Therapeutic potential of adipose-derived stem cells and macrophages for ischemic skeletal muscle repair

Aim: Progressive ischemia due to peripheral artery disease causes muscle damage and reduced strength of the lower extremities. Autologous cell therapy is an attractive treatment to restore perfusion and improve muscle function. Adipose-derived stem cells (ASCs) have therapeutic potential in tissue repair, including polarizing effects on macrophages (MPs). **Materials & methods:** Co-culture systems of ASCs and MPs were analyzed for gene and protein expression modifications in ASC-conditioned MPs. Co-transplantation of MPs/ASCs *in vivo* led to improved skeletal muscle regeneration in a mouse model of peripheral artery disease. **Results:** ASCs/MPs therapy restored muscle function, increased perfusion and reduced inflammatory infiltrate. **Conclusion:** Combined MPs/ASCs cell therapy is a promising approach to restore muscle function and stimulate local angiogenesis in the ischemic limb.

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Peripheral artery disease (PAD) refers to partial or complete obstruction of one or more peripheral arteries. Globally over 200 million people are living with PAD. Over the last decade, there was a 30% increase in PAD diagnoses in developing countries and 13% increase in the developed world [1,2]. It is estimated that around 20–40 million people (10–20% of total PAD diagnoses) experience intermittent claudication, with 100 million people (50% of PAD patients) presenting with atypical leg symptoms, the remaining patients with ischemia experience significant pain, loss of mobility and diminished quality of life [3]. Past studies looking at the progression of PAD suggest that clinical evolution of PAD is rather stable [4] with significant advancement over 4.6 years [5]. The presence of cardiovascular risk factors, diabetes mellitus and smoking significantly increases the chance of developing critical ischemia and possibility for limb amputation [1,5–6]. As such, prompt diagnosis

and early intervention are essential in PAD treatment. There are several treatment options currently in practice for PAD. Tobacco cessation and healthy lifestyle practices are often effective in reversing disease after early diagnosis. Available medications include statins, blood thinners and vasodilators to lower cholesterol synthesis and improve tissue blood supply. Physical interventions include balloon angioplasty, endoluminal stenting and atherectomy [6]. The search for alternative, less invasive and more effective interventions is ongoing in hopes to minimize the incapacitating physical manifestations of PAD.

Patients with PAD experience repeated ischemia/reperfusion insults, leading to skeletal muscle injury, peripheral neuropathy, functional decline and mobility loss [7]. It is believed that both impaired vasculature and skeletal muscle pathophysiology contribute to functional decrements in PAD [7,8]. Interestingly, functional decline is also observed

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in asymptomatic and atypical PAD patients in the absence of classical intermittent claudication. As such, there is a need for the development of therapeutic interventions to prevent and treat functional problems leading to mobility loss in patients with PAD [7,8].

Basic research on cell-mediated therapies shows significant promise. Adipose-derived stem cells (ASCs) are known for their powerful angiogenic and anti-inflammatory properties [9–18], while ASC-conditioned macrophages (MPs) are recognized for their role in immunomodulation and tissue regeneration [19–22]. The strategy of using cells to elicit complex tissue-specific responses has obvious advantages over the delivery of individual biological factors [23]. We have previously published on the role of inflammatory M1 (LPS/IFN- γ) MPs in skeletal muscle regeneration from ischemia/reperfusion injury [24]. In addition, Jetten *et al.* [25,26] showed that injection of exogenous, inflammatory M1 MPs and anti-inflammatory M2 MPs had positive effects on reperfusion recovery, concluding that polarization of MPs is sufficient to induce collateral vessel formation. Unfortunately, cell preconditioning strategies require significant cell culture times, may alter relevant biologic characteristics of cells and do not meet minimal cell manipulation standards set forth by the US FDA (21CFR1271.3). As such, serial cell culture is undesirable for clinical application.

Aside from being powerful inducers of neoangiogenesis, ASCs are potent modulators of MP function [19,22]. In this report we focus on the role of ASCs in modulating MPs polarization status and evaluate the potential of this dual-cell therapy approach in a mouse model of PAD.

Materials & methods

Animals

Female C57BL/6 mice (3–6 months) were used for this study (Jackson Laboratories, Bar Harbor, ME, USA). Animals were housed with *ad libitum* access to food and water, and maintained on a 12-h light/dark cycle. All experimental procedures were approved and conducted in accordance with the guidelines set by The University of Texas at Austin IACUC (Protocol # AUP-2014-00259).

Cell lines

Human ASCs (hASCs; PT-5006; Lonza) were commercially purchased and cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% Glutamax I (Life Tech) and 1% penicillin-streptomycin. The cell culture media were changed every 2–3 days. Cells were passaged using 0.25% trypsin/EDTA (Lonza), collected by centrifugation at $600 \times g$ for 7 min, and counted with Trypan blue exclusion. For all experiments, cells were seeded at a

density of 5000 cells/cm². Passage 5–8 cells were used in this study.

