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Aging-Related effects of Bed Rest followed by Eccentric Exercise Rehabilitation on Skeletal Muscle Macrophages and Insulin Sensitivity

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Abstract

The pro- and anti-inflammatory macrophages are associated with insulin sensitivity and skeletal muscle regeneration. Infiltrating macrophages in skeletal muscle during a period of physical inactivity and subsequent reloading/rehabilitation in older adults is unknown, but may provide insight into mechanisms related to the development of metabolic disease and changes in muscle cell size. The purpose of this study was to determine if skeletal muscle macrophage infiltration is modulated differently between young and older adults after bed rest and exercise rehabilitation and if these responses are related to muscle and insulin sensitivity changes. 14 young and 9 older adults underwent 5-days of bed rest followed by 8-weeks of lower limb eccentric exercise rehabilitation (REHAB). Dual-energy X-ray absorptiometry, magnetic resonance imaging and

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Supplemental Figures 1–8 and Table 1–3 are available from the “supplemental digital content” link in the online posting of the article.

Authors' contributions

MJD, RLM, MAS, PCL, CSF designed the research; PTR, CCL, AIM, RLM, MAS, PAL, MJD conducted research; PTR, CCL, AIM, CSF, RLM, MAS, PAL, MJD reviewed the manuscript; PTR, CCL, AIM, CSF, MJD analyzed data; PTR, CCL, CSF, MJD wrote the manuscript and had primary responsibility for final content. All authors read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest.

myofiber analysis were used to identify muscle morphology and CLIX-IR and CLIX- β were used to assess insulin sensitivity. Skeletal muscle macrophages, CD68 (pan), CD11b (M1), CD163 (M2), CD206 (M2), were characterized using immunohistochemistry and gene expression. Insulin sensitivity, independent of age, decreased ~38% following bed rest and was restored following REHAB. We found robust age-related differences in muscle atrophy during bed rest, yet older and younger adults equally hypertrophied during REHAB. Interestingly, there were age-related differences in macrophage content (CD68⁺CD11b⁺ and CD68⁺CD11b⁻ cells) but both young and old similarly increased macrophages with REHAB. Satellite cell changes during rehab corresponded to macrophage content. Muscle tissue resident macrophages and gene expression, were not associated with changes in insulin sensitivity following bed rest and REHAB. These data suggest that muscle macrophages are modulated as a result of exercise rehabilitation following bed rest and may more associated with muscle regrowth/hypertrophy rather than insulin sensitivity in young or older adults.

Keywords

aging; atrophy; immune cells; M1; M2; muscle-resident; rehabilitation; regrowth; recovery; eccentric exercise; training

1. Introduction

Bed rest during hospitalization contributes to severe functional decline in older adults and is associated with adverse outcomes such as increased rates of repeated hospitalization and death (Brown and others 2004; Gill and others 2004). We have shown that after 5-days of bed rest, older adults experience a significantly increased muscle loss compared to young subjects. Following 2-months of rehabilitation consisting of eccentric exercise, these older adults were able to reverse muscle mass loss and strength deficits due to short-term bed rest (Tanner and others 2015).

The process of aging involves physiological and functional decline that can be described by various cellular changes including the accumulation of inflammatory cells within skeletal muscle. Macrophages in skeletal muscle modulate the process of regeneration and repair following exercise and muscle disuse in rodents, which is dependent upon their polarization state (Tidball and Wehling-Henricks 2007). Following injury, neutrophil invasion peaks in skeletal muscle within 24h. Neutrophils recruit M1 macrophages to infiltrate the muscle within 2 days and this subpopulation of macrophages produces pro-inflammatory cytokines that promote the clearance of necrotic debris. M2 macrophages peak around 4 days post injury and produce anti-inflammatory cytokines that enhance regeneration of muscle fibers and fibrosis (Tidball and Wehling-Henricks 2007). However, there is a lack of evidence in human skeletal muscle suggesting that macrophages can be altered after disuse and following recovery and if there are any age-related differences.

Macrophages have also been shown to modulate insulin sensitivity *in vitro*. CD11c expression was increased in skeletal muscle of mice fed a high fat diet and conditioned media from FFA-treated bone-marrow cells induced insulin resistance in L6 myotubes (Nguyen and others 2007). In an *in vitro* model using a human cell line, pro-inflammatory

M1 macrophages inhibit insulin-stimulated glucose disposal, whereas M2 macrophages enhance this response (Groshong 2013). It is well known that bed rest results in insulin resistance (Hamburg and others 2007), which is primarily located in skeletal muscle (Stuart and others 1988). Therefore, the insulin resistance associated with bed rest may be modulated by the inflammatory infiltrate and in turn contribute to the impaired muscle regrowth post bed rest.

Our goal was to elucidate the effects of bed rest and exercise rehabilitation on macrophage polarization within skeletal muscle of younger and older healthy adults while also determining if a relationship exists between macrophage infiltration and insulin resistance.

