Macrophages support healing of ischemic injury by transdifferentiating towards mural cells and adopting functions important for vascular support

Kristel Parv  
Uppsala University

Carmen Herrera Hidalgo  
Uppsala University

Feifei Xu  
Uppsala University

Catarina Amoedo-Leite  
Uppsala University  https://orcid.org/0000-0002-7556-4826

Antoine Giraud  
Uppsala University  https://orcid.org/0000-0002-8559-5781

Daniel Holl  
Karolinska Institutet

Cedric Seignez  
Uppsala University

Christian Goeritz  
Karolinska Institut  https://orcid.org/0000-0003-0799-766X

Gustaf Christoffersson  
Uppsala University

Mia Phillipson  (mia.phillipson@mcb.uu.se)  
Uppsala University

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Abstract

Sterile inflammation following injury is important for tissue restoration. In injured human and mouse tissues, macrophages were recently found to accumulate perivascularly. This study investigates if macrophages adopt a mural cell identity important for restoration following ischemic injury. Single-cell RNA-sequencing of fate-mapped macrophages from ischemic mouse muscles demonstrates an identity switch of a subpopulation of macrophages with downregulated myeloid cell genes and upregulated mural cell genes. This macrophage-to-mural cell switch was further strengthened when including unspliced transcripts in the analysis. Induction of macrophage-specific PDGFRβ-deficiency prevented the perivascular macrophage phenotype, impaired vessel maturation and increased vessel leakiness, which ultimately reduced limb function. In conclusion, macrophages in adult ischemic tissue were demonstrated to undergo a transdifferentiation program to morphologically, transcriptomically and functionally resemble mural cells while losing their macrophage identity. The macrophage-to-mural cell switch is crucial for restored tissue function, and warrants exploration for future immunotherapies to enhance healing following injury.

Introduction

The unique properties of macrophages include their ability to traffic to and accumulate at distinct locations upon infection or tissue injury to exert their effector functions. In injured, ischemic tissues, these effector functions encompass clearing the site from dead and damaged cells (Tauber, 2003), as well as contributing to the formation of new vessels by paracrine release of growth factors and enzymes (Christoffersson et al., 2010, 2012; Eming et al., 2017a; Phillipson & Kubes, 2019). The first cells to arrive to the injured tissues are neutrophils, monocytes and macrophages, where the short-lived neutrophils are most numerous during the first couple of days post-insult, whereas macrophages remain at the site and polarize into different subsets with distinct functions in response to environmental instructions (Hidalgo et al., 2019; Varol et al., 2015). Different macrophage subsets are therefore believed to contribute to restoration of tissue perfusion by separate means, e.g. by clearing the site of injured cells, as well as by producing distinct growth factors to promote angiogenesis and vessel stabilisation (Arras et al., 1998; Hong & Tian, 2020; Wynn & Vannella, 2016). To date, the wide range of known macrophage functions are attributed to their ability to switch into subsets within their lineage. Whether macrophages of adult tissues can adopt functions of other cells by initiating transdifferentiation have not been demonstrated.

Manifestations of cardiovascular diseases are caused by impaired tissue perfusion, and subsequent injury and loss of tissue function. Rapid re-establishment of functional blood flow is critical following an ischemic event to limit the extent and severity of tissue damage, as well as allowing for healing and regaining function. Formation of new blood vessels from existing ones, angiogenesis, is promptly initiated following ischemia onset, and occur in parallel with an inflammatory response provoked by the damaged tissue (Hong & Tian, 2020). Angiogenesis is a highly complex process that involves degradation of the capillary basement membrane, endothelial cell proliferation and directed migration, followed by tube formation and vessel fusion (Adair & Montani, 2010). Macrophages contribute to
angiogenesis by producing Vascular Endothelial Growth Factor A (VEGF-A), and by degrading extracellular matrix (Christoffersson et al., 2012, 2017; Massena et al., 2015; Vågesjö et al., 2021). To regain functional perfusion, these vessels then need to stabilize and mature, which requires recruitment of perivascular mural cells (pericytes and smooth muscle cells) in a platelet-derived growth factor BB (PDGF-BB) (secreted by endothelial cells) and platelet-derived growth factor receptor beta (PDGFRβ) (on the surface of mural cells) -dependent manner. In a model of renal injury, macrophages have been demonstrated to support the recruitment of pericytes by secreting PDGF-BB (Spiller et al., 2014). During homeostasis, pericytes and vascular smooth muscle cells regulate vessel permeability and blood flow, respectively, and genetic models of mural cell-loss result in haemorrhagic and dilated vessels leading to lethality before birth (Hellström et al., 1999, 2001; Levéen et al., 1994). So far, therapeutic means to improve the formation of functional blood vessels by upregulating growth factors or chemokines at the site of ischemic injury has proven limited clinical success (Dragneva et al., 2013; Eming et al., 2017a; Phillipson & Kubes, 2019; Tauber, 2003; Wynn & Vannella, 2016).

Using patient samples of the injured muscle following myocardial infarction and peripheral arterial disease, as well as ischemic mouse muscles, we recently found that macrophages accumulate at perivascular locations (Vågesjö et al., 2021). The current study investigated if the perivascular macrophages in the ischemic muscle acquire a mural cell phenotype and genotype important for re-establishing perfusion. Using two different mouse models of tissue ischemia, in combination with macrophage fate-mapping, intravital microscopy and single-cell RNA-sequencing (scRNA-seq) analyses, this study demonstrates that macrophages of adult mice can initiate transdifferentiation while losing their macrophage identity. The macrophages thereby shift towards a mural cell fate and undertake several mural cell functions important for healing of ischemic injury. This study uncovers a novel role for perivascular macrophages in injured tissue, which may provide a new potential target to promote the formation of functional blood vessels in ischemic disease.

**Online Methods**

**Experimental animals and lineage tracing experiments**

C57Bl/6J mice (Taconic Bomholt), Cx3cr1<sup>CreERT2</sup> (Yona et al., 2013), Rosa26-tdTTomato mice (Madisen et al., 2010), Pdgfrβ<sup>EGFP</sup> mice, Cx3cr1<sup>GFP/+</sup> mice (Jung et al., 2000), Pdgfrβ<sup>flx/flx</sup> kindly provided by Professor Betsholtz, Uppsala, Sweden) and Ng2<sup>dsRed</sup> mice (Zhu et al., 2008) were used in the current study. CreERT<sup>2</sup>-mediated recombination was initiated by oral gavage of 2 mg of tamoxifen (Sigma) in 90% of corn oil and 10% of Gethanol given once every 24 hours for 5 days for lineage tracing, and every 12 hours for 10 days to the Pdgfrβ<sup>flx/flx</sup> mice. All experiments were approved by the Uppsala Region Laboratory Animal Ethics Board (numbers C81/14 and C12740/20).