U-937 cells (CRL-1593.2™) (ATCC), a human monocyte/macrophage cell line (hMPs), were cultured in suspension in RPMI-1650 growth media supplemented with 10% FBS and 1% penicillin-streptomycin. For macrophage differentiation, 100 nM of 12-O-tetradecanoylphorbol-13-acetate (TPA; Cell Signaling Technology) was added to the growth media and the cells were cultured for 48–72 h. Cells were grown under standard cell culture conditions (37°C, 5% CO₂).

Mouse adipose-derived stem cell isolation

Gonadal (periuterine) fat pads were isolated from 6-month-old donor C57BL/6 females. Explanted tissue was rinsed in phosphate-buffered saline (PBS), mechanically digested with sterile scissors and added to sterile suspension of 0.1% collagenase type I/1% BSA solution in PBS. Tissue was digested at 37°C for 90 min with occasional mixing. Following enzymatic processing, adipose tissue cell suspension was centrifuged and top layer containing adipocytes was removed via aspiration. The red-colored pellet was plated in standard tissue culture flasks (75 cm², Corning) in high glucose DMEM supplemented with L-glutamine and 10% FBS (Invitrogen) with 1% penicillin-streptomycin (Invitrogen). Single T75 flask was prepared from digested (~1 gm) fat tissue collected from three animals. After 48 h, adherent cells were washed with PBS to remove loose cellular debris. Cell culture medium was replaced every 48–72 h. P1–P3 passage numbers were used in all experiments. For differentiation studies, mouse adipose-derived stem cell (mASCs) P3–P5, were grown to 80% growth confluency and incubated in either osteogenic or adipogenic growth medium. Osteogenic culture medium was made following the addition of 20 mM glycerol phosphate (Sigma), 50 ng/ml thyroxine (Sigma), 1 nM dexamethasone (Sigma), 50 μ M ascorbate 2-phosphate (Sigma). Adipogenic culture media was made with 5 μ g/ml insulin (Sigma), 50 μ M indomethacin (Sigma), 1 μ M dexamethasone (Sigma) and 0.5 μ M 3-isobutyl-1-methyl xanthine (Sigma). Cells were cultured in these media formulations for 2 weeks. Cells were fixed in 10% formalin and stained with Alizarin Red S, pH 4.1 to identify calcium deposition or 0.5% Oil Red O to visualize neutral lipid vacuoles (Supplementary Figure 1A) [27].

Mouse bone marrow macrophage isolation & polarization

Lower limb bones from 6-month-old female C57BL/6 animals were isolated. Associated muscle and connective tissue were trimmed off the bones in sterile conditions. Bones were quickly rinsed in 70% ethanol and kept in PBS, on ice during further processing. Bone marrow

from bone shafts of femurs and tibias was flushed out using 27G needle attached to PBS-containing syringe. Cell aspirate was centrifuged at 400 *g* for 5 min, filtered and resuspended in 10% FBS high-glucose DMEM with 10 ng/ml macrophage colony-stimulating factor (M-CSF) at 2×10^6 cells/ml in 6-well plates. Cells were cultured for 7 days (**Supplementary Figure 1B**). To polarize mouse bone marrow macrophages (mMPs), cells were stimulated with IFN- γ (Invitrogen) at 20 ng/ml for 48 h. M2 mMPs were produced after 48-h treatment with 20 ng/ml IL-4 (Invitrogen).

Femoral artery excision surgery

Unilateral femoral artery excision (FAE) was performed on 3-month-old female C57BL/6 mice under 2% isoflurane inhalation anesthesia. The femoral artery was separated from the femoral vein and nerve, and tied off (ligated) using 7–0 Vicryl suture (Ethicon, NJ, USA) at the inguinal ligament and the popliteal bifurcation. The portion of artery between the two ligation sites was removed. The wound was closed using 5–0 Prolene suture (Ethicon, NJ, USA). For cell transplantation 2×10^6 mMPs, 2×10^5 mASCs or 2.2×10^6 mMPs/mASCs mix (10:1) were injected in 60 μ l PBS into ischemic gastrocnemius muscle 24 h after FAE surgery. PBS injection alone was used as control.

Hypoxic cultivation & analysis of conditioned media

Severe forms of PAD are characterized by critical ischemia. Early interventions involve transferring cells into a hypoxic tissue microenvironment. As such, we wanted to investigate how low oxygen conditions modify ASC's and MP's proangiogenic secretome. Human ASCs were plated at a concentration of 20,000 cells/cm² in growth media (10% FBS) and allowed to adhere overnight under normal cell culture conditions (95% O₂, 5% CO₂). Following overnight incubation, the media was replaced with serum-free cell culture media following washing with PBS without calcium and magnesium. The cells were placed in either a normoxic (20% O₂) or a hypoxic (1% or 2% O₂) environment for 48 h. The media were collected every 24 h and replaced with serum-free media. The media were stored at -80°C until further use.