2. Methods

2.1. Subjects

We recruited 23 healthy community dwelling subjects from the Salt Lake City area in two age groups of 18–35 and 60–75 years and with a BMI <30 kg/m² for this study: 14 young (7 male, 7 female) and 9 older adults (2 male, 7 female). Most of these subjects were used in a prior study assessing the role of muscle protein synthesis after bed rest and following rehabilitation (Tanner and others 2015). Subjects were recruited through campus and community flyers, radio announcements, health fairs, ClinicalTrials.org and word-of-mouth. Subjects were screened through personal interview, physician evaluation and a blood panel to meet an extensive set of inclusion/exclusion criteria. The exclusion list contained various physical, mental, muscle or metabolic dysfunctions that may impact on protein metabolism or contraindicated for bed rest. These included uncontrolled hypertension, diabetes, HIV, hepatitis B and C, hyper/hypothyroidism, cardiovascular, kidney, respiratory, vascular and liver disease, history of DVT, neurological disorders, recent cancer treatment (within 1 year of enrolling), osteopenia, depression, and alcohol/substance abuse. Physical activity status, medication usage of the subjects and menstrual status of young female subjects are previously described (Tanner and others 2015). Of the 14 young subjects that underwent bed rest, 7 young subjects were randomly assigned (restricted randomization in blocks of 4–1:1 ratio) to continue onto the rehabilitation phase (REHAB) with a protein supplement whereas the remaining 7 conducted the REHAB but did not have supplementation. We did not find any differences during the REHAB phase in the young subjects according to protein supplementation so we collapsed the young subject groups for the majority of the outcomes. However, we present a few of the outcomes in young subjects according to protein supplement for use as supplemental data (Supplemental Figure 1). All subjects read and signed the informed consent form. The study was approved by the University of Utah Institutional Review Board (no. 00050933) and conformed to the Declaration of Helsinki and Title 45, US Code of Federal Regulations, Part 46, “Protection of Human Subjects”.

2.2. Experimental design: General

The experimental design can be summarized as follows: Body composition and lower extremity power was assessed before bed rest (PRE), after bed rest (BED REST) and after 8 weeks of exercise rehabilitation (REHAB). Bed rest consisted of a 5-day, 4-night (Monday - Friday) physical inactivity experiment within the University of Utah’s clinical research unit.

REHAB encompassed 8 weeks of high intensity eccentric resistance exercise (3× per week) of both legs coupled with post-exercise branch chained amino acid-enriched protein supplementation. A biopsy sample was collected at PRE (bed rest day 1) and repeated after bed rest (day 5) and after 8 weeks of exercise rehabilitation (REHAB).

2.3. Tissue composition and power testing

Two weeks before bed rest, and after an overnight fast, lean tissue was determined using a dual-energy X-ray absorptiometry (DXA) scan (Berg et al. 1993) by a trained technician at the University of Utah's Centre for Clinical and Translational Sciences, Clinical Research Unit. Subjects also underwent MRI testing for their lower legs before and after bed rest and after rehabilitation. Finally, subjects underwent knee extension power testing using the Leg Extension (Nottingham) Power Rig (Queen's Medical Centre, Nottingham, UK) as described previously (Marcus and others 2011). Following five practice trials, each leg was tested individually and data were reported as peak power (Watts) from the average of maximal efforts from both legs. Power testing was monitored by a research assistant. Tissue composition and power testing were repeated after BED REST and after REHAB.

A habitual dietary assessment was measured approximately 2-wk prior to bed rest and was recorded over a 5–7 day period. Caloric intake was later calculated using the Food Processor Nutrition Analysis software (Salem, OR, USA). There were no caloric and macronutrient differences between young and older adults at baseline (Tanner and others 2015).

2.4. Biopsy study

After an overnight fast, application of 1% lidocaine and using aseptic technique, a muscle biopsy was sampled from the *vastus lateralis* using a modified Bergstrom needle approach with manual suction (Evans et al. 1982). Muscle tissue was immediately washed with cold saline and dissected of visible non-muscle tissue, flash-frozen in liquid nitrogen, or mounted in isopentane-cooled OCT and stored at –80°C for later analysis. Biopsy samples used for these analyses were taken before bed rest (PRE), after bed rest (BED REST) and following exercise rehabilitation (REHAB).