**Hindlimb ischemia**
Mice were anesthetized (Isoflurane, 2.5%, Forene, Abbott Laboratories) and the fur was removed from the left leg. Hindlimb ischemia (HLI) was induced as described before (Niiyama et al., 2009). Briefly, the left femoral artery was separated from the femoral vein and nerve, followed by ligation and cutting of the femoral artery above the superficial epigastric artery branch. Post-operative pain relief was achieved with sub-cutaneous administration of carprofen (5 mg/kg). Laser speckle flowmetry was carried out on day 1 to confirm hindlimb ischemia. Functional recovery of limb function was scored over time based on the following Tarlov scoring system: 0: no movement; 1: barely perceptible movement, no weight bearing; 2: frequent and vigorous movement, no weight bearing; 3: supports weight, may take 1 or 2 steps; 4: walks with only mild deficit; 5: normal but slow walking; 6: full and fast walking (Westvik et al., 2009). Healthy controls are defined as animals who have not undergone hindlimb ischemia induction.

**Laser speckle flowmetry**

Blood flow in footpads was measured as previously described in (Vågesjö et al., 2021). Briefly, anesthetized (spontaneous inhalation of 2.5% isoflurane, Forene, Abbott Scandinavia, Stockholm) mice were placed in a prone position with paws resting on tubing with circulating water (Laser Speckle Contrast Analysis, 785 nm laser, 20 µm resolution, PeriCam HR PSI System, Perimed). After baseline registration, the circulating water temperature was increased, resulting in an approximately 10°C temperature increase on the dorsal side of the footpad. Blood flow was recorded for 2 min after reaching the plateau, at 10 images/s and analyzed in PIMSoft (Perimed).

**Pancreatic islet isolation and transplantation**

Islets were isolated from donor C57Bl/6J mice as described previously (Bohman et al. 2006). Briefly, mice were euthanized by cervical dislocation, and thereafter an ice-cold collagenase A solution from Clostridium histolyticum (2.5 mg/mL; # 10103578001, Roche Diagnostics) in HBSS was injected into the pancreas via the common bile duct. The pancreas was then removed and placed in a 37°C water bath for 18 minutes. Following tissue digestion, islets were separated from the exocrine fraction by density gradient centrifugation (Histopaque-1077 and RPMI1640, Sigma-Aldrich) and hand-picking of intact islets as evaluated by edge clarity. The free-floating islets were incubated overnight at 37°C in islet culture medium (GlutaMAX RPMI 1640 (Gibco) with added D-glucose (11.1 mM), FBS (10%) and penicillin-streptomycin solution (#L0022; Biowest).

Immediately before transplantation, islets were fluorescently labelled with the intracellular probe Celltracker Blue CMAC (#C2110, Invitrogen) according to manufacturer’s instructions. Labelled islets were transplanted through a butterfly needle to the abdominal external oblique muscle of syngeneic Cx3cr1\(^{GFP/+}\) Ng2\(^{dsRed}\) mice anesthetized isoflurane (2.5%, Forene, Abbott Laboratories) (Christoffersson et al., 2010).

**Macrophage depletion and PDGFR\(\beta\) inhibitor treatment**

Macrophage depletion was achieved by administration of clodronate liposomes (Liposoma Research) in the tail vein (500 µg) and/or intramuscularly (125 µg). Control liposomes were given to the control group...
following the same protocol. Efficiency of depletion was determined by in vivo detection (intravital microscopy) of CX3CR1+ cell density at the site of islet engraftment, and by flow cytometry-based quantification of macrophages (CD45+CD11b+CX3CR1+Ly6C−) in the gastrocnemius muscle. Inhibition of PDGFRβ signalling was achieved by intramuscular injections of 20 µg (in saline, 100 µl final volume) of PDGFRβ inhibitor (CP673,451; Tocris) 2 to 6 days after ischemia induction.

**Single cell isolation**

The mice were euthanized by cervical dislocation, after which the gastrocnemius muscle or the islet graft was removed and minced into very small pieces with a scalpel. The tissue was then mechanically and enzymatically dissociated as follows: the muscle was incubated in collagenase II (500 U/ml) (#17101015; Gibco) in RPMI1640 in 37°C for 30 minutes during intermittent pipetting. After washing the tissue with ice-cold DPBS, it was incubated for further 30 minutes with collagenase IV (15 U/ml) (#17104019; Gibco) and dispase (2.4 U/ml) (#17105041; Gibco) in RPMI1640, with intermittent pipetting. Thereafter, the tissue suspension was passed through a 23G 0.6 mm needle 8–10 times and filtered through a 70 µm cell strainer.

**Flow cytometry**

For flow cytometry, single-cell suspensions were subjected to debris removal (#130-109-398; Miltenyi Biotec), followed by incubation with 10% FBS in RPMI-1640 for 20 minutes on ice. Next, extracellular antigen fluorochrome-conjugated primary antibodies were added for 15 minutes on ice, with appropriate control antibodies (Table 1). Last, live/dead cell staining was carried out using CellTrace Calcein Violet, AM (C34858; Invitrogen) or LIVE/DEAD Fixable Violet Dead Cell Stain Kit (L34964; Invitrogen), according to manufacturer’s instructions. Cells were analysed using CytoFlex S (Beckman Coulter) flow cytometer with CytExpert software (Beckman Coulter) and data analysis was performed using FlowJo software (BD).

For FlowSight, single-cell suspension was stained with CellTrace Calcein Violet, AM (C34858; Invitrogen) according to manufacturer’s instructions. The FlowSight imaging flow cytometer (EMD Millipore) was used to acquire images of the cells. Cells were gated from debris using a plot of bright-field channel area versus bright-field channel aspect ratio. Image analysis was performed in IDEAS software (EMD Millipore).

**Single-cell RNA-seq, data processing and analysis**

A total of 755 tdTomato+PDGFRβ+/− (tdTomato+PDGFRβ+ and tdTomato+PDGFRβ−) cells and 372 tdTomato−PDGFRβ+ cells from ischemic muscles at day 21 post-ischemia onset, and a total 190 tdTomato+PDGFRβ+/− cells and 192 tdTomato−PDGFRβ+ cells from healthy muscle were FACS sorted into SMART-Seq2 plates in two batches using BD FACSaria III Cell Sorter in a four-laser configuration (405, 488, 561, 633 nm) (BD Biosciences). RNA libraries were prepared using the Smart-seq2 protocol (Picelli et al., 2013) and sequenced with Illumina HiSeq 2000 at Eukaryotic Single Cell Genomics Facility at SciLifeLab, Stockholm.
The raw sequencing data was converted to demultiplexed FASTQ files using deindexer (https://github.com/ws6/deindexer) based on the Nextera index adapters and the 384 well layout. An average depth of 0.6 million reads (43 bp, single end) per cell was obtained. Reads were then aligned to the mouse genome (GRCm38) using STAR v2.7.7a (Dobin et al., 2013), and FeatureCounts v2.0.1 (Liao et al., 2014) was subsequently used to extract raw gene expression count matrix from the alignments.