For the co-culture experiments, hASCs were seeded in 6-well transwell inserts (8 μ m pore size) at a concentration of 20,000 cells/cm² in DMEM containing 10% FBS and cultured under standard cell culture conditions over night to allow for adherence. In addition, U-937 hMPs were TPA treated for 48 h and seeded in 6-well plates at a concentration of 400,000 cells/well in RPMI media containing 10% FBS and cultured under standard cell culture conditions over night to allow for adherence. Following overnight culture, the

media were replaced with serum-free DMEM or 10% FBS containing RPMI media for stem cells and U937 hMPs, respectively. The transwell inserts containing hASCs were transferred to the 6-well plates containing U937 hMPs. The co-cultured cells were then incubated under normoxic or 1% hypoxic conditions for 48 h, after which the media were collected and RNA was isolated. U937 hMP cultures were used as controls.

For direct co-culture experiments, primary bone marrow derived mMPs were seeded into 6-well plates at 5×10^5 cells/well, the mASCs were added at 1×10^5 cells/well to achieve 5:1 ratio, respectively. Cells were allowed to adhere and were either left under normoxia (5% CO₂, 20% O₂) or transferred into hypoxic incubator (5% CO₂, 1–2% O₂) for 48 h at 37°C. BM-MPs alone cultures were used as controls. To separate cells following direct co-culture, cells were trypsinized for 2 min leading to detachment of mASCs. mASCs were removed and remaining MPs were washed twice with PBS and processed for RNA isolation. Purity of MPs was >93% as assessed by fluorescence-activated cell sorter (FACS). For FACS analysis, both populations were removed from the plate and analyzed based on the expression of F4/80 and CD206 surface markers.

ELISA-based cytokine array

For a broad screening of human ASC's paracrine secretions that can affect angiogenesis and cross-talk with U937 hMPs, a customized RayBio® Custom C-Series Human Array (Raybiotech, Inc.) was used. The following proteins were analyzed: MCP-1, bFGF, IFN- γ , PDGF-BB, VEGF, TGF β 1, IGF-I, MMP-1, 2, and 9, IL-1 α and β , 10, 13, 4, 6 and 8. The membranes were incubated with the media samples overnight at 4°C, followed by addition of biotinylated antibody and HRP-conjugated streptavidin reagents for 2 h each at 4°C. The chemiluminescent signal was imaged using FluorChem Q (ProteinSimple) and signal quantification was analyzed using AlphaView software.

Flow cytometry analysis

Prior to staining, cells were blocked in 1% BSA/PBS, pH7.2 with addition of Fc-block (#14-0161-82, eBioscience). Cell preparations were stained with anti-CD206-Alexa 647 (#141712, Biolegend) and anti-F4/80 APC.Cy7 (#123117, Biolegend) antibodies along with recommended isotype controls.

Immunophenotyping of mASCs was performed using anti-CD45-APC.Cy7, anti-CD90-PE, anti-Sca1-Alexa-647 and anti-F4/80-APC.Cy7 antibodies as well as corresponding isotype controls (Biolegend) (**Supplementary Figure 1A**). Data were analyzed using FlowJo software. Gating strategy was determined based on isotype control staining.

Real-time PCR

RNA was extracted from adherent cells using Trizol Reagent (Invitrogen) and Direct-zol RNA Mini-Prep Kit (Zymo Research and reverse transcribed using SuperScript IV Kit (Invitrogen) according to manufacturers’ instructions. Resulting cDNA was subjected to real-time PCR analysis using Bio-Rad iCycler IQ5 after addition of validated primers purchased from RealTimePrimers.com (Table 1), and SYBR-green Master Mix (Bio-Rad). Relative gene expression was determined using the $\Delta\Delta C_t$ method.

Histology & immunofluorescence

Frozen, OCT-embedded gastrocnemius muscle samples were sectioned on a cryostat (Leica CM1900; Leica Microsystems, Inc., IL, USA). Hematoxylin & eosin (H&E) staining was performed as previously described [28], and slides were observed with a light microscope (Nikon Diaphot, Nikon Corp., Tokyo, Japan) with the 20× objective lens. Images were taken using a mounted digital camera (Optronix Microfire; Optronix, CA, USA). Myofiber cross-sectional area (CSA) was measured using ImageJ software. Anti-mouse CD31 antibody (1:25; #550274; BD Pharmingen, CA, USA) and anti-mouse CD45 (1:10; #550539; BD Pharmingen) were used to identify endothelial cells and immune cells, respectively. Antirat secondary antibody (1: 200) Vectastain ABC Kit (Vector Laboratories, CA, USA) and DAB substrate (Thermo Scientific, IL, USA) were used as detecting agents according to manufacturers’ instructions. Primary rabbit polyclonal to MyoD antibody (1:100; ab203383; Abcam, MA, USA) was used to examine myogenesis, detected with secondary donkey-antirabbit Alexa-568 (1:200; ab175694) and counterstained with DAPI (1:1000; Molecular Probe, OR, USA, D1306). Images were captured using 40×oil objective, (2 × 2 tiles; 10% overlap) with Zeiss 710 Laser Scanning Confocal & SIM and quantified using ImageJ software.