2.5. 5-day bed rest

Prior to bed rest and after an overnight fast, subjects arrived at the University of Utah Center for Clinical and Translational Sciences (CCTS) Clinical Research Unit and underwent a DXA scan for assessment of body composition and an oral glucose tolerance test (OGTT). Approximately 1–2 wk later, subjects participated in a 5-day/4-night bed rest protocol at the CCTS. After the completion of the first biopsy study, subjects adhered to five continuous days of bed rest as previously described in detail (Tanner and others 2015). Adherence to bed rest was monitored by nursing staff 24 h a day who also performed standard of care (Tanner and others 2015) for hospitalized/bed-ridden patients while monitoring subject safety. The night before entering the Clinical Research Unit, subjects were given a pre-packaged dinner to consume consisting of a caloric and macronutrient density based on the individuals' body weight. Total caloric intake during bed rest was pre-determined by the research dietician using the Harris–Benedict equation adjusted for no physical activity. Additionally, subjects consumed a diet that provided a daily protein amount of 1.0 g protein

kg⁻¹·day⁻¹ protein intake. Subjects were also provided water ad libitum throughout the 5 days of bed rest. On day 4, the DXA scan and OGTT test were repeated under the same conditions as before. On day 1 and day 5 of bed rest and after an overnight fast, a second (Post) muscle sample was collected on the opposite leg as on day 1. Leg power and MRI was repeated afterwards before beginning exercise rehabilitation.

2.6. Exercise rehabilitation

We used high-intensity eccentric contractions for rehabilitation as previously described (Tanner and others 2015) as this is an effective rehabilitation strategy to enhance lower limb strength and hypertrophy in older adults (LaStayo and others 2014). Exercise rehabilitation was conducted three times per week with 48 h between each session and the protocol was increased progressively over 8 weeks to maintain intensity. The progressive resistive eccentric exercise training of the knee and hip extensor muscles was performed using a recumbent stepper-ergometer (Eccentron, Baltimore Therapeutic Equipment, Hanover, MD). Total work (kJ) was recorded after each exercise session to monitor progress. Following each exercise session, 17g BCAA-enriched whey protein; BCAA Pepform BCAA Peptide, Glanbia Nutritionals, Twin Falls, ID, USA) was consumed by the subject in the presence of an investigator to those randomized to receive the supplement. Exercise sessions were supervised and recorded by a research assistant to monitor compliance and progress. All subjects were asked to maintain their day-to-day habitual activities (e.g. walking) and normal daily living and dietary habits during the exercise rehabilitation period. Adherence was indicated by missing no more than 2 of the 24 training sessions (92%). After exercise rehabilitation, subjects completed the last biopsy study, approximately 3–5 days following the last rehabilitation training session. Finally, subjects repeated the, MRI, OGTT, DXA scan, and leg power tests following the biopsy study.

2.7. Serum analysis and insulin sensitivity

Serum collected during the OGTT was assessed for glucose using a standard glucose analyzer (YSI, Yellow Springs, OH). Serum insulin (EMD Millipore, Billerica, MA), serum creatinine (Crystal Chem #80189), and C-Peptide (EMD Millipore #E6020-K) were determined for each OGTT time point according to manufacturers' instructions. C-peptide and serum creatinine values were utilized to calculate the CLIX-IR and CLIX-β equations as measures of insulin sensitivity (Anderwald and others 2007). The equations are as follows:

$$\text{CLIX-IR: Serum creatinine (0.85 male, 0.1 female)} / (\text{mean AUC of glucose} \times \text{mean AUC of C-peptide}) \times 6600$$

$$\text{CLIX-}\beta\text{: AUC of C-peptide} / (\text{AUC of glucose} - 6000) \times \text{CLIX-IR} \times 10$$

2.8. Magnetic resonance imaging

Magnetic resonance imaging (MRI; Siemens Trio, Siemens Medical, Erlangen, Germany) was performed for determination of quadriceps cross-sectional area (CSA) and fat content as previously described in detail (Addison and others 2014). A subgroup of the participants (9 young; 9 old) underwent this procedure as this was a late addition to the protocol.

2.9. Immunofluorescence

Samples were removed from the cork at -25°C in a ThermoFisher Cryostat (Fisher Scientific HM 525X) where they were cut in $6\text{ }\mu\text{m}$ cross-sections. PRE, BED REST and REHAB samples for the same subject were placed on the same slides Fisherbrand Superfrost®/Plus microscope slides (Fisher Scientific, USA). Several slides were generated per subject, one for analysis of myofiber myosin heavy chain (MHC) typing with WGA analysis of cross-sectional area (CSA), one for fiber-type-specific satellite cell content and then one slide for each of the three macrophage stains. Following cutting, a hydrophobic marker (Vector, H-4000, Burlingame, CA) separated the sections, which were dried at room temperature (RT) and then stored at -20°C until staining.

Myofiber MHC type and CSA was determined as follows: Slides were incubated for at least 1 h at RT and then overnight at 4°C with primary antibodies, mouse anti-myosin heavy chain (MHC) type I (BA.D5 IgG2b, 1:75, Developmental Studies Hybridoma Bank, Iowa City, IA) and Dystrophin (Abcam ab3149, 1:200) in PBS. On day 2, slides were rinsed with PBS followed by 1 h incubation with secondary antibodies Alexa Fluor 647 conjugated goat anti-mouse IgG2b (for MHC I: 1:250, #A21242, Invitrogen) and Alexa Fluor 488 conjugated goat anti-mouse IgG1 (for dystrophin: 1:500, #A21121 Invitrogen) at RT in the dark. After a 5 min post-fix in methanol, slides were mounted with fluorescent mounting media (Vector, H-4000) and dried before imaging. Images for fiber typing and CSA were captured at $20\times$ magnification using a fluorescence microscope (a fully automated Nikon Ti-E inverted widefield microscope using a high sensitivity Andor Clara CCD camera) with Nikon NIS-Elements multi-platform acquisition software. Image processing and analysis was done using FIJI (Image J) and SMASH (Smith and Barton 2014). For each image, the number of muscle fibers for pure MHC type I (purple- converted to blue in FIJI for analysis in SMASH) and MHC II (negative stain) were counted, and cross sectional areas (CSA) for MHC type I and II fibers were measured. A total of 446 ± 37 muscle fibers per section (MHC I: 234 ± 47 ; MHC II: 209 ± 20) were analyzed for fiber type distribution and CSA.