Scanpy v1.7.1 package (Wolf et al., 2018) was used to convert the cell by gene count matrix to AnnData object and for further analysis of the scRNA-seq data. Cell libraries were filtered out if: 1) the percentages of mitochondrial and ribosomal gene families were above 10%; 2) the number of unique genes in a cell was below 500; 3) predicted doublet by Scrublet v0.2.3 (Wolock et al., 2019). In total, 1,425 cells were retained for downstream analysis. Genes detected in less than three cells along with mitochondrial genes were excluded from the analysis, leaving 38,178 genes in the count matrix.

The Python version of Velocyto v0.17.17 (La Manno et al., 2018) run_smartseq2 command was used to count unspliced transcripts from the alignments masking out expressed repeat annotation downloaded from UCSC genome browser. The resulting loom file was read and the unspliced counts were added to AnnData object in ‘layers’ using scvelo v0.2.4 (Bergen et al., 2020).

Raw counts were then normalized and log-transformed, which were further processed for identification of highly variable genes using flavour ‘cell_range’ (Zheng et al., 2017), and only the HVGs were used for downstream dimensionality and visualization computation. Data were then scaled to be zero centred and linear data compression using principal component analysis (PCA) from which the top 40 principal components (PCs) explaining about 70% of the variance in the dataset were chosen for downstream analysis. Graph construction was also run on the top 40 PCs using approximate nearest neighbour (KNN) (Arya et al., 1998) search with k = 15. Non-linear dimensionality reduction was run on the top 40 PCs using UMAP (McInnes et al., 2018) and embedded into two final dimensions. Since the single cell data were obtained and sequenced at two different time points, we used Scanorama v1.7 (Hie et al., 2019) to integrate the two datasets in order to correct for the introduced batch effect.

Unsupervised graph clustering was run using the Leiden method (Traag et al., 2019), with modularity resolution parameter (res = 0.4). Macrophage cluster was further split into cluster 0, 1 and 2 using Leiden method with res = 0.3. Cell type annotation from (Oprescu et al., 2020) was used as reference and R Seurat v3 ‘FindTransferAnchors’ and ‘TransferData’ methods were applied for cell type prediction (Stuart et al., 2019). The marker genes used in the reference were further investigated to validate the predictions. Boundary cases where the cluster and prediction did not agree, cell types annotations were changed to follow the majority cells in the cluster.

Differential gene expression analysis between interested clusters was performed using Scanpy ‘rank_genes_groups’ with Wilcoxon rank-sum test based on normalized and logarithmized raw gene expression. Retrieved differentially expressed genes (pvals_adj ≤ 0.05) were divided into up- and down-regulated genes and the corresponding gene names were loaded to STRING v11.0b (Szklarczyk et al., 2019) for gene set enrichment analysis. Trajectory inference analysis was performed using PAGA (Wolf et
Pseudotemporal orderings were constructed by selecting cluster 0 cells as root. Diffusion pseudotime (DPT) was calculated for all the remaining cells relative to the root. Cellular trajectories were assembled for paths through specified clusters, with cells ordered by DPT values.

Gene unspliced ratio is calculated as dividing the unspliced counts by the sum of the unspliced counts and the spliced counts from featureCounts, which is then aggregated over cells within a given cluster and the mean value was taken. Histogram of the mean unspliced ratios revealed a cutoff at 0.3, when taking genes with $\geq 0.3$ mean unspliced ratio omitting lowly expressed genes in the majority of the cells in the cluster. The total sum of the unspliced counts of a given cluster divided by the total sum of the unspliced and spliced counts, were then calculated for the remaining genes. This score was used as the final gene unspliced ratio, and a cutoff at $\geq 0.8$ was applied to define genes with high unspliced ratio per cluster. The list of genes with high unspliced ratio from cluster 2 was loaded to STRING v11.0b (Szklarczyk et al., 2019) for gene set enrichment analysis.

scRNA-seq raw and processed data are deposited at GEO under accession number GSE211550. Data processing and analysis are compiled into a Python notebook available at personal Github deposit https://github.com/feifei/macro_smartseq2.

In vivo imaging

Anti-CD31 antibody tagged with Alexa Fluor 647 (#102416; Biolegend) was intravenously administered to the mice via the tail vein. Thereafter, mice were anesthetized (Isoflurane, 2%, Forene, Abbott Laboratories) and the left gastrocnemius muscle or abdominal external oblique muscle was exposed and mounted using the in-house developed light vacuum window system (Ahl et al., 2019) for intravital microscopic imaging (Leica SP8).

Immunohistochemistry

For quantifying vessel perfusion, 50 µg of lectin SBA from Glycine Max (#L32462; Invitrogen) was injected intravenously. After 15 minutes, the mouse was euthanized using cervical dislocation. Whole gastrocnemius muscle was removed and snap-frozen using liquid nitrogen. Thereafter, 20 µm thick cryosections were prepared and fixed for 10 min in ice-cold methanol and incubated for 60 min with 10% FBS in DPBS. Following, sections were stained with anti-CD31 antibody (Table 1). For detection of tdTomato and GFP proteins, whole gastrocnemius muscles were processed as described before (Giordani et al., 2018). Briefly, dissected muscles were fixed and permeabilized for 2 hours at room temperature in 2% PFA-0.1% Triton X, followed by incubation in 15% sucrose overnight at 4 degrees. The following day, the muscles were snap-frozen using liquid-nitrogen/2-methylbutane double-bath as described before (Meng et al., 2014). Thereafter, 20 µm thick cryosections were prepared and incubated for 60 min with 10% FBS in DPBS, and stained (Table 1). Vessel permeability was assessed in 0.5% Triton X-permeabilized 20 µm thick cryosections of gastrocnemius muscles as extravascular IgG (AF568 donkey-anti-mouse IgG; ThermoFisher; #A10037). All confocal imaging was performed with a LSM700 (Zeiss).
Table 1  
Flow cytometry and immunofluorescence staining antibodies.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody</th>
<th>Concentration</th>
<th>Isotype control</th>
<th>Secondary antibody (if applicable)</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>#103137 (Biolegend)</td>
<td>1 ng/µl</td>
<td>#400646 (Biolegend)</td>
<td>NA</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>#149033 (Biolegend)</td>
<td>1 ng/µl</td>
<td>#400266 (Biolegend)</td>
<td>NA</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>CD11b</td>
<td>#562287 (BD)</td>
<td>1 ng/µl</td>
<td>#562308 (BD)</td>
<td>NA</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Ly6C</td>
<td>#560596 (BD)</td>
<td>0.4 ng/µl</td>
<td>#560571 (BD)</td>
<td>NA</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>#ab6586 (Abcam)</td>
<td>10 µg/ml</td>
<td>#ab172730 (Abcam)</td>
<td>#A31573 (Invitrogen)</td>
<td>IHF</td>
</tr>
<tr>
<td>CD31</td>
<td>#16-0311-85 (Invitrogen)</td>
<td>10 µg/ml</td>
<td>#ab18450 (Abcam)</td>
<td>#A21247 (Invitrogen)</td>
<td>IHF</td>
</tr>
</tbody>
</table>

Statistical analysis

Data are presented as mean ± SEM and statistical analysis was performed using Prism 9 (GraphPad Software). Outliers were identified using Grubb’s test (Alpha = 0.05) and excluded. Shapiro-Wilk’s test was used to assess normal distribution. F-test and Brown-Forsythe tests were used to determine equal variance when analysing 2 or more groups, respectively. In case of < 6 data points per group, a non-parametric test was used. Unpaired two-tailed Student’s t-test was used for comparing two normally distributed groups with equal variance. One-way ANOVA with Dunnett’s post-hoc test was used for comparing data with more than two normally distributed groups. Mann-Whitney U test was used for comparing 2 groups with either non-normal distribution or non-equal variance. When comparing more than 2 groups, Kruskal-Wallis with Dunn’s multiple-comparison post-hoc test was used. P-values ≤ 0.05 were considered statistically significant.

