SUPPLEMENTAL MATERIAL

Ischemic-trained monocytes improve arteriogenesis in a mouse model of hindlimb ischemia

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Supplemental Materials and Methods

Mouse study design

Male and female C57Bl/6J wild type (stock No: 000664) and C57Bl/6 tomato (mT/mG, mTmG stock No: 007676) mice were obtained from the Jackson Laboratory. In our animal facility, animals were housed in collective cages, with temperature and humidity-controlled, on a 12h light - dark cycle, allowed *ad libitum* access to standard laboratory diet and water.

Flow Cytometry Analysis

The sub-population of injected BM-Mono mice were analyzed by flow cytometry. Briefly, one million cells were stained with PerCP anti-mouse/human CD11b Antibody (cat no. 101230) and APC anti-mouse Ly-6C Antibody, (cat. no. 128016), both from BioLegend. BM-Mono were incubated with antibodies diluted 1/100 in FACS buffer (5% fetal bovine serum, 1mM EDTA, 0.1% sodium azide in phosphate-buffered saline) for 45 min on ice in the dark, then washed once, and fixed in FACS buffer plus 1% paraformaldehyde. Fixable Viability Dye eFluor 520 was used to irreversibly label dead cells prior to analysis. Labeled cells were analyzed using the BD LSRII from BD Biosciences. The LSRII is equipped with 4 lasers and 15 detectors and runs the FACS Diva v.8 acquisition software.

Circulating Cytokines and Chemokines

Circulating levels of cytokines/chemokines were evaluated in 24h trained and sham donor mice. Blood was extracted by cardiac puncture in anesthetized mice and collected in heparin vials. Samples were centrifuged at room temperature (15 minutes at 2,500 rpm), then the plasma was transferred into a clean vial, properly labeled, and stored at -80°C until used. Cytokine and chemokine levels in the plasma were analyzed using a Mouse Cytokine Discovery Assay (Eve Technologies, Calgary, AB Canada).

Gene Expression of Circulating GLUT-1 in Erythrocytes

Circulating levels of GLUT-1 were assessed in 24h trained, 24h ischemia, and sham donor mice. A cardiac puncture was performed in anesthetized mice and blood was collected in heparin vials. Blood was centrifuged at room temperature for 15 minutes at 2,500 rpm. The plasma was removed, and the erythrocytes were washed three times with PBS and stored in RNA at -80°C until used. RNA extraction, cDNA, and qRT-PCR were performed as described in the methods session of this manuscript.

Tomato Cell Tracing in Hindlimb Muscle after Adoptive Transfer

Recipient mice were subjected to hindlimb ischemia one day prior to the BM-Mono adoptive transfer from tomato donor mice (24h trained and sham). Recipients were euthanized 24h after the adoptive transfer was performed (via tail injection), and the calf muscle was harvested. Calf samples were then fixed in 0.5% paraformaldehyde, 1% glutaraldehyde, and 20% sucrose in 100-mM sodium phosphate buffer (pH 7.4) for 24h and transferred to 20% sucrose for 24h. Tissues were embedded and frozen in OCT cutting compound, cut into 5-μm frozen sections, and then

DAPI staining was performed. Briefly, slides were heat at 37°C for 30 min and then put in cool acetone for 10 min. After that, they were removed from acetone, let dry for 10 min, and then rinsed three times with PBS. Slides were incubated with DAPI solution (1µg/ml) for 5 min. After being rinsing three times with PBS, the slides were mounted with polyvinyl alcohol mounting medium (Sigma, cat. 10981). Tissue sections were examined with a fluorescence microscope Olympus IX71 (Olympus America, Melville, NY). Data were captured and processed using the cellSens Standard Imagining Software v. 1.9 (Olympus Corporation).

Lipid Profile of Isolated BM-Mono

Isolated BM-Mono from sham and 24h trained group were rinsed 3 times with PBS. After the adjustment of million cells per 100µl, they were instantly frozen and stored at -80oC until analysis. Liquid chromatography-mass spectrometry platform was used to detect and quantify the sterol lipids present in the samples. The analysis was performed by Creative Proteomic using pmol/million cells.