Fiber-type-specific satellite cells were determined as previously described (Arentson-Lantz and others 2016; Reidy and others 2017a; Reidy and others 2017b). Images were captured at $100\times$ magnification using a fluorescence microscope (a fully automated Nikon Ti-E inverted widefield microscope using a high sensitivity Andor Clara CCD camera) with Nikon NIS-Elements multi-platform acquisition software. Image processing and analysis was done using FIJI (Image J). Following counting of Pax7⁺DAPI⁺ cells within an image, fiber number was quantified manually to express the number of Pax7 cells per fiber specific to each fiber type (MHC I or II). Pax7⁺DAPI⁺ per myofiber were determined from 663 ± 41 muscle fibers per section (MHC I: 305 ± 21 ; MHC II: 358 ± 26) muscle fibers in each section.

Skeletal muscle macrophage markers for CD68/CD11b (M1), CD68/CD163 (M2) and CD206 (M2) co-labeling were performed as follows: Sections were fixed in -20°C acetone for 10 min followed by 3×3 min rinses in PBS. Sections were incubated for 7 min 3% H_2O_2 in PBS treatment to block endogenous peroxidases. Following three 3 min washes in PBS, sections were blocked for 1 h in 2.5% normal horse serum (NHS, Vector, S-2012) at RT and subsequently incubated overnight at 4°C with primary antibodies diluted in 2.5% NHS

against rabbit dystrophin (Santa Cruz #sc-15376, 1:100) and mouse CD68 (Dako #M0814, 1:100). On day 2 sections were incubated for 1 h with secondary antibodies: Alexa Fluor 647 conjugated goat anti-rabbit IgG (Invitrogen #21245, 1:500, for dystrophin) and biotin-SP-conjugated goat anti-mouse IgG (Jackson Immuno Research #115-065-205, 1:1000, for CD68) diluted in 2.5% NHS at RT in the dark. After 3×3 min rinses with PBS, sections were incubated for 1 h with streptavidin-horseradish peroxidase conjugate (SA-HRP, Invitrogen, S-911, 1:500 in PBS) and rinsed again with PBS were repeated following this step. Sections were incubated for 12 min with Alex Fluor 488 (Invitrogen #T20932, 1:200 in amplification diluent included in kit) and followed by 3×3 min rinses. Sections were blocked for 45 min in M.O.M. Ms IgG blocking buffer (Vector #MKB-2231; 1 drop/ml PBS) at RT and then blocked again in 2.5% NHS for 1 h at RT. Sections were incubated overnight at 4°C with one of the following mouse primary antibodies in 2.5% NHS against CD11b (Cell Sciences #MON1019-1, 1:100) or CD163 (Santa Cruz #sc-20066, 1:200). On day 3 of staining, sections were rinsed with PBS for 3×3 min before and after 1 h incubation with goat anti-mouse IgG biotin-SP-conjugated (Jackson Immuno Research #115-065-205, 1:1000) in 2.5% NHS at RT. Sections were exposed to a 1 h incubation of SA-HRP (1:500 in PBS), washed in 3×3 min intervals in PBS, and incubated for 15 min in Alexa Fluor 568 (Invitrogen #T20932) in amplification diluents. Following three 5-min washes in PBS, sections were mounted in DAPI containing medium mounting media (Vector, H-1200) and allowed to air dry. This staining protocol for CD11b/CD68 and CD163/CD68 identification resulted in DAPI positive nuclei (staining blue), dystrophin (staining purple), CD68 (staining green) and CD11b or CD163 (staining red). For CD206 staining Sections were fixed in -20 C acetone for 7 minutes followed by three 3-minute rinses in PBS. Sections were blocked for 1 h in 2.5% NHS (Vector, S-2012) at RT and subsequently incubated overnight at 4C with primary antibodies diluted in 2.5% NHS against mouse dystrophin (Abcam #ab1808, 1:200) and goat CD206 (R&D, cat#AF2534, 1:200). On day 2 of staining, sections were rinsed with PBS for 3×3 min before and after 1 h incubation with Rb anti-goat IgG biotin-SP-conjugated (Vector labs, Cat #BA-5000, 1:1000) and donkey anti-mouse IgG Alexa Fluor 647 (Jackson, 1:500, for dystrophin) in 2.5% NHS at RT. Following washes in PBS, sections were exposed to a 1 h incubation of SA-Dylight 549 (Vector Labs, Cat # SA-5549, 1:200 in PBS) and then washed in 3×3 min intervals in PBS before sections were mounted in DAPI containing medium mounting media (Vector, H-1200) and allowed to air dry before imaging. Muscle macrophage content was quantified via two methods; 1) per fiber (range: 0.02–0.30 cells/myofiber) and 2) density per unit area (range: 10–70 cells/mm²). 397 ± 25 muscle fibers were counted and the average area was 1.75 ± 0.12 mm² per section. Also, in the case of the CD68/CD163 stain we also counted the pockets of macrophages located in the perimysium (Supplemental Figure 2) expressed as density per unit area because there were high concentrations of cells in these areas (range: 100–2000 cells/mm²) and because they may play a physiologically important role (Liu and Gordon 2013). The average area of these sections was 0.022 ± 0.002 mm² per section. We did measure the area of analyzed perimysium tissue, but there were no statistical differences between groups or time points due to high variability between sections.