Results

Macrophages adopt mural cell-like characteristics in ischemic muscle

Macrophages (F4/80⁺, CD11b⁺, CX3CR1⁺) accumulated in the gastrocnemius muscle at day 7 following induction of hindlimb ischemia (HLI, Extended Data Fig. 1A). Using whole-mount imaging and
immunofluorescence, we found that macrophages were located preferentially at perivascular positions where they presented an elongated phenotype mimicking a mural cell-like morphology (Fig. 1A), as previously shown (Vågesjö et al., 2021). Interestingly, the number of F4/80⁺, CD11b⁺, CX₃CR1⁺ cells which also expressed the mural cell markers PDGFRβ and Neural/glial antigen 2 (NG2) increased significantly at day 7 post-ischemia as determined by flow cytometry (Fig. 1B). To decipher if this observation was due to macrophages upregulating mural cell markers or vice versa, we genetically lineage traced macrophages and studied their response to ischemic injury. For this, Cx3cr1-CreERT² mice were crossed with R26-TdTomato mice, resulting in a heritable expression of tdTomato in myeloid cells and tissue resident macrophages upon tamoxifen administration (Extended Data Fig. 1B, C). For simultaneous in situ detection of PDGFRβ expression, the Cx3cr1-CreERT²xR26-TdTomato line was crossed with the PdgfrβEGFP reporter line. Tamoxifen-mediated recombination was followed by a 5-day washout period prior to induction of hindlimb ischemia to ensure labelling specificity (Robinson et al., 1991) (Extended Data Fig. 1C). This approach resulted in 29.3 ± 2.4% and 33.7 ± 4.0% labelled macrophages in circulation and muscle, respectively, assessed at the time of ischemia induction (day 5 of washout; Extended Data Fig. 1E). Characterization of tdTomato⁺ cells in healthy muscles using flow cytometry (day 5 of washout; see Extended Data Fig. 1D for gating strategy) showed that all recombined cells are indeed macrophages (CD45⁺, CX₃CR1⁺ and CD11b⁺; Extended Data Fig. 1F). Importantly, we also found that PDGFRβ⁺ cells did not express leukocyte markers prior to ischemia induction (Extended Data Fig. 1F), validating our lineage tracing strategy.

Consequently, we induced ischemia in mice with lineage-traced macrophages (Fig. 1C for experimental outline), and found through in vivo imaging that tdTomato⁺ macrophages took on perivascular positions in the ischemic muscle (at day 7 and day 21 after ischemia induction; Fig. 1D). Notably, flow cytometry of single cell suspensions of muscles revealed that 31.0 ± 5.2% tdTomato⁺ macrophages downregulated the expression of the myeloid markers CD45 and CX₃CR1 (Fig. 1E), while 13.0 ± 1.5% upregulated the mural cell marker PDGFRβ at 21 days after HLI (Fig. 1F), suggesting a macrophage-to-mural cell adaptation in response to ischemia. Immunofluorescent evaluation of tissue sections demonstrated that three weeks after HLI induction, tdTomato⁺ cells were located within the collagen IV⁺ endothelial basement membrane (Fig. 1G), a position shared with pericytes (Armulik et al., 2011). Interestingly, while the numbers of PDGFRβ⁻ lineage-traced macrophages remained constant during the three weeks following onset of ischemia, PDGFRβ⁺ lineage-traced macrophages accumulated in ischemic hindlimbs (Fig. 1H).

Downregulation of myeloid markers was specifically detected in PDGFRβ⁺ lineage-traced macrophages (Fig. 1I). Very few lineage-traced macrophages could be detected at 3 months after ischemia induction (319.2 ± 95.6 cells per gram of muscle) when the ischemic injury was healed and the number of muscle macrophages was similar to that of unaffected, healthy muscle (356.4 ± 142.6 compared to 7822.0 ± 2604.0, respectively). However, the few lineage-traced cells identified in the healed muscle had downregulated their expression of both macrophage- and mural markers (CD45, CX₃CR1, and PDGFRβ), while retaining their CD11b expression (Fig. 1J). These data demonstrate that macrophages adopt mural cell-like characteristics during the healing phase of ischemic muscle injury.
scRNA-seq reveals an intermediate macrophage subtype with a mural cell expression profile

To assess the extent of macrophage-to-mural cell shift following ischemia, we performed scRNA-seq of FACS-sorted tdTomato+ (predominantly of macrophage origin) and tdTomato−PDGFRβ+ (predominantly mural cells and fibroblasts) cells using healthy and ischemic (day 21 post-ischemia) gastrocnemius muscle from lineage traced Cx3cr1-CreERT2 x R26-tdTomato mice (Fig. 2A, Extended Data Fig. 2A).

Among the 1425 cells that passed the quality assessment, there were 148 and 737 tdTomato+PDGFRβ+ cells and 177 and 363 tdTomato−PDGFRβ+ cells from the healthy and ischemic muscles (day 21 post-ischemia), respectively (Fig. 2B, C). Antigen-presenting cells (APCs) (132 cells) and macrophages (744 cells, three sub-clusters 0, 1 and 2) formed distinct clusters among tdTomato+ cells, and mural cells (118 cells), fibro/adipogenic progenitors (FAPs) (274 cells), tenocytes (64 cells) and endothelial cells (63 cells) formed distinct clusters among tdTomato− cells (Fig. 2D, E, Extended Data Fig. 2B, 3). Furthermore, 21 cells that were tdTomato− were identified as macrophages or APCs (Fig. 2B, D), indicative of incomplete recombination efficiency with tamoxifen treatment, as previously shown in Extended Data Fig. 1E.

Macrophages of healthy muscle predominantly belonged to macrophage cluster 1, while the majority of macrophages of ischemic muscle were found in cluster 0, indicating an ischemia-induced expression signature (Fig. 2C, D, Extended Data Fig. 2B). Interestingly, macrophage cluster 2 appeared only in the ischemic muscle, suggesting that these macrophages emerged after the ischemia induction. Among the differentially expressed genes (DEGs) between cluster 2 and 0 in ischemic muscle, there were 168 up- and 1068 down-regulated genes at three weeks after ischemic injury (Fig. 3A, Extended Data Table 1). Genes upregulated in cluster 2 were associated with mural cells, while downregulated genes were macrophage-associated (Fig. 3A, B). Adding unspliced transcripts on top of the spliced transcripts, it became visually more apparent that the upregulated genes in cluster 2 associated with mural cells (*Pdgfrb, *Adamts2, *Ano1, *Sema5a, *Cacna1c and *Myh11, * in Fig. 3B, which all have high fraction of unspliced transcripts), while macrophage-associated genes generally had a low fraction of unspliced transcripts (with the exception of *Cd86, + in Fig. 3B), reflecting an ongoing induction of gene expression and therefore the future state of the cells (La Manno et al., 2018). Cluster 2 contained a high proportion of unspliced transcripts at 73% compared to an average of 26% unspliced sequences (Fig. 3C), and the average unspliced level is in agreement with the general observation in scRNA-seq data (La Manno et al., 2018). This high fraction of unspliced transcripts was also maintained at cellular level (Fig. 3D).