Major Resources Table – Supplemental

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mouse	The Jackson Laboratory	C57Bl6J	both	https://www.jax.org/strain/000664

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	mouse	The Jackson Laboratory	C57Bl6J	mT/mG , mTmG	https://www.jax.org/strain/007676
Parent - Female	mouse	The Jackson Laboratory	C57Bl6J	mT/mG , mTmG	https://www.jax.org/strain/007676

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
Cd11-b	BioLegend	101230	1/100		https://www.biolegend.com/en-us/products/percp-anti-mouse-human-cd11b-antibody-4315?GroupID=BLG10427
Ly6C	BioLegend	128015	1/100		https://www.biolegend.com/en-us/products/apc-anti-mouse-ly-6c-antibody-6047?GroupID=BLG7242
SMA	Aligent-DaKo`	M0851	1/200		https://www.agilent.com/en/product/immunohistochemistry/antibodies-controls/primary-antibodies/actin-(smooth-muscle)-(concentrate)-76542

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Mouse BM-monocytes	Primary culture	Both sex C57Bl6J	

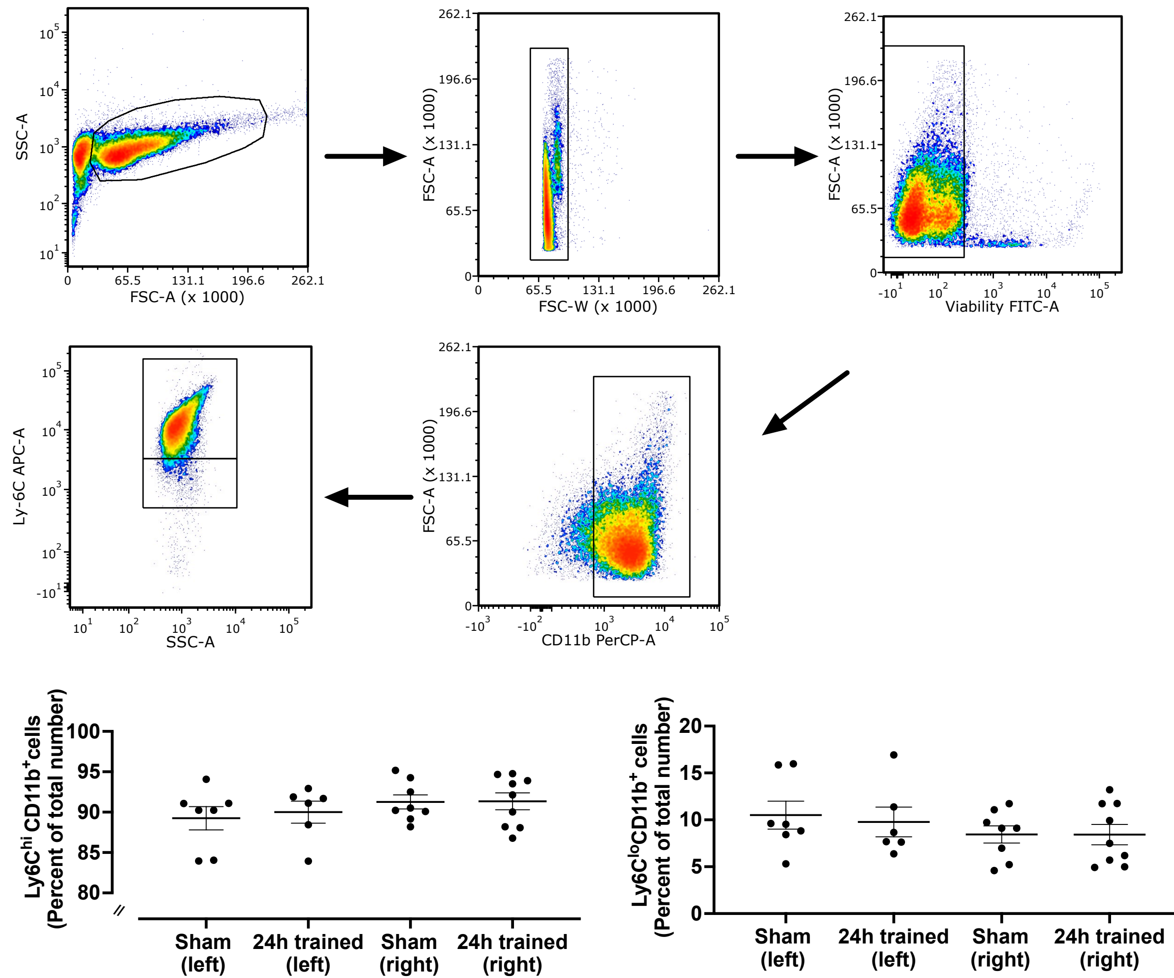

Figure S1. Flow cytometry analysis of isolated BM-Mono. Isolated BM-Mono from right (non-ischemic) and left (ischemic) legs of 24h trained and sham donors were analyzed for Ly6C^{hi}CD11b^{+} (inflammatory monocytes) and Ly6C^{lo}CD11b^{+} (patrollers monocytes) markers. There was no difference in the percentage of inflammatory vs. patrollers monocytes between the groups. Data were compared to sham (left) using Brown-Forsythe ANOVA test with Dunnett's multiple comparisons test.