2.10. Gene expression

Step-by-step methods for each of these procedures can be found in a previous publication (Drummond et al. 2010). Total RNA was isolated by homogenizing 10–15 mg tissue with a handheld homogenizer in a solution containing Tri reagent LS (Molecular Research Centre, Cincinnati, OH, USA). The RNA was separated and precipitated using chloroform and isopropanol. Extracted RNA was washed with ethanol then suspended in nuclease-free water with EDTA. RNA concentration was determined using the EPOCH (Take3; BioTek) spectrophotometer. RNA was DNase-treated and cDNA was synthesized using commercially available kits (DNA-free, Ambion, Austin, TX, USA; iScript, BioRad, Hercules, CA, USA). All isolated RNA and cDNA samples were stored at -80°C until analysis. Real-time PCR was carried out with a CFX Connect real-time PCR cycler (BioRad) under similar protocol conditions as reported previously (Drummond and others 2014; McKenzie and others 2016) using either TaqMan fluorescence pre-designed or SYBR green custom designed primers. TaqMan PCR assays (Thermo Fisher Scientific, Waltham, MA) were performed under manufactures instruction guidelines. The following TaqMan primers were used: tumor necrosis factor alpha (TNF α), HS01113624; interleukin 10 (IL-10), Hs00961622; transforming growth factor beta 1 (TGF β 1), Hs00998133; cyclooxygenase 2 (COX-2), Hs00153133; interleukin 1 beta (IL-1 β), Hs00174097; interleukin 4 receptor alpha (IL-4R α), Hs00166237 and nitric oxide synthase 3 (NOS3), Hs01574659. Self-designed primers for beta 2-microglobulin (β 2M), and cluster of differentiation 68 (CD68) have been previously described (Kwon and others 2015b). Cycle threshold values of target genes were normalized to β 2M then fold change from PRE values were calculated (2^{-C_t}). β 2M remained stable across interventions and between age groups.

2.11. Statistical Analysis

Values are the raw values or change scores expressed as Mean $\pm$ SEM. Primary outcome data were evaluated for equal variances and normality, and no major violations of model assumptions were found, except for correlation analyses so non-parametric Spearman tests were used. Baseline characteristics were compared with students t-test. A priori, we set the primary comparisons of interest; the changes from PRE to BED REST, BED REST to REHAB or PRE to REHAB to test for an effect of age using individual t-tests to address the specific question if there is an effect of age with that particular change. The comparison PRE to BED REST examined the atrophy from inactivity, the comparison from BED REST to REHAB examined the muscle regrowth following inactivity and the comparison from PRE to REHAB examined the ability to return to baseline values. Statistics were conducted with a 2-way ANOVA with either Sidak or Tukey Post Hoc tests as appropriate to test for age effects. To test for effects of time independent of age a 1-way ANOVA for each age group was also used. Significance was set as $P \leq 0.05$ and trends as $0.05 < p < 0.10$. All analyses were conducted with Graph Pad Prism 6.0f (La Jolla, CA). All figures were generated with the same program.

3. Results

3.1. Subject Characteristics

There were no baseline differences in the subject characteristics (height, weight, WBLM, LLM and power, but the older adults tended ($p=0.064$) to have a greater BMI compared to the younger adults (Table 1). Total work (kilojoules) conducted during the rehabilitation was greater ($p<0.001$) in the young ($1.19 \times 10^6 \pm 1.22 \times 10^5$) compared to the old adults ($6.76 \times 10^5 \pm 6.44 \times 10^4$) and was not different ($p>0.10$) between the young control ($1.25 \times 10^6 \pm 2.26 \times 10^5$) and young protein ($1.13 \times 10^6 \pm 1.09 \times 10^5$) groups. When total work during rehabilitation was normalized to leg muscle mass, there were no differences between young and old (Tanner and others 2015).