Genes upregulated in cluster 2 compared to cluster 0 were also highly expressed in mural cells (Fig. 3A, B). *Ano1, also known as *Tmem16a, is a calcium-activated chloride channel previously demonstrated to be required for peripheral blood vessel contractility causing disturbances in blood pressure following gene-inactivation in mice (Heinze et al., 2014). *Myh11 is known to be expressed in mature smooth muscle cells and pericytes to generate smooth muscle contractile protein myosin heavy chain 11 (myosin-11) (Kumar et al., 2017). Other mural cell-associated genes, including *Pdgfrb, *Sema5a, *Adamts2 and
Cacna1c, were also upregulated in cluster 2 (He et al., 2016) (Fig. 3A, B) in agreement with our observations of elevated protein levels of PDGFRβ in ischemic muscles (Fig. 1F). Expression of other mural cell markers, including Rgs5, Nes, Acta2 and Des, were nevertheless not significantly upregulated in cluster 2 (Fig. 3A, B) (He et al., 2016). Regarding genes associated with immune cell functions, Ptprc and Cx3cr1 were both downregulated in cluster 2 compared to cluster 0 (Fig. 3A, B), again confirming our observations at the protein level (Fig. 1I). In addition, downregulation of various hallmark innate immunity genes was observed in cluster 2, including toll-like receptors (Tlr2 and Tlr7), which play key roles in the detection of pathogens and consecutive macrophage activation. Expression of the gene encoding MMR (Mrc1), which is considered a marker of M2 macrophages modulating macrophage polarization, was also reduced in cluster 2 (Fig. 3A, B). In addition, Ebf1, encoding Early B-cell factor-1 transcription factor, was not among the significantly upregulated genes in cluster 2 (Fig. 3A), but had a high fraction of the unspliced transcripts (Fig. 3B). EBF1-expressing perivascular cells have been suggested to represent pericytes, and EBF1 has a functional role in the cell fate commitment toward the pericytes phenotype (Pagani et al., 2021).

Trajectory inference revealed the pseudotemporal ordering of the ischemic muscle cells, which identified cluster 2 as an “intermediate” population between cluster 0 macrophages and mural cells (Fig. 3E). Analysis of known macrophage and mural cell markers along this transdifferentiating path reveals a decreasing expression of macrophage associated markers and increasing expression of various mural cell markers discussed above (e.g. Pdgfrb, Ano1 and Myh11) (Fig. 3F). Inclusion of unspliced transcripts in this analysis further highlighted the increased expression of mural cell markers (Fig. 3F). Gene set enrichment analysis performed using STRING (Szklarczyk et al., 2019) with genes upregulated in cluster 2 (versus 0) yielded various GO biological process terms (referred to as GO terms) associated with mural cell functions (Teng et al., 2021) in the ischemic muscle (Fig. 4A), including blood circulation, blood vessel development, cell junction assembly, anatomical structure morphogenesis and tube development. In total, 73.5% of GO terms enriched in cluster 2 overlapped with those enriched in mural cells clusters. In contrast, of the GO terms depleted in cluster 2, 72.7% were associated with those enriched in cluster 0 macrophages, including immune system process, positive regulation of immune system process, immune response, myeloid cell activation in immune response, cytokine secretion, and leukocyte differentiation (Fig. 4B). Various overlapping terms between cluster 2 and those of tenocytes (tendon fibroblasts) and FAPs, previously shown to be related to mural cells and the production and remodelling of ECM, were also revealed (Fig. 4, Extended Data Figs. 4 and 5) (Biferali et al., 2019; De Micheli et al., 2020). Genes with high unspliced numbers of transcripts from cluster 2 were also enriched in mural cell associated biological processes (68.9%, Fig. 4A), indicating an ongoing expression shift of cluster 2 towards a mural cell identity.

In summary, the scRNA-seq data demonstrate that 3 weeks after ischemia induction, a sub-population of muscle macrophages acquire a gene expression profile characteristic of mural cells, while simultaneously losing the expression profile associated with macrophages.
Macrophages adopt mural-cell functions in the ischemic hindlimb and in the transplanted islet grafts

Next, we asked if the macrophage-to-mural cell shift was necessary for vessel maturation. To this end, we developed a macrophage depletion protocol where macrophages were depleted prior to losing their macrophage characteristics and at a time point where the ischemia-induced angiogenesis is already initiated, as macrophages are known to also induce angiogenesis. Thus, clodronate liposomes were administered daily between days 3–7 post-ischemia induction, which resulted in a 67 ± 8% depletion of macrophages in ischemic muscles (Fig. 5A, B and Extended Data Fig. 6 for gating strategy), while vessel density was not affected (day 7 post-ischemia, Fig. 5C). This macrophage-depletion strategy resulted in decreased number of perfused vessels (shown as reduced lectin:CD31 ratio; Fig. 5D), increased vessel permeability (Fig. 5E) and reduced basal tissue perfusion (Fig. 5F), demonstrating a role of macrophages in vessel maturation post-ischemia.

The model of hindlimb ischemia ensures strong hypoxic stimuli and consequent angiogenic response in gastrocnemius muscle, but the anatomical localization of the muscle makes in vivo visualization of newly formed vessels a challenge. To enable in vivo observations of macrophages and mural cells interacting with newly formed vessels, we utilized our previously developed and characterized model of syngeneic pancreatic islet transplantation to abdominal muscle (Christoffersson et al., 2010, 2012, 2017). This model results in localized hypoxia within the isolated and transplanted islet grafts, followed by de novo islet revascularization by vessels originating from muscle tissue (Christoffersson et al., 2010; Grapensparr et al., 2018). By transplanting islets into macrophage- and pericyte-reporter mice (Cx3cr1GFP/+; Ng2dsRed mice, Fig. 6A), we found that the newly formed intra-islet vessels were fully covered by mural cells by day 5 after transplantation (Fig. 6B, Extended Data Fig. 7). Further, macrophages from the recipient mice were promptly recruited to the transplanted islets (Christoffersson et al., 2017; Fig. 6C). In agreement with our observations in the hindlimb ischemia model, 16.5 ± 4.8% of the recruited macrophages co-expressed the mural cell marker NG2 and the macrophage marker CX3CR1 at day 5 following islet transplantation (Fig. 6D). To determine if macrophages contribute to mural cell coverage, we administrated clodronate liposomes to deplete macrophages before and after islet transplantation. Clodronate liposome treatment caused a 92.8 ± 0.1% depletion of macrophages in transplanted islets compared islets transplanted to mice receiving control liposomes (Fig. 6E). While the treatment did not affect total vascular volumes per islet volume (11.2 ± 2.1% (control), 16.6 ± 3.8% (clodronate)) (Fig. 6F), macrophage depletion resulted in severe deficiency of perivascular NG2+ mural cells at the sites of islet transplantation (4.9 ± 1.1% (control), 1.3 ± 0.4% (clodronate)) (Fig. 6G), along with increased vessel diameter indicative of immature vessels (Fig. 6H). These results demonstrate that in a model of localized hypoxia, as well as in the model of hindlimb ischemia, macrophages take on a mural cell phenotype and function at sites of ischemia.