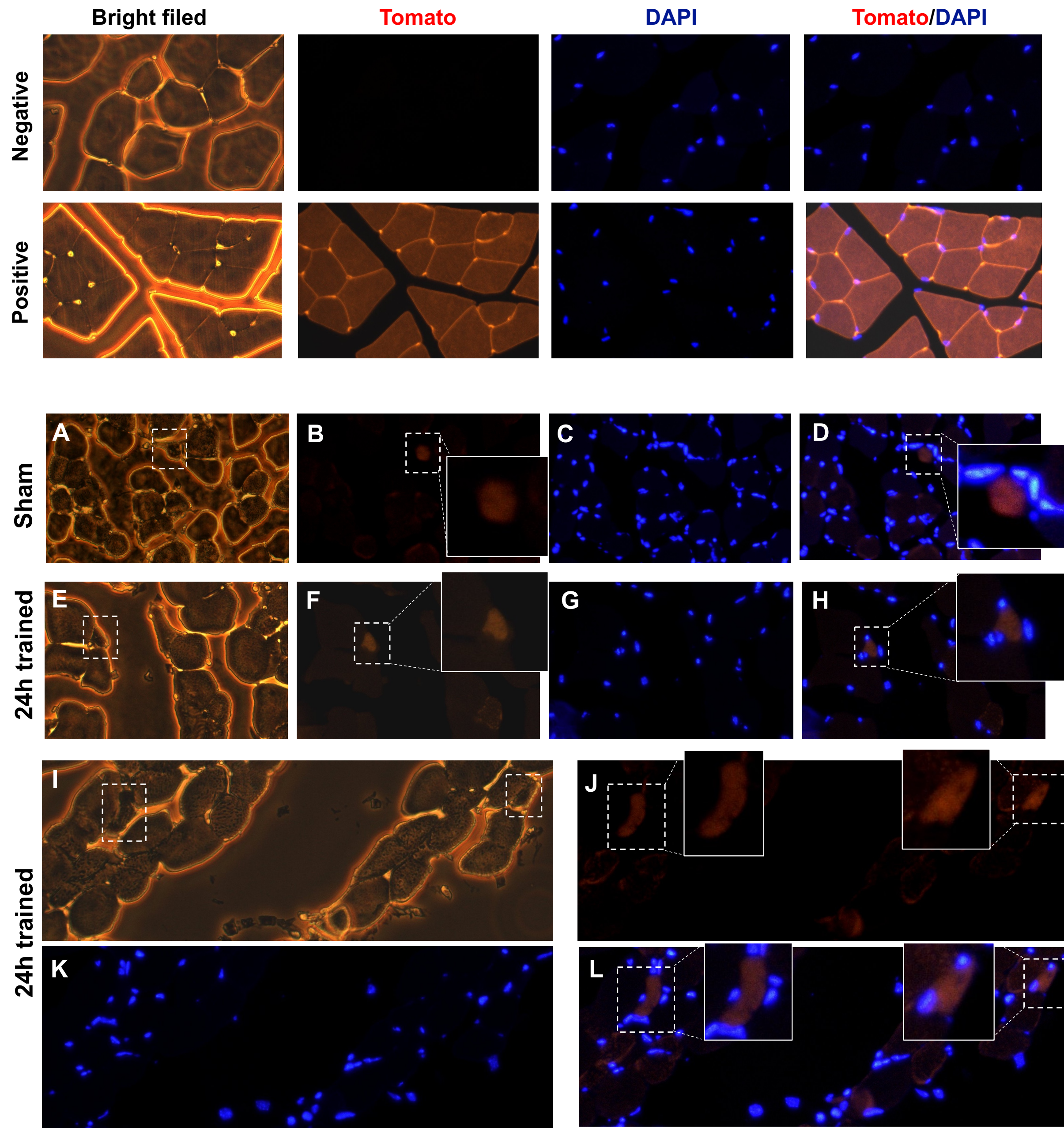

Figure S2 - Cell tracing after adoptive transfer of tomato donors BM-Mono in recipients' ischemic muscle. BM-Mono from 24h trained and sham tomato donor mice were injected in wild type recipient mice subjected to hindlimb ischemia. Upper panel demonstrates negative control (calf of wild type recipient) and positive control (calf of tomato donor). Tomato fluorescence (B, F and J) indicates the presence of donors' BM-Mono in the wild type recipient ischemic calf using immunofluorescence microscopy. Images A, E and I represent bright filed, and nuclei were counterstained with DAPI (blue; C, G and K). Tomato/DAPI staining (D, H and L) confirms that the donor cells were found in both, 24h trained and sham ischemic calf of recipient mice. Representative photomicrographs were taken using 20x lenses, and in images B, D, F, H, J and M, the square is 2x magnified.