3.2. Quadriceps CSA, Intra-Muscular Fat and Body Composition and Power

Whole muscle cross-sectional area (CSA) of the mid-quadriceps (Figure 1A & 1B, Supplemental Figure 3A) tended ($p=0.082$) to be lower in older compared to younger adults at PRE (Table 1). The young adults demonstrated significant quadriceps muscle hypertrophy ($p<0.01$) from PRE to REHAB ($5.61 \pm 1.64\%$) and BED REST to REHAB ($4.13 \pm 0.95\%$). A trend ($p=0.075$) for an effect of age on quadriceps CSA was shown at REHAB. In regards to BED REST, we did not identify any differences in quadriceps CSA between young or older adults, yet examination of the older adult data revealed that all but one subject decreased from PRE to BED REST ($-2.07 \pm 1.47\%$) and removal of the one outlier revealed significant muscle atrophy ($p=0.034$) in the older adults ($-3.14 \pm 1.14\%$). When examining the change from PRE to BED REST, there was a significantly greater ($p=0.049$ with outlier, $p=0.017$ without outlier) whole muscle atrophy in the old vs. young adults. Both groups demonstrated a significant increase ($p<0.05$) in quadriceps CSA from BED REST to REHAB (Young: $4.13 \pm 0.95\%$; Old: $3.23 \pm 1.44\%$). When examining the change from PRE to REHAB there was a significantly greater ($p=0.036$) quad muscle hypertrophy in the young vs. older adults (Figure 1A & Supplemental Figure 3A). There was no effect of protein supplementation on whole muscle hypertrophy in the younger adults from BED REST to REHAB (data not shown).

Lean mass (Table 2) assessed via DXA, as an estimate of muscle mass, demonstrated similar results to the MRI data. A trend ($p=0.088$) for loss in whole body lean mass (WBLM) from PRE to BED REST was driven by a decrease ($p<0.001$) in the older adults. The older adults reduced ($p<0.001$) trunk lean mass (TLM) (Figure 1I & 1J, Supplemental Figure 3E, Table 2) from PRE to BED REST ($-5.89 \pm 0.72\%$) and this decrease was greater ($p<0.001$) than with the younger adults ($-1.36 \pm 0.72\%$). Older ($p<0.001$) and younger ($p=0.052$) adults demonstrated increases in TLM from BED REST to REHAB (Young: $1.70 \pm 0.82\%$; Old: $4.33 \pm 1.39\%$). The older adults did not regain their losses in TLM as there was a weak trend ($p=0.098$) for a reduction from PRE to REHAB, which is supported by an age effect ($p=0.041$). Arm (ALM) and leg lean mass (LLM) are more precise estimates of muscle mass compared to WBLM and TLM since WBLM is diluted by non-contractile lean mass. ALM (Table 2) was unchanged over time and had no influence of age. However, the older adults reduced ($p=0.019$) LLM (Figure 1C & 1D, Supplemental Figure 3B, Table 2) from PRE to BED REST and this change ($-3.82 \pm 0.74\%$) was more ($p=0.012$) compared to the younger

adults ($-0.30 \pm 0.67\%$). Both groups demonstrated an increase ($p < 0.05$) in LLM from BED REST to REHAB (Young: $2.63 \pm 1.09\%$; Old: $3.78 \pm 1.40\%$). Although no significant changes or age related differences were evident from PRE to REHAB there was a weak trend ($p = 0.099$) for younger adults to demonstrate LLM hypertrophy ($2.31 \pm 1.23\%$). There was no effect of protein supplementation on any measure of lean mass in the younger adults from BED REST to REHAB (Supplemental Figure 1A).

Absolute fat mass (Table 2) was not different following BED REST and REHAB, however, it was higher in older adults compared to younger adults at baseline ($p < 0.001$). Relative fat mass was higher in older adults compared to younger adults ($p < 0.001$) and was increased in older adults from PRE to BED REST ($p = 0.007$) to a greater degree than younger adults ($p = 0.015$). There was a trend ($p = 0.082$) for protein supplementation to reduce RFM in the younger adults from BED REST to REHAB (PRO: $-0.66 \pm 0.37\%$; CON: $0.22 \pm 0.28\%$). Intra-muscular fat as determined via MRI, (data not shown) was not different across age or over time.

Knee extensor power (watts) (Figure 1G & 1H, Supplemental Figure 3D) (Young: PRE 164.74 ± 4.74 , BEDREST 157.97 ± 5.57 , REHAB 204.59 ± 6.17 ; Old: PRE 150.22 ± 5.41 , BEDREST 137.03 ± 4.68 , REHAB 169.44 ± 5.50) changed over the experiments ($p < 0.001$), but was not different by age. There was a decrease ($p < 0.001$) from PRE to BED REST (Young: $-12.51 \pm 3.98\%$; Old: $-8.47 \pm 3.78\%$), and an increase ($p < 0.005$) from PRE to REHAB (Young: $26.25 \pm 7.50\%$; Old: $14.08 \pm 6.67\%$) and BED REST to REHAB ($p < 0.002$) (Young: $42.66 \pm 11.05\%$; Old: $25.16 \pm 6.67\%$). There was no effect of protein supplementation on leg power in the younger adults from BED REST to REHAB (Supplemental Figure 1C).