**PDGFRβ-mediated signalling instructs macrophages to transdifferentiate into mural cells in the ischemic muscle and is required for functional recovery of limb function**
PDGFRβ-mediated signalling is central to the recruitment of mural cells to blood vessels during angiogenesis and mediates their close contact to the endothelium during homeostasis. To study if PDGFRβ-mediated signalling also controls the perivascular positioning of macrophage-derived cells, we administrated the PDGFRβ inhibitor CP673,451; (20 µg; or sham injection for control ischemic muscles) intramuscularly daily between day 2 and 6 after ischemia induction (Fig. 7A). *In vivo* imaging of the ischemic muscles revealed that the numbers of macrophages in sham- and inhibitor-treated muscles were comparable (Fig. 7B, C). However, pharmacological inhibition of PDGFRβ-signalling resulted in loss of the elongated macrophage shape observed in untreated ischemic muscles (Fig. 7B, D) and reduced macrophage coverage of blood vessel by 30% (Fig. 7B, E).

To further study the functional role of PDGFRβ in macrophages, we generated macrophage-specific PDGFRβ knockout mice (*Cx3cr1*-CreER<sup>T2</sup> x *Pdgfrb<sup>flx/flx</sup> mice, from here on referred to as PDGFRβ cKO mice), in which tamoxifen administration leads to a deletion of PDGFRβ specifically in CX<sub>3</sub>CR1<sup>+</sup> macrophages (Fig. 7F, G). We have previously found that 88 and 94% of the CX<sub>3</sub>CR1<sup>+</sup> cells express the macrophage marker F4/80 in the healthy and ischemic gastrocnemius muscle, respectively (Vågesjö et al., 2021). Macrophage-specific PDGFRβ knockout resulted in loss of the elongated macrophage shape in the ischemic muscle (Fig. 7N and O), similarly to the results shown with pharmacological inhibition, demonstrating PDGFRβ-mediated signalling contributes to the mural cell morphology of macrophages. Further, vessel densities and permeability were both increased in the gastrocnemius muscle of PDGFRβ cKO mice 7 days post-ischemia (Fig. 7H-J), demonstrating that PDGFRβ deletion in macrophages results in formation of immature vasculature in the ischemic muscle. Consequently, blood perfusion of footpads of the affected leg was demonstrated to be further impaired in PDGFRβ cKO mice 7 days post-HLI induction (Fig. 7M). Furthermore, assessment of the functional recovery of the limb using Tarlov scoring revealed reduced recovery of function in PDGFRβ cKO mice (Fig. 7K, L), demonstrating an important role of macrophage-PDGFRβ in the healing of ischemic injuries. Together these results demonstrate that macrophages in ischemic muscles initiate a transdifferentiation program into mural cells, which is crucial for blood flow recovery following injury.

**Discussion**

In the present study, we report that macrophages in adult, ischemic tissue undergo a differentiation program to morphologically, transcriptomically, and functionally resemble mural cells while losing their macrophage identity. This phenotype switch was dependent on macrophage upregulation of PDGFRβ, as impaired macrophage-specific PDGFRβ-signalling strongly compromised the recruitment of macrophages to vasculature, which consequently impaired blood vessel maturation and function at the affected site. Thus, macrophage phenotype conversion was crucial for the re-establishment of functional tissue perfusion necessary for healing, and prompts exploration when developing immunotherapies to restore tissue function following ischemic insults.
Macrophages are dynamic tissue resident immune cells with a wide arsenal of important functions in both host defense and tissue restoration following injury (Sica & Mantovani, 2012). The local microenvironment greatly influences macrophage effector functions, resulting in that the same cell can exert different tasks at different time points (Sica & Mantovani, 2012). Thus, macrophages entering an infected or injured area adopt an inflammatory phenotype following activation by interferon gamma and/or PAMPs and DAMPs (pathogen- or danger-associated molecular patterns, respectively), facilitating clearance of bacteria and cell debris. When this acute phase is over, the inflammatory phenotype of macrophages is dampened as they shift into a restorative phenotype driven by specific cytokines (IL-4/-13) (Su et al., 2015), tissue specific defense collagens (Minutti et al., 2017) or efferocytosis of apoptotic neutrophils (Bosurgi et al., 2017). In addition, the influence of metabolic factors on macrophage activation is increasingly recognized (Eming et al., 2017b). Stabilization of HIF-1α during hypoxia coordinates transcription of proinflammatory and glycolytic pathways in macrophages (Cramer et al., 2003). In contrast, IL-4/IL-13-activated macrophages undergo a metabolic switch by upregulating genes important for fatty acid oxidation, which occur in parallel with a functional shift from proinflammatory macrophages into tissue restorative at the injured site (Vats et al., 2006). In injured muscle from patients with myocardial infarction or peripheral arterial disease, as well as in mouse models of hindlimb ischemia, macrophages have been found to chaperone the vasculature and attain a mural cell morphology (Vågesjö et al., 2021). In the current study, we found that these perivascular macrophages upregulated proteins associated with mural cell identity, while downregulating those associated with macrophages. Intriguingly, two studies of embryonic development have identified mural cell marker expression of macrophages in skin and brain using immunohistochemistry (Yamamoto et al., 2017; Yamazaki et al., 2017). Thus, Yamazaki et al. showed that F4/80+ embryonic myeloid progenitors contribute to the mural cell pool of the skin in mouse embryos (Yamazaki et al., 2017). In addition, a capacity of CD31+F4/80+ macrophages in the central nervous system to differentiate towards NG2/PDGFRB/desmin-expressing cerebrovascular mural cells early during development has been demonstrated (Yamamoto et al., 2017). Importantly, these observations of embryonic tissues were merely based on immunohistochemistry and not further characterized at transcriptomic level, which precludes conclusions of potential transdifferentiation.