Skeletal muscle contractile studies

At 21 days after FAE, experimental animals were subjected to 2% isoflurane inhalation anesthesia. Calf

muscles were surgically exposed and subjected to *in situ* functional measurements [28]. The Achilles tendon was tied to the muscle lever arm of a servomotor (model 305B, Cambridge Technologies) interfaced with a computer equipped with an A/D board (National Instruments). The muscle was electrically stimulated to shorten (contract) using an Isolated Pulse Stimulator (Model 2100; A-M Systems) with leads positioned on the muscle belly. Maximal twitch tension at 0.5 Hz stimulation was used to determine optimal muscle length. At optimal length, a150 Hz stimulus was applied to elicit the peak tetanic tension (P_0). Two minutes of rest was used between the contractions. Muscle temperature was maintained throughout the procedure using warm mineral oil and radiant heat lamp. Data were stored and analyzed using LabView software (National Instruments).

Speckle imaging

Laser speckle imaging was used to quantify blood flow to the lower hind limb. Briefly, a diode laser (785 nm, 50 mW; Thor Labs) was used to evenly illuminate the footpad of a mouse under 2% isoflurane inhalation anesthesia. The speckle images of blood perfusion were captured by a Basler 1920 × 1080 monochrome CCD with a zoom lens (Zoom7000; Navitar) mounted on a microscope boom stand and quantified using Matlab and MetaMorph. Relative perfusion was expressed as a ratio of left (ischemic) to right (uninjured) limb.

Statistics

Data were analyzed using two-tailed Student’s *t*-tests and one-way ANOVA (Tukey or Dunnett’s *post hoc* tests) where appropriate ($\alpha = 0.05$). Values are represented as mean ± SEM.

Results

Human ASCs modify polarization state of U937 hMPs *in vitro* following hypoxic cultivation

We cultivated human ASCs and U937 hMPs under low oxygen conditions and evaluated their protein secretions (Supplementary Figure 2). Modified levels

Table 1. Real-time PCR primer sequences.		
Gene	Forward (5'–3')	Reverse (5'–3')
Arg1	GTGAAGAACCACGGTCTGT	CTGGTTGTCAGGGGAGTGTT
Nos2	TGACGGCAAACATGACTTCAG	GCCATCGGGCATCTGGTA
Tnfa	CCCACTCTGACCCCTTACT	TTTGAGTCTTGATGGTGGT
Il1b	CCCAACTGGTACATCAGCAC	TCTGCTCATTACGAAAAGG
Ccr2	GGAGAAAAGCCAACCTCTTC	AGGCAGTTGCAAAGGTACTG
Ppia	AGCTCTGAGCACTGGAGAGA	GCCAGGACCTGTATGCTTTA
Il10	AGTGGAGCAGGTGAAGAGTG	TTCGGAGAGAGGTACAAACG

of secreted proteins (i.e., IL-6, MCP-1, bFGF, VEGF, MMP-1 and IL-8) were detected by an ELISA-based cytokine array (Figure 1A) and evaluated using heat-map analysis of the relative percent changes (Figure 1A). The heat map was colorized based on the percentage levels, with red denoting an increase and blue a decrease of hypoxic conditioned media relative to normoxic. The data shown were semiquantitatively derived from chemiluminescent images based on the signal intensity. Hypoxic cultivation of hASCs led to large increases in production of IL-6, IL-8 and CCL2 (Figure 1A). In U937 hMPs, hypoxia stimulated production of IL-6, IL-10 and VEGF, while CCL2 and IL-8 were slightly downregulated (Figure 1A). Interestingly, significant hASC-induced changes in U937 hMPs gene expression were observed following transwell co-culture under hypoxia (Figure 1B). The U937

hASC-conditioned hMPs upregulated genes characteristic for both M2 (*Arg1*, arginase) and M1 (*Ccr7*, chemokine receptor) polarized hMPs. To eliminate the possibility of bias due to the use of cell lines, we undertook further characterization of ASC/MP cross-talk using direct cultivation approaches using primary mouse-derived cell populations.

Mouse-derived mASCs induce significant upregulation of mannose receptor on the surface of bone marrow-derived mMPs after direct co-culture

We isolated mouse ASCs from gonadal fat pads of C57Bl/6 mice. mASCs expressed stem cell antigen (Sca-1) and CD90 surface markers in the absence of common leukocyte antigen (CD45) and mMP-specific marker (F4/80) expression (Supplementary Figure 1A). Cells

Figure 1. Hypoxia-induced changes in individual human adipose-derived stem cell and U937 macrophages secretomes, and adipose-derived stem cells-induced alterations of functional macrophage phenotype in co-cultures. (A) Modified secretion of soluble factors by hASCs cultured under 1–2% oxygen for 48 h. The hASCs conditioned media was collected following 1–2% oxygen hypoxic cultivation and analyzed using an ELISA-based array kit. Representative blotted membranes are shown for hASCs on the left. The heat maps were colorized based on the percentage change relative to normoxia controls. The data shown were semi-quantitatively derived from chemiluminescent images based on the signal intensity. (B) U937 human macrophages were cultured in the bottom of a transwell insert under normoxia and hypoxia, alone or in the presence of hASCs. Values are displayed as mean $\pm$ standard deviation.