3.3. Vastus Lateralis MHC Fiber Type Composition

Vastus lateralis were composed of MHC I myofibers at the following percentages for younger and older adults, respectively: PRE ($51.6 \pm 2.9\%$, $49.3 \pm 3.1\%$), BED REST ($48.1 \pm 3.3\%$, $48.6 \pm 4.5\%$), and REHAB ($59.4 \pm 2.9\%$, $57.2 \pm 5.6\%$). There was an increase in MHC I composition from PRE to REHAB ($p < 0.05$) and BED REST to REHAB ($p < 0.007$), independent of age.

3.4. Vastus Lateralis Myofiber Cross Sectional Area (Table 3)

Similar to quadriceps CSA, older adults tended ($p = 0.070$) to have reduced myofiber size ($-10.02 \pm 11.78\%$) from PRE to BED REST as compared to the younger adults ($8.42 \pm 5.63\%$) (Figure 1C & Supplemental Figure 3C). In contrast to the quadriceps CSA and leg lean mass data, only the older adults increased ($p = 0.002$) myofiber CSA from BED REST to REHAB and this change was significantly greater ($p = 0.007$) in the older adults ($45.53 \pm 12.16\%$) versus the younger adults ($-0.49 \pm 6.17\%$). There was a trend ($p = 0.051$) for lower mean myofiber CSA in the older adults as compared to the younger adults after BED REST. For MHC I myofiber CSA, older adults demonstrated a significant increase ($p = 0.003$) from BED REST to REHAB, but there were no effects of age. When examining the changes in MHC I myofiber CSA, the older adults displayed a similar pattern as the mean myofiber data. For MHC II myofiber CSA, older adults demonstrated an increase ($p = 0.019$) from

BED REST to REHAB, and they had smaller ($p=0.045$) myofibers as compared to the younger adults after BED REST. There was no effect of protein supplementation on Mean, MHC I or MHC II myofiber CSA in the younger adults from BED REST to REHAB (Supplemental Figure 1B).

3.5. Insulin Sensitivity (Figure 2)

CLIX-IR and CLIX- β , measures of insulin sensitivity, were decreased ($p<0.05$) following BED REST and returned to PRE levels after REHAB ($p<0.05$). There was no influence of age.

3.6. Vastus Lateralis Satellite Cell Content (Figure 3)

Vastus lateralis myofiber Pax7⁺ satellite cell content expressed as per fiber (Figure 3A) and also density (Figure 3B) was increased ($p<0.002$) following REHAB compared to PRE (Young: $60.69 \pm 13.19\%$; Old: $37.03 \pm 11.30\%$) and REHAB compared to BED REST (Young: $66.88 \pm 14.10\%$; Old: $68.68 \pm 13.47\%$) with no effect of age. When examining the fiber-type 20 specific response in young adults, both MHC I and MHC II myofibers increased ($p<0.007$) following REHAB compared to PRE and compared to BED REST and there were no differences between fiber types. Examination of the fiber-type specific response in older adults revealed that both MHC I and MHC II myofibers increased ($p<0.002$) following REHAB compared to PRE and compared to BED REST and that Pax7 content was greater ($p<0.03$) in MHC I compared to MHC II myofibers at PRE and REHAB. The older adults exhibited a trend ($p=0.062$) to decrease MHC I Pax7 content from PRE to BED REST ($-21.8 \pm 7.2\%$). Regardless of how the data were expressed (density or per fiber) there was no effect of protein supplementation on Pax7 content in the younger adults from BED REST to REHAB. Example immunohistochemical images of Pax7⁺ cells can be found as a supplement (Supplemental Figure 4).

3.7. Skeletal Muscle Macrophages

The muscle macrophage milieu was modulated following bed rest in young and older adults (Figure 4). The data were similar when expressed as both per fiber and density per unit area so we have reported the data as per fiber. CD68⁺CD11b⁻ cells (as both per fiber and density per unit area) were significantly greater ($p<0.05$) in older adults as compared to younger adults across all time points. In addition, CD68⁺CD11b⁻ cells increased ($p<0.05$) following REHAB as compared to PRE and compared to BED REST. CD68⁻CD11b⁺ cells (per fiber) were of low abundance (range: 0.01 – 0.07 cells per myofiber) and were significantly ($p<0.05$) greater in younger adults as compared to older adults across all time points. CD68⁻CD11b⁺ cells (density per unit area) were not different across time or by age. All CD68⁺ cells were increased ($p<0.05$) following REHAB as compared to PRE and compared to BED REST with no effect of age. All CD11b⁺ and CD68⁺CD11b⁺ cells (as both per fiber and density per unit area) were significantly ($p<0.05$) greater in younger adults as compared to older adults across all time points, yet there were no significant differences ($p>0.10$) across time. CD206⁺ cells (as both per fiber and density per unit area) were increased ($p<0.05$) following REHAB as compared to PRE and BED REST. Older adults demonstrated an increase in CD206⁺ cells ($p<0.001$) following REHAB as compared to PRE and BED REST and were significantly greater than in young adults at REHAB ($p<0.05$).