Cellular transdifferentiation can be studied by genetic fate mapping followed by single cell sequencing of lineage traced cells. Using this approach, we identified a subpopulation of lineage-traced macrophages which had initiated transdifferentiation into mural cells 3 weeks after ischemia induction while downregulating their innate immunity signature. The collective term mural cells refers to pericytes and smooth muscle cells as they cannot be separated by specific and stable marker genes, and have functional overlap in regard to vessel maturation and function (Armulik et al., 2011; Muhl et al., 2020). A closer investigation of the gene expression profiles revealed that the subpopulation of macrophages in which various mural cell genes were progressively upregulated (e.g. Pdgfrb, Sema5a and Adamts2), in parallel downregulated characteristic macrophage markers (e.g. Ptprc, Cx3cr1, Mrc1). Interestingly, iNOS expression was not significantly induced in the mural cell-like macrophages, indicating that the recent observation of macrophages adopting blood flow regulation in the ischemic muscle refers to another set
of macrophages (Vågesjö et al., 2021). Further, the transcriptomic shift of macrophages into mural cells were further enhanced when unspliced transcripts were included, which was selectively high in proportion in the macrophage population expressing mural cell genes. Unspliced reads can reflect an ongoing induction of gene expression and future cell state (La Manno et al., 2018), and could therefore indicate that this macrophage population has not reached its final state and is further transitioning towards the mural cell phenotype. Mural cell ontogeny is known to be heterogeneous and varies depending on tissue and context (Chen et al., 2016). The predominant view is that local proliferation of pericytes is induced following injury, as shown by upregulation of genes involved in mitotic cell division (Teng et al., 2021). Likewise, neointimal smooth muscle cells have been shown to undergo phenotypic switching and consequent cell proliferation in response to vascular injury (Herring et al., 2014; Nemenoff et al., 2011). However, the ability of macrophages to transdifferentiate into mural cells, or in fact any another cell type, of adult tissues has not previously been demonstrated.

Mural cells promote vessel maturation, as well as provide structural support and integrity to the vessel wall, which are crucial for vascular function. In the developmental setting, the PDGF-BB:PDGFRβ axis is central for mural cell recruitment to angiogenic vessels, and knockout mouse models for either gene present with severe mural cell deficiency and concomitant vascular dysfunction with leaky and dilated vessels that results in embryonic or perinatal lethality (Hellström et al., 1999, 2001; Levéen et al., 1994; Soriano, 1994). Here we show that the same signaling pathway is important to retain PDGFRβ+ macrophages at a perivascular location in the ischemic muscle, and that depletion of macrophages during post-ischemic angiogenesis results in formation of dilated vessels with reduced mural cell coverage. Further, when PDGFRβ was depleted in macrophages after initial stages of angiogenesis in the hindlimb ischemia model to not affect vessel densities, vessel perfusion was reduced and leakiness increased. This demonstrates that macrophages take on the function of mural cells following ischemic injury. As others and we have found that macrophage recruitment to angiogenic vessels occurs in a CXCL12-dependent manner (Grunewald et al., 2006; Vågesjö et al., 2021), we now propose that macrophage upregulation of PDGFRβ and concomitant PDGFRβ signaling is necessary for proper adhesion and organization of macrophages along the vessels, as reported for pericytes (Lindblom et al., 2003). In addition, for the first time to our knowledge, we have demonstrated that macrophage-specific expression of PDGFRβ is an important regulator of vascular density and integrity in the site of ischemic injury. During tissue restoration following ischemic injury, the macrophages thereby both shift towards a mural cell fate, as well as undertake several mural cell functions of importance for healing of ischemic injuries.

Peripheral vascular disease and ischemic heart disease are the most common cardiovascular disorders, and develops as a result of loss of tissue function due to inadequate restoration of tissue perfusion following the ischemic events (Timmis et al., 2018). Efficient treatment strategies are still lacking, and several different approaches are currently being assessed. Local stimulation of angiogenesis to re-establish perfusion of the injured tissue has been thoroughly investigated by local upregulation of growth factors and chemokines including VEGF, HGF, SDF-1, alone or co-delivered with e.g. PDGF-BB, but has so
far showed limited clinical success as the new blood vessels are leaky and immature (Banfi et al., 2012; Gianni-Barrera et al., 2016; Reginato et al., 2011; Thurston et al., 2000). More recently, immune-based therapeutic approaches encompassing biomaterial, biologics and targeted cell and gene therapy have been explored to limit injury and stimulate regain of tissue function by modifying specific immune responses important for the endogenous repair program. Of these, in vivo-generated antifibrotic chimeric antigen receptor (CAR) T cells have demonstrated promising results in mouse models of cardiac injury (Aghajanian et al., 2019; Rurik et al., 2022). Whether the current findings of an endogenous macrophage-to-mural cell switch being important for the healing of ischemic injury can be therapeutically enhanced warrants further explorations.

Together, our data reveal that a subpopulation of lineage traced macrophages in the ischemic muscle initiate a gene and protein expression profile extensively overlapping with the functional characteristics of mural cells, while losing a macrophage gene signature. This cellular switch was demonstrated to be central for vascular maturation and function, and pinpoint macrophages as a potential target for therapeutically enhancing vascular integrity and healing of ischemic injuries.

Declarations

CONTRIBUTIONS

KP and CHH designed, developed, performed, analysed experiments and wrote the manuscript. FX analysed scRNA-seq data and wrote the manuscript. CS performed and analysed experiments. AG performed experiments. GC and CL designed, performed and analysed experiments. DH and CG designed the lineage tracing experiments and advised on the scRNA-seq experiments. MP conceived, designed and supervised all experiments, and wrote the manuscript. All experiments were performed in the Phillipson laboratory, and all authors critically revised the manuscript.

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**Figures**
**Figure 1**

**Macrophages accumulating in the ischemic muscle adopt a mural-cell-like phenotype.** A) Representative confocal whole mount image of CX3CR1⁺ macrophages in the ischemic hindlimb (day 7) demonstrates a mural cell-like morphology. B) The number of macrophages expressing mural cell makers PDGFRβ⁺ and NG2⁺ increases at day 7 post-ischemia. C) Schematic representation of the lineage tracing strategy using...
the Lin-Cx3cr1CreERT2 Rosa26-tdTom mouse model where heritable tdTomato-labelling of CX3CR1-expressing macrophages is induced by tamoxifen treatment. D) Representative images of lineage-traced macrophages (day 7 and day 21 after ischemia induction) demonstrate their mural cell morphology. E) While the percentage of tdTomato+ cells that express CD45 and CX3CR1 is reduced with time following ischemia, F) the percentage of PDGFRβ expressing tdTomato+ cells increases in ischemic muscles. G) Representative image of a tdTomato+ cell localized within the collagen IV+ endothelial basement membranes in ischemic muscles. H) The number of tdTomato+ cells that express PDGFRβ increased over time at sites of ischemic muscle (day 21) while the number of tdTomato+ cells that did not express PDGFRβ was not different. I) Myeloid markers are reduced with time following ischemia in the tdTomato+PDGFRβ+ population but not in the tdTomato+PDGFRβ− cells. J) 3 months post-ischemia, the lineage-traced cells expressed predominantly CD11b, while other myeloid and mural cell markers were reduced. Kruskal-Wallis followed by Dunn’s post-hoc test (E, F). Mann-Whitney U test (B, H (left), I (%CX3CR1, %CD45 (right), %CD11b (right)). Unpaired student’s t test (H (right), I (%CD45 (left), %CD11b (left)). Data shown as average ± SEM.
Figure 2

Single-cell RNA profiling of tdTomato\(^+\) and tdTomato-PDGFR\(\beta\)^+ cells in healthy and day 21 post-ischemic muscle. A) Experimental layout of the scRNA-seq experiment, where Lin- Lin-Cx3cr\(^{1}\) CreERT2 Rosa26-ttdTom; \(Pdgfrb\)^eGFP mice received tamoxifen treatment for 5 days, followed by a 5-day washout period prior to induction of HLI. Single cells were isolated from healthy mice and 21 days post-ischemia induction, and
thereafter sorted based on tdTomato and PDGFRβ eGFP signal. UMAP embedding of scRNA-seq data showing the cells colored by B) tdTomato signal, C) time point (Healthy or Day 21 post-ischemia) and D) assigned cell types with macrophage sub-clustering. E) Dotplot of known marker genes for the assigned cell types with the number of cells in each cell type in bar plot to the right.

**Figure 3**
A subset of macrophages upregulates mural cell associated genes, and downregulates immune cell associated genes. A) Volcano plot of differentially expressed genes comparing cluster 2 to 0. Each dot represents a gene, x-axis represents the log2 fold change of gene expression in comparison, y-axis represents the -log10-transformed adjusted p-values. Genes with False Discovery Rate (FDR) adjusted p-value < 5% were considered to be significant, with up- and downregulated genes marked green and red, respectively. B) Dot plot of known macrophage- and mural cell-associated gene expressions without or with unspliced transcripts in different clusters, with * marking genes with ≥80% unspliced transcript fraction and + marking genes with ≥40-80% unspliced transcript fraction. C) Proportions of spliced and unspliced transcripts in all the clusters, with mean value expressed as the black line, showing that cluster 2 has a high proportion of unspliced transcripts. D) Clustered cells colored by fraction of unspliced transcripts, with cluster 2 standing out as having a high proportion of unspliced transcripts. E) Trajectory inference analysis by PAGA and F) expression of known macrophage- and mural cell-associated genes in pseudotemporally ordered cells (left: spliced transcript only, right: spliced and unspliced transcripts combined). All the analysis performed here are based on Day 21 post-ischemia cells.

Figure 4

Enrichment of GO terms associated with mural cell functions and depletion of those associated with immune cell function in cluster 2. A) Enrichment and B) depletion of GO terms in tdTomato+ macrophage sub-clusters 1 and 2, and PDGFRβ+ clusters of mural cells, compared to cluster 0; and genes of high unspliced fraction in cluster 2 (only in A).
Figure 5

**Macrophages of the ischemic muscle adopt mural cell function.** A) Schematic representation of the experimental layout. B) While the number of macrophages is reduced in the clodronate liposome treated ischemic muscles, the CD31 positive area is unaffected (C, expressed as ratio to average of control liposomes treated group) while the percentage of perfused vessels is reduced (D, % of total CD31+ vessels stained *ex vivo* which are double stained with lectin administered *in vivo*). E) Macrophage depletion also increased vessel leakage measured as extravascular IgG staining, and reduced basal perfusion of ischemic muscles (F). Unpaired student’s t-test (B,D-F) and Mann-Whitney U test (C). Data shown as average ± SEM.
Figure 6

Macrophage depletion results in acute mural cell deficiency along new vessels following islet engraftment. A) Experimental layout of the ischemic model of syngeneic islet transplantation: Isolated islets are transplanted into the abdominal muscle of recipient mice (red arrowhead) to enable in vivo imaging of the newly formed vasculature and the cells recruited to the hypoxic sites (black arrowhead). Control or clodronate liposome administration is indicated by the blue arrowhead. B) Representative
images of engrafted islets 5 days after transplantation where the newly formed intra-islet vasculature are lined by NG2⁺ mural cell. C) Representative images of islet transplantation sites of control and macrophage-depleted (clodronate) mice. D) Flow cytometry and FlowSight imaging identifies CX₃CR1-expressing NG2⁺ cells at islet transplantation sites at different time points (black arrow heads in panel A). E) While the positive areas for CX₃CR1 is reduced following clodronate treatment, the CD31⁺ areas do not change (F). However, macrophage depletion reduced the NG2⁺ areas in transplanted islets (G) and increased the average vessel diameter (H). Unpaired student's t-test (F-G), Mann-Whitney U test (E, H). Data shown as average ± SEM.s
Lack of PDGFRβ prevents the perivascular positioning of macrophages in ischemic muscles, leads to vessel leakiness and disturbs functional recovery of the leg following HLI. A) Experimental layout of the inhibition of PDGFRβ in the HLI model: PDGFRβ inhibitor CP673,451 was injected from day 2 to 6 d post-ischemia (HLI indicated by a red arrowhead), and in vivo imaging of the gastrocnemius muscle was carried out on day 7 (black arrowhead). B) Representative confocal images of macrophages (CX3CR1⁺)
and blood vessels (SBA-lectin 647) in sham and PDGFRβ inhibitor treated ischemic gastrocnemius muscles, demonstrating a change in macrophage morphology and perivascularity in inhibitor-treated group. C) Number of macrophages (n=3-4) and D) macrophage sphericity (sphericity index; n=3-4) in sham and PDGFRβ inhibited ischemic gastrocnemius. E) Vessel coverage by macrophages (n=3-4). F) Experimental layout: deletion of PDGFRβ in macrophages is induced by tamoxifen treatment starting 3 days before HLI, and continuing through the experiment. Control mice are age-matched mixed background (C57Bl/6J and 129S) mice that lack CreER\textsuperscript{T2} expression. G) Schematic representation of the CX\textsubscript{3}CR1\textsuperscript{+} cell specific PDGFRβ knock-out mouse model where heritable labelling by tdTomato and deletion of PDGFRβ in CX\textsubscript{3}CR1-expressing macrophages is induced by tamoxifen treatment. Imaging of CD31 in control and KO mice 7 days post-ischemia (gastrocnemius muscle whole-mounts) (H) revealed increased vessel volume (I) in KO mice (n=2 mice (control), n=6 mice (KO). J) Vessel leakage 7 days post-ischemia measured as extravascular IgG staining. Deletion of PDGFRβ in CX\textsubscript{3}CR1\textsuperscript{+} cells results in reduced functional recovery of the ischemic leg as assessed by the Tarlov score (K, L), as well as reduced perfusion of the hind-limb assessed by laser speckle flowmetry (M). N) Representative confocal images of labeled macrophages (tdTomato\textsuperscript{+}) and blood vessels (CD31) in ischemic gastrocnemium muscles from control mice or mice where PDGFRβ was deleted in their CX3CR1-expressing cells, demonstrating a change in macrophage morphology in KO group. O) Cell sphericity in mice lacking PDGFRβ in their CX3CR1\textsuperscript{+} cells. In C-D, I-J and O, each dot represents an area analyzed from tile-scan image of muscle whole-mounts. Unpaired student’s t-test (D, M). Mann-Whitney U test (C, E, I-L, O). Data shown as average ± SEM.

**Supplementary Files**

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