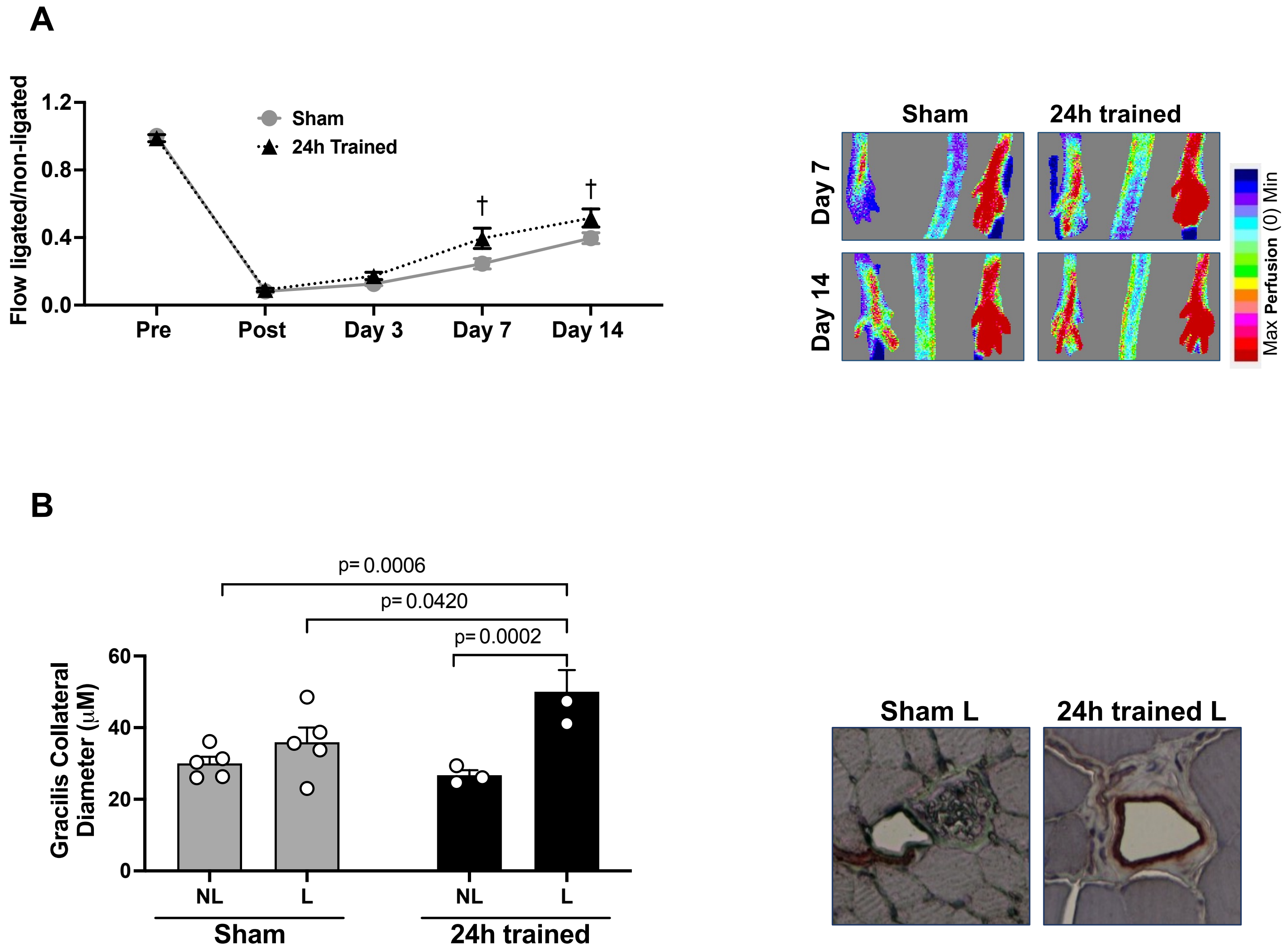

Figure S3 – Adoptive transfer of BM-Mono that were subjected to ischemia training (or sham) one-week prior to isolation/injection. (A) LDI demonstrates a significant improvement of blood flow recovery in recipient mice who received ischemic-trained BM-Mono (n=8). **(B) I**schemic limbs of recipient mice who received ischemic-trained monocytes show remarkably effective arteriogenesis of the gracillis collaterals. NL: non-ligated, L: ligated. The data were analyzed using two-way ANOVA followed by Sidak multiple comparison tests. Errors bars represent SEM (†p<0.01 vs. same timepoint – Sham).

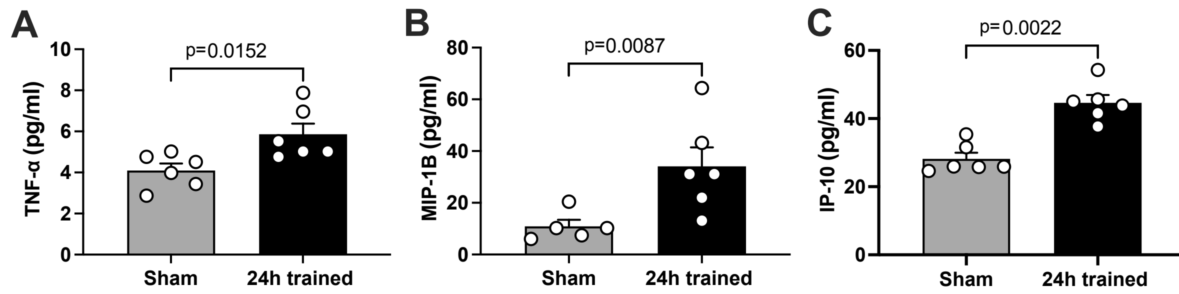

Figure S4. Circulating pro-inflammatory cytokines/chemokine levels in donor mice. Plasma samples from 24h trained donor mice demonstrate upregulation of cytokines (A) TNF-α and chemokine (B) MIP-1B and (C) IP-10 (median; 95% CI) by Mann-Whitney test.

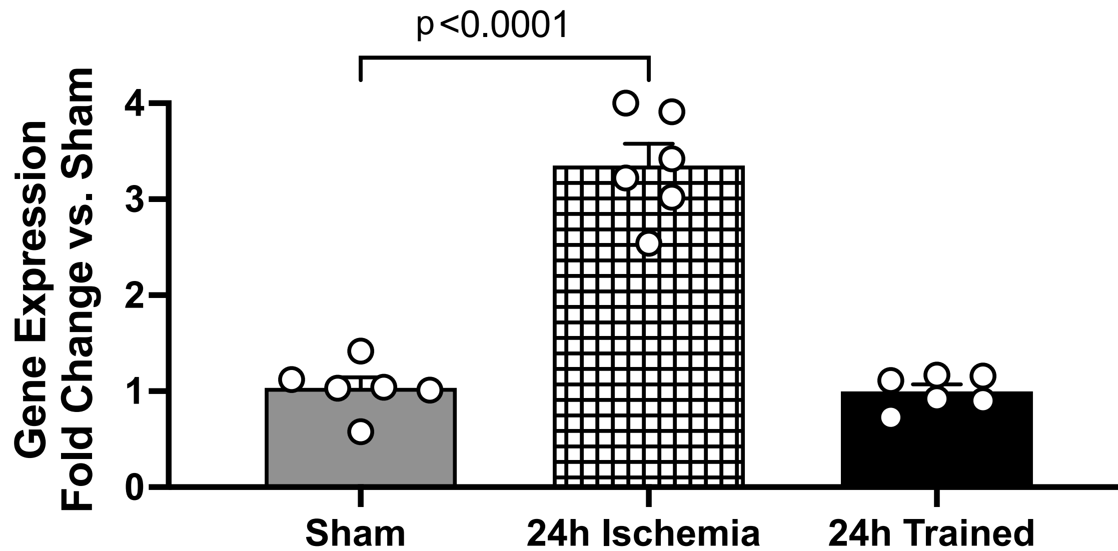

Figure S5. Gene expression of GLUT-1 in the circulation. Red blood cells (erythrocytes) were isolated from 24h ischemia (when FA was still occluded after 24h of ligation); 24h trained (when FA was occluded for 24h but already opened for 2 days); and sham. GLUT-1 gene expression was quantified by q-RT-PCR. Erythrocytes from 24h ischemia group demonstrate upregulation of GLUT-1 (more than 3fold), which is known as one of the most important HIF-1α target genes. The data were compared to sham group using 1-Way ANOVA test followed by Dunnett's multiple comparisons test. Errors bars represent SEM.

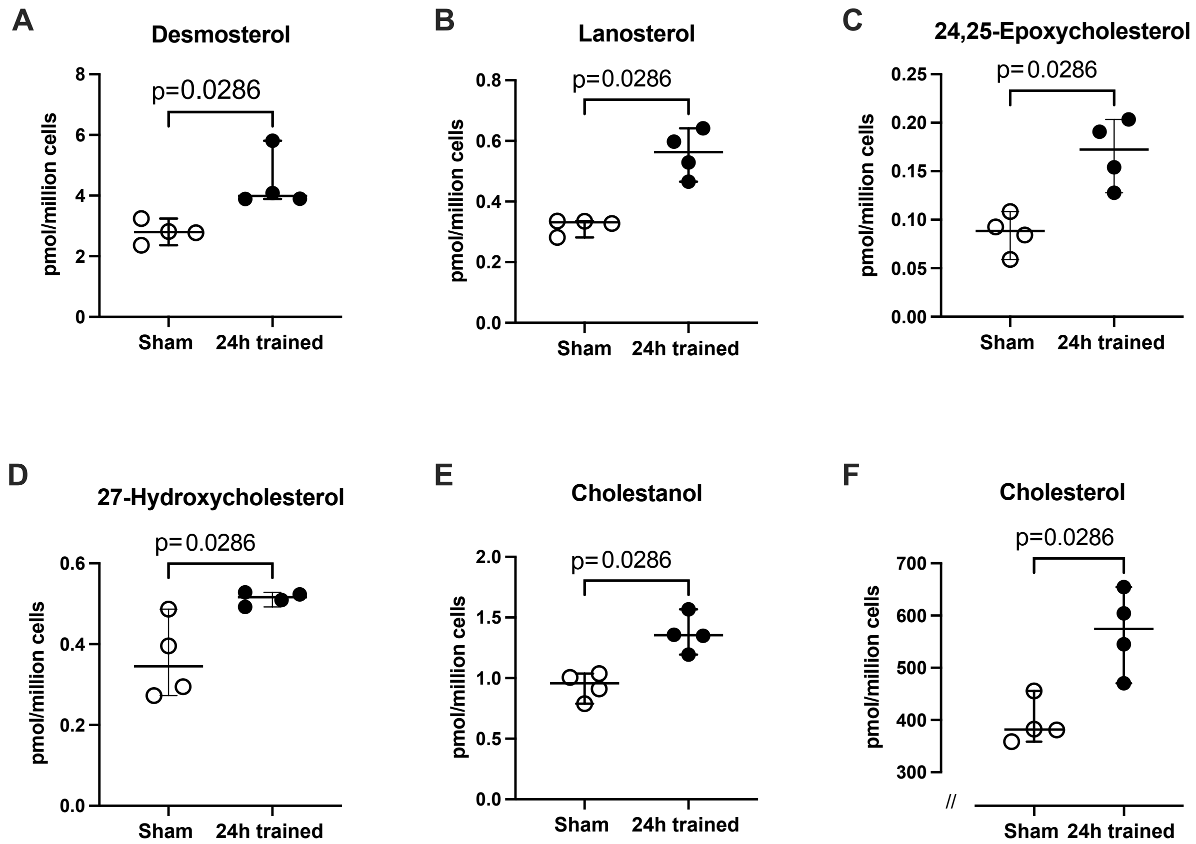

Figure S6. Sterol analysis of BM-Mono. Isolated BM-Mono of both legs show up-regulation of (A) desmosterol; (B) Lanosterol; (C) 24,25-Epoxycholesterol; (D) 27-Hydroxycholesterol; (E) Cholestanol, and (F) Cholesterol in the 24h trained compared to Sham (median; 95% CI) by Mann-Whitney test.

Table S1. Top monocytes genes regulated by ischemia training

Gene	Symbol	ENSEMBL ID	*p* value	FDR	Fold Change
Apolipoprotein E	**ApoE**	ENSMUSG00000002985	8.06E-09	0.0001	***- 2.388***
Family with sequence similarity 71 member A	Fam71a	ENSMUSG00000091017	3.39E-07	0.0017	3.275
PNN interacting serine and arginine rich protein	Pnisr	ENSMUSG00000028248	8.19E-07	0.0034	- 1.452
Terminal nucleotidyltransferase 5A	Tent5a	ENSMUSG00000032265	1.01E-06	0.0040	- 1.463
Joining chain of multimeric iga and igm	Jchain	ENSMUSG00000067149	3.33E-06	0.0108	- 2.259
Squalene epoxidase	**Sqle**	ENSMUSG00000022351	4.14E-06	0.0120	***- 2.221***
O-linked N-acetylglucosamine (glcnac) transferase	Ogt	ENSMUSG00000034160	4.90E-06	0.0134	- 1.463
Myristoylated alanine rich protein kinase C substrate	Marcks	ENSMUSG00000047945	8.17E-06	0.0205	- 1.579
Chemokine (C-C motif) ligand 6	Ccl6	ENSMUSG00000018927	1.14E-05	0.0253	- 1.662
Adhesion G protein-coupled receptor E4	Adgre4	ENSMUSG00000032915	1.24E-05	0.0264	- 1.787
BCL3 transcription coactivator	Bcl3	ENSMUSG00000053175	1.57E-05	0.0321	- 1.717
Thioredoxin interacting protein	Txnip	ENSMUSG00000038393	2.03E-05	0.0379	- 1.774
Lipin 1	**Lpin1**	ENSMUSG00000020593	2.05E-05	0.0379	***- 1.809***
24-dehydrocholesterol reductase	**Dhcr24**	ENSMUSG00000034926	2.41E-05	0.0417	***- 2.200***
Calcium/calmodulin dependent protein kinase I	Camk1	ENSMUSG00000030272	2.50E-05	0.0420	- 1.607

Table shows all genes down-regulated or up-regulated by ≥1.5-fold. Genes were considered to be statistically different from sham group with a false discovery rate p-value (FDR) ≤ 0.05 determined by applying the Benjamini-Hochberg multiplicity correction method (n=4).