CD68⁺CD163⁺ cells (per fiber and density per unit area) were of low abundance and were not different by age or across time. CD68⁺CD163⁻ cells (per fiber) were of low abundance and were not different by age or across time, but when expressed as density per unit area the young to increase these cells from PRE to REHAB. CD68⁺CD163⁺ cells (as both per fiber and density per unit area) were ($p < 0.05$) greater at REHAB compared to BED REST (per fiber) and compared to PRE. All CD68⁺ cells responded similarly as CD68⁺CD163⁺ cells while all CD163⁺ cells also demonstrated a similar pattern that was increased at REHAB compared to BED REST (per fiber) but not when expressed as density per unit area. Finally, we found that the perimysium tissue macrophages were not different over time or by age with the exception of CD68⁺CD163⁻ cells, which displayed a tendency to have greater amounts in older adults at PRE ($p = 0.09$) that decreased ($p = 0.07$) following REHAB. (Supplemental Table 3). Example immunohistochemical images of macrophages can be found as supplemental images (Supplemental Figure 2,5–7).

3.8. Vastus Lateralis mRNA expression of inflammatory and macrophage markers

At PRE, mRNA expression of IL-10, IL-4R α and CD68 was higher in older adults compared to younger adults ($p < 0.05$) (Table 4). CD68 mRNA (Figure 5A) was increased from BED REST to REHAB ($p < 0.05$) with no effect of age. Older adults, independent of younger adults, decreased CD68 mRNA expression ($p < 0.001$) from PRE to BED REST followed by an increase ($p = 0.032$) from BED REST to REHAB. IL-10 mRNA (Figure 5B) expression was increased following REHAB ($p < 0.05$) from PRE and BED REST with no effect of age. IL-10 expression in older adults (independent of young) tended to decrease ($p = 0.064$) from PRE to BED REST followed by an increase ($p = 0.054$) from BED REST to REHAB. TNF α (Figure 5C) mRNA expression was increased from PRE to REHAB ($p < 0.05$) with no effect of age. Whereas young adults demonstrated increased TNF α mRNA expression at BED REST ($p = 0.046$) compared to PRE. Young adults demonstrated increased NOS3 mRNA (Figure 5E) expression at BED REST ($p = 0.020$) and REHAB ($p < 0.001$) compared to PRE. There was a tendency ($p = 0.076$) for NOS3 mRNA expression to be higher in younger compared to older adults after BED REST. TGF β 1 (Figure 5F) mRNA was increased from PRE and BED REST to REHAB ($p < 0.05$) with no effect of age. IL-1 β (Figure 5H), IL-4R α (Figure 5D) and COX-2 (Figure 5G) mRNA expression were unaltered by age or time. However, analysis in older adults, independent of younger adults, revealed that IL-4R α expression increased ($p = 0.048$) following REHAB compared to BED REST.

3.9. Correlational Analysis

There were few and/or weak relationships between insulin sensitivity (CLIX-IR or CLIX- β) and CD68⁺CD163⁺, CD206⁺, CD163⁺ cells or perimysium tissue macrophages ($r < 0.30$, $p > 0.10$). When all time points were pooled, there was a positive relationship between CLIX-IR and CD11⁺ cells ($r = 0.37$, $p = 0.003$) and CD68⁺CD11b⁺ cells ($r = 0.44$, $p < 0.001$), at PRE ($r = 0.51$ – 0.42), $p = 0.016$ – 0.050) and REHAB ($r = 0.38$ – 0.49 , $p = 0.085$ – 0.022). Yet, those relationships were driven by four outliers (2 old, 2 young) during REHAB. There was a trend for the increase in insulin sensitivity from BED REST to REHAB to be positively correlated ($r = 0.38$, $p = 0.080$) to the amount of CD68⁺ cells and between perimysium CD68⁺CD163⁻ cells at REHAB ($r = 0.53$, $p = 0.014$). However, the increase in insulin sensitivity from BED REST to REHAB was positively correlated ($r = 0.46$ – 0.48 , $p < 0.028$)

with the increase in ALM or LLM during the same period and negatively ($r=-0.44-0.46$, $p<0.038$) associated with the loss of ALM following BED REST. Interestingly, the participants with greater insulin sensitivity at PRE displayed the greatest reductions ($r=-0.48$, $p<0.001$) following BED REST. Also, Pax7⁺ satellite cell content and macrophages (CD68 and CD206) content ($r=0.50$, $p<0.001$) were positively correlated (Supplemental Figure 8).

4. Discussion

The primