 ng/ml VEGF was used as a positive control. HUVEC tubule formation was quantified after 14 days using ImageProPlus software.

Luminex quantification of secreted cytokines
A custom Luminex assay (R&D Systems, UK) for murine PIGF-2, VEGF, MMP9, IL-1β and IL-10 was used to quantify secreted protein levels in conditioned media collected from nTie2-iBMM and eTie2-iBMM cell cultures at days 3, 7, 14 and 21. The assay was carried out according to manufacturer’s instructions, and data captured using a Bio-Plex MAGPIX system (BioRad, UK).

Vegfa and MCP-1 ELISA
The secretion of Vegfa and MCP-1 by Ang-1/Ang-2-stimulated Tie2-iBMMs was assessed using ELISA (R&D Systems, UK) according to manufacturer’s instructions. Briefly, either nTie2-iBMMs or eTie2-iBMMs were stimulated with 200ng/ml Ang-1 or Ang-2 for 24 h. Media was then replaced with serum-free iBMM media for a further 24 h and conditioned media subsequently collected for analysis.

Animal source and husbandry
This study complied with ethical regulations stipulated by U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, and the study protocol approved by the Home Office. Male C57BL/6 mice aged 8–10 weeks were procured from Charles River Laboratories. All animals were randomised prior to experimentation and during acquisition of data, observers were blinded to these allocations. Animals were maintained in individually ventilated cages, and their health status monitored throughout the course of the experiment.

In vivo biofluorescence
Tie2-iBMMs were stained with VivoTrack680 biofluorescent dye (Perkin Elmer, UK) according to manufacturer’s instructions. Cells were then either directly injected into the adductor muscle of mice undergoing hindlimb ischaemia surgery, or encapsulated in alginate prior to implantation in operated mice. Each mouse received 1 × 10\(^6\) Tie2-iBMMs. Radiance efficiency was quantified using an IVIS Spectrum In Vivo imaging system (Perkin Elmer) at days 0, 3, 7, 14, 21 and 28 using a 60 s exposure time to assess changes in fluorescence intensity using Living Imaging v4.5 software.
Statistical analysis
All statistical analysis was performed using GraphPad Prism 7 software. Technical and experimental repeats were conducted to ensure that experiments were powered to at least 80%. Statistical significance was analysed by one- or two-way ANOVA and appropriate post-hoc tests, or by Mann Whitney/Kruskal Wallis test, as specified in the figure legends. A threshold of $P < 0.05$ was defined as statistically significant. Data are presented as mean ± SD.

Reporting summary
Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
The data that supports the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS
We would like to acknowledge Professor Michele De Palma and Dr. Mario Leonardo Squadrone for providing the Tie2-BMM cell used in this study. This work has been carried out with the support of a research grant from the British Heart Foundation (PG/12/83/29917).

AUTHOR CONTRIBUTIONS
Co-first authors: F.E.L. and A.P. contributed equally to this study. Co-senior authors: B. M. and A.S. contributed equally to this study. F.E.L., A.P., G.D., J.C., and J.F. carried out all experiments described in this study. F.E.L., G.D., and A.P. analysed all data generated. F.E.L., A.P., G.D., Q.X., S.N.J., A.S., and B.M. conceived the study and contributed to experimental design. F.E.L., A.P., G.D., S.N.J., A.S., and B.M. contributed to manuscript preparation. B.M. is guarantor for this study.

ADDITIONAL INFORMATION
Supplementary information accompanies the paper on the npj Regenerative Medicine website (https://doi.org/10.1038/s41536-019-0068-5).

Competing interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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