* $p < 0.05$ compared with normoxic condition.

hASC: Human adipose-derived stem cell.

were also negative for CD11b, CD31 and CD34 (data not shown). Moreover, we confirmed the transdifferentiation potential of mASCs by inducing osteogenic and adipogenic phenotypes (Supplementary Figure 1A). Likewise, mMPs were generated by culturing mouse bone marrow with M-CSF for 7 days. F4/80 expressing mMPs were identified by flow cytometry (Supplementary Figure 1B).

We performed *in vitro* polarization studies on isolated mMPs using IFN- γ to induce M1 'classical' inflammatory phenotype and IL-4 to promote M2 'alternative' activation. As expected, IFN- γ polarized M1 mMPs significantly upregulated the relative expression of IL-1 β (*Il1 β* ; ~sixfold) and TNF- α (*Tnfa*; ~tenfold) over control M-CSF induced mMPs (Figure 2A; top). There was a nonsignificant upregulation of IL-10 (*Il10*) and slight upregulation of arginase (*Arg1*) (Figure 2A; top). Alternatively, IL-4 treatment of mMPs led to downregulation of TNF- α (*Tnfa*) and IL-10 (*Il10*), consistent with previous report [29] and large upregulation of arginase (*Arg1*; ~1000-fold) (Figure 2A; bottom). Interestingly, CCR2

(*Ccr2*) chemokine receptor showed a slight upregulation after M1 polarization, consistent with reported increased trafficking characteristic of M1 mMPs [30–32], while there was significant reduction of CCR2 expression after IL-4 treatment (Figure 2A). The expression of mannose receptor (CD206) on mMPs followed *in vitro* polarization trend, with decreased expression after M1 polarization and significant upregulation after IL-4 treatment. These data are consistent with existing literature reporting CD206 expression on M2 MPs *in vivo* [32–35].

In the absence of exogenously added polarizing factors, direct co-cultures of mMPs and mASCs (5:1) (Supplementary Figure 2) induced upregulation of CD206 on the surface of mMPs, consistent with previously published data [19] and known characteristic of ASCs to promote M2 polarization of MPs [21]. In addition, we show for the first time that the upregulation of CD206 mannose receptor on mMPs is significantly higher than that induced by IL-4 treatment alone (Figure 2B).

Figure 2. Gene-expression profile and CD206 cell surface marker expression of *in vitro* polarized bone marrow macrophages. (A) Bone marrow MPs were cultured with either IFN- γ (20 ng/ml) or IL-4 (20 ng/ml) for 48 h. MPs maintained in M-CSF were used as controls (=1). RNA was isolated and gene expression was quantified using SYBR real-time polymerase chain reaction and double delta Ct method; *p < 0.05, Student's t-test. (B) Flow cytometry was used to characterize the expression of mannose receptor (CD206) on the surface of bone-marrow derived MPs after *in vitro* polarization and following direct co-culture with mouse ASCs. Values are displayed as mean $\pm$ standard deviation. *p < 0.05 relative to M-CSF treated MPs (control).

#p < 0.05 relative to IFN- γ polarized MPs.

&p < 0.05 relative to IL-4 polarized MPs.

ASC: Adipose-derived stem cell; MP: Macrophage; M-CSF: Macrophage colony-stimulating factor.

Figure 3. Direct correlation between the number of mouse adipose-derived stem cells and expression levels of mannose receptor on the surface of mouse bone marrow macrophages in direct co-cultures. Flow cytometry was used to characterize the expression of mannose receptor (CD206) after varying percent mouse ASCs (mASCs) in co-cultures from 0 (0:1; mASC:mouse bone marrow MPs [mMPs]) to 20% (1:5; mASC/mMPs). Strong correlation exists between percent (%) mASCs in direct co-culture and subsequent percent (%) MPs expressing CD206.

ASC: Adipose-derived stem cell; CC: Co-culture; MP: Macrophage.

Upregulation of CD206 on the surface of mMPs is tightly correlated to the quantity of mASCs in co-culture

We varied the ratio of mASC: mMPs in direct co-cultures from 0:1 (no ASCs) or 1:80 (1 mASC per 80 mMPs) to 1:5 (1 mASC per 5 mMPs) to show that there is a strong correlation between the presence of mASCs and status of CD206 expression on mMPs (Figure 3). There is a linear progression in upregulation of CD206 surface marker on mMPs up to 1:10 (mASC:mMPs) ratio, suggesting that quantity of mASCs determined the extent of mMP modulation. The ratio of 1:5 mASC: mMPs shows the highest level of expression, as such, it was further used in our gene-expression studies. The ratio of 1:10 mASC: mMPs was used in co-delivery studies *in vivo* as a minimal

cell ratio with significant CD206 upregulation on the surface of mMPs (Figure 3).

Gene-expression profiling of mMPs after direct co-cultures with mASCs under normoxia & hypoxia

Direct co-cultures of mASCs and mMPs lead to mixed polarization phenotypes of MPs. Under normoxia ASC-conditioned MPs increased the expression of both M1 (TNF- α ; ~four-fold) and M2 (arginase [~40-fold], IL-10 [~fourfold]) genes relative to normoxic mMPs culture alone, with no changes in IL-1 β expression (Figure 4; top). After hypoxic cultivation, mASC-conditioned mMPs exhibited very large increases in arginase expression (~10,000-fold) along with nonsignificant increase in TNF- α (Figure 4; bottom). Curiously,

expression of chemokine receptor CCR2 on mMPs was upregulated in normoxic co-culture and increased even further under hypoxic cultivation (~20-fold), which may indicate that mASC-conditioned mMPs possess increased migratory capabilities.

Combined mMPs/mASCs treatment strategy leads to improved skeletal muscle regeneration in the mouse model of PAD

We wanted to evaluate the effects of combined mMPs/mASCs therapy on muscle tissue regeneration in a mouse model of PAD (Supplementary Figure 2). According to our data (data not shown), FAE injury leads to significant infiltration of ischemic muscle by neutrophils and inflammatory monocytes at 24 h. Recruitment of inflammatory cells gradually increases and peaks at 3 days. We wanted to administer our cell

therapy early, in order to dominate cellular response to ischemic injury. As such, we transplanted cells acutely, 24 h after FAE, during critical limb ischemia in order to characterize effects of cell treatment on revascularization and restoration of muscle function.

We analyzed muscle tissue at 21 days after surgery by measuring average myofiber size, muscle mass and force. Our findings show that there are significant decreases in average myofiber size in saline (PBS) control, mMPs alone and mASCs alone groups (Figure 5A) at the 3-week time point post-FAE. There is also significant decrement in muscle mass in the mASCs-treated group (Figure 5B; top). We did not detect statistically significant differences in central nucleation among groups (percent [%] of centrally nucleated fibers: PBS – 82 ± 8%; mMPs – 84 ± 5%; mASCs – 64 ± 24%; mASCs/mMPs – 47 ± 33%). We were not surprised

Figure 4. Gene-expression profile and CD206 surface expression of mouse bone marrow macrophages after direct co-culture with mouse adipose-derived stem cells under normoxic and hypoxic conditions. (A) Mouse bone marrow MPs (mMPs) were co-cultured with mouse ASCs (mASCs) under normoxia (20% oxygen) for 48 h. MPs cultured alone were used as controls (=1). Expression levels of mannose receptor (CD206) were confirmed by flow cytometry. RNA was isolated and gene expression was quantified using SYBR real-time polymerase chain reaction. **(B)** mMPs were co-cultured with mASCs under hypoxia (1–2% oxygen) for 48 h. MPs cultured alone were used as controls (=1). Values are displayed as mean ± standard deviation.

*p < 0.05 Student's t-test.

ASC: Adipose-derived stem cells; MP: Macrophage.

Figure 5. Co-delivery of mouse bone marrow macrophages/mouse adipose-derived stem cells after femoral artery excision injury leads to improved muscle histopathology and contractile function at 3 weeks of recovery.

(A) Representative images of hematoxylin and eosin staining (20X; scale bar: 100 μm) and quantification of average myofiber sizes in contralateral controls and treatment groups; (B) Characterization of muscle contractile properties by evaluating muscle mass (mg), tetanic tension (N) and normalized force (N/mg). $n = 5-9$ for each group; three images/animal. Values are expressed as mean $\pm$ standard deviation.

* $p < 0.05$ compared to contralateral side.

$p < 0.05$ compared with M0 group.

* $p < 0.05$ compared with PBS group using one-way ANOVA with *post hoc* Tukey honest significant difference.

ASC: Adipose-derived stem cell; MP: Macrophage; PBS: Phosphate-buffered saline.

that at 3 weeks skeletal muscle is still in the process of remodeling for all treatment groups.

Combined mMPs/mASCs treatment significantly increases the tetanic tension (N) and normalized force (N/gm) showing near complete recovery of muscle contractile function (Figure 5B; middle and bottom). Interestingly, treatment with mASCs alone also shows improved contractile force recovery at 21 days after normalization by mass despite reduced tetanic force and average myofiber sizes. This suggests that ASCs may facilitate reductions in CSA helping to acceler-

ate metabolic adaptation of muscle to reduced blood flow conditions, while mMPs/mASCs treatment could provide additional stimulus for accelerated muscle recovery and growth (Figure 5).

Combined mMPs/mASCs treatment provides a stimulus for collateral revascularization

Timely restoration of blood flow following critical ischemia is currently an important clinical goal to halt the progression of PAD. We evaluated blood perfusion in our animal model using speckle imag-

ing and histological quantification of CD31⁺ capillaries (Figure 6A & B). One day after FAE surgery blood flow was significantly reduced (23% of uninjured side). Saline treatment had marginal effect on blood perfusion at 21 days (40% of uninjured side), while combined mMPs/mASCs delivery led to efficient restoration of relative blood flow at 3 weeks post-excision surgery (85% of uninjured side). Both mMPs alone (67% of uninjured side) and mASCs alone (77% of uninjured side) increased relative perfusion, albeit without statistical significance (Figure 6C). Quantification of capillaries per myofiber ratio supports blood flow data showing significantly higher capillaries per myofiber in combined mMPs/mASCs treatment group and increase in mASCs alone group (Figure 6D). These data are in agreement with immunohistological

images of muscle pathology (Figure 5A) and supported by muscle contractile studies (Figure 5B).

Decreased inflammatory infiltrate after mASCs & mMPs/mASCs cell treatments

In the absence of adequate blood supply, skeletal muscle tissue undergoes continuous degeneration in order to metabolically adapt to reduced flow of nutrients. During muscle degeneration, immune cells are continuously recruited into muscle to assist with immune surveillance and debris clearance. In the resting state, very few immune cells are present in the muscle tissue [36–38]. Here we show that mASCs and combined mMPs/mASCs treatments led to reduced inflammatory cell presence in the muscle at 21 days (Figure 7A & B), which correlated well with the func-

Figure 6. Skeletal muscle angiogenesis is significantly improved 3 weeks after mouse bone marrow macrophages/mouse adipose-derived stem cell co-delivery into femoral artery excision injured muscles. (A) Representative images of laser speckle and (B) CD31 staining of ischemic limbs (20X), scale bar 100 μm. Quantification of (C) blood flow relative to contralateral, uninjured side. (D) capillary per myofiber ratio. n = 3–5 for each group; three images/animal. Values are expressed as mean ± standard deviation.

*p < 0.05 compared to D1 post-FAE using one-way analysis of variance with *post hoc* Dunnett's test.

*p < 0.05 compared to contralateral side.

#p < 0.05 compared with M0 group.

+p < 0.05 compared with PBS group using one-way analysis of variance with post-hoc Tukey honest significant difference.

ASC: Adipose-derived stem cell; FAE: Femoral artery excision; MP: Macrophage; PBS: Phosphate-buffered saline.

Figure 7. Therapeutic transplantation of mouse bone marrow macrophages/mouse adipose-derived stem cells exhibit immunomodulatory function at 3 weeks after treatment and increases MyoD expression 4 days post-femoral artery excision. Therapeutic transplantation of mouse bone marrow MPs/mouse ASCs exhibits immunomodulatory function at 3 weeks after treatment and increases MyoD expression 4 days post-femoral artery excision. **(A)** Representative images of CD45-specific staining (20X) in brown, scale bar: 100 μ m. **(B)** Quantification of the number of inflammatory cells in ischemic muscles per field of view 3 weeks post-femoral artery excision, n = 5 for each group; 3 images/animal. **(C)** Representative images of MyoD staining (40X) in injured skeletal muscle (MPs/ASCs group), scale bar: 50 μ m. **(D)** Quantification of percent of MyoD+ nuclei out of total DAPI+ cells, n = 3 for each group; 5–7 images/animal. Values are expressed as mean $\pm$ standard deviation.

*p < 0.05 compared with PBS group.

*p < 0.05 compared with mouse bone marrow MPs group using one-way analysis of variance with *post hoc* Tukey honest significant difference.

ASC: Adipose-derived stem cell; MPs: Macrophage; PBS: Phosphate-buffered saline.

tional and revascularization data presented above. As such, both mASCs and mMPs/mASCs cell treatments provide immunomodulatory benefits in our animal model of PAD, which can be easily attributed to the anti-inflammatory properties of ASCs [9,13,39–41].

Combined mMP/mASCs treatment provides additional stimulus for muscle regeneration by increasing the number of MyoD+ myogenic precursors at the early stages of muscle repair

We have seen that mASC-mediated treatment helps in skeletal muscle regeneration by inducing collateral angiogenesis and modulating inflammatory responses. However, despite increased normalized force (N/mg) in the mASCs alone group, combined mMPs/mASCs treatment prevented myofiber atrophy and signifi-

cantly increased tetanic tension (Figure 5), both of which may indicate a faster muscle repair in this treatment group. We have previously seen that co-cultures of mMPs/mASCs are characterized by increased expression of TNF- α , arginase, IL-10 and CCR2 on mASC-conditioned mMPs (Figure 4). Arginase was previously shown to protect MyoD transcription factor from degradation in the highly inflammatory setting [38,42], while TNF- α was shown to be beneficial for proliferation of myoblasts [43]. The CCR2–CCL2 axis has been long recognized as a primary axis for the recruitment of MPs into injured skeletal muscle [35,44–45] and facilitation of their interactions with myoblasts [30–32,46–48]. IL-10 is known to be a potent immunomodulatory cytokine [49–51]. Taken together, these factors may provide strong influences

on the extent of acute myogenesis. We chose to quantify MyoD nuclear expression, as an indicator of early myogenesis on D4 after FAE surgery. As expected, we detected significantly higher percentage of MyoD⁺ nuclei in muscles injected with mMPs/mASCs relative to other groups (Figure 7C & D). These findings explain increases in muscle force and myofiber CSA in mMPs/mASCs treatment group relative to mASC treatment alone at 21 days.

Discussion

In this report we showed that dual mMPs/mASCs cell therapy can be used as an intervention in the treatment of critical ischemia. Local injection of both cell types leads to accelerated recovery of muscle function and histopathology, reduced inflammation and improved perfusion of regenerating ischemic limb. We also presented experimental evidence that mASCs are powerful modulators of mMPs functional status as evidenced by upregulation of CD206 and gene profiling.

We have previously published on the use of polarized mMPs to treat ischemia/reperfusion muscle injury. According to our studies, early delivery of M1 mMPs promotes faster muscle repair [24]. Other reports also showed the benefit of M1 mMPs in accelerating tissue recovery post-injury [25–26,51–52]. Interestingly, M2 mMPs are not as beneficial when delivered early [HSIEH PL *ET AL.*, UNPUBLISHED DATA]. This leads to the conclusion that mASC-educated mMPs exhibit a unique phenotype, which cannot be easily grouped with existing *in vitro* classifications.

ASCs are known as highly secretory cells, with proangiogenic secretome and powerful immunomodulatory properties. We showed that following hypoxic subculture hASCs are capable of upregulating a number of proangiogenic factors (Figure 1). Specifically, IL-6 and CCL2 (MCP-1) have been previously shown to increase survival of myeloid cells as well as promote their polarization toward M2-like phenotype [53]. The upregulation of CCR2 on mMPs after co-culture with mASCs is also very interesting. In the alternative, IL-4 driven M2 polarization, expression of CCR2 is significantly downregulated, which is consistent with previous reports on MPs trafficking [30] as well as the notion that M2 MPs may downregulate their chemokine receptor expression in order to maintain contact with regenerating fibers [32] persisting within regenerating tissue [54]. The CCL2–CCR2 axis has been long recognized as a primary axis for MPs recruitment into injured skeletal muscle [35,44–45] as well as its role in MP trafficking toward myoblasts [30–31,47]. According to our data, this could also be an important pathway mediating mASC–mMP as well as mMP–myoblast cross-talk *in vivo*.

Although the idea of ASC-educated MPs is not novel [22], the concern of whether these cells are ‘operational’ *in vivo* remains [22]. We used a co-delivery approach to show that injection of mMPs/mASCs is much better at promoting tissue recovery post-FAE than utilization of mMPs and mASCs individually. Despite the fact that the mASCs alone treatment led to improved contractile muscle function, combined mMPs/mASC treatment was significantly better in a number of criteria, including myofiber CSA, muscle mass and tetanic force (Figure 5). It appears that co-delivery of mASCs/mMPs leads to high variability in central nucleation (data not shown), which combined with myofiber CSA data is indicative of faster maturation process at 3 weeks after dual-cell delivery. Evaluation of early myogenesis following FAE injury showed that combined cell delivery led to higher percentage of MyoD⁺ nuclei, explaining accelerated muscle recovery in this group. In activated MPs, arginase can modulate nitric oxide production [55]. Although expression of iNOS has been shown to be essential for muscle regeneration [56], high levels of NO could interfere with myogenesis via reduction in MyoD expression [38,42]. Therefore, high levels of arginase expression by MPs following ASC preconditioning may contribute to MyoD stabilization and myogenesis during acute phases of tissue degeneration. Interestingly, TNF- α was also reported to play a role in MyoD modulation [38,43], further emphasizing the complexity of regenerative responses. Overall, MPs were shown to actively interact with myoblasts *in vitro* [47] and regenerating muscle fibers *in vivo* [31–32,48]. As such, ASC-educated MPs may be superior for the treatment of muscle injuries due to their ability to effectively integrate signals from environmentally conditioned ASCs.

Conclusion

Our data suggest that combined mMPs/mASCs cell therapy is a promising approach to restore muscle function and stimulate local angiogenesis in regenerating ischemic limb. It is clinically attractive because it requires minimal cell processing, does not alter relevant biological characteristics of cells and eliminates the need for extensive subculture. Basic research work should be carried out to better characterize pathways involved in ASC–MP cross-talk and identify target protein mediators responsible for accelerated skeletal muscle tissue repair.

Supplementary data

To view the supplementary data that accompany this paper please see online at: www.futuremedicine.com/doi/full/10.2217/rme-2016-0094

Authors' contribution

V Rybalko: Manuscript writing, conception and design, collection and assembly of data, data analysis and interpretation. P-L Hsieh: Conception and design, collection and assembly of data, data analysis and interpretation. LM Ricles: Collection and assembly of data, data analysis and interpretation. E Chung: Collection and assembly of data, data analysis and interpretation. RP Farrar: Financial support, administrative support, conception and design, final approval of manuscript. LJ Suggs: Financial support, administrative support, conception and design, final approval of manuscript.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Adipose-derived stem cells exert strong immunomodulatory effect on macrophages in direct and indirect co-cultures under normoxia and hypoxia.
- Adipose-derived stem cell-conditioned macrophages exhibit M2-like polarization status characterized by increased arginase gene expression and upregulation of CD206.
- Dual mouse adipose-derived stem cell (mASCs)/mouse bone marrow macrophages (mMPs) cell therapy in a mouse model of peripheral artery disease leads to significant improvements in muscle histopathology, contractile function and revascularization at 3 weeks post-ligation.
- Combined mASCs/mMPs therapy results in increased MyoD expression in ischemic skeletal muscle during acute stages of tissue repair and accelerated resolution of inflammation at 3 weeks.
- Co-delivery of mASCs/mMPs has several advantages over single cell type therapy. The cross-talk between mASCs and mMPs likely contributes to better regenerative outcome via effects on early myogenesis and inflammation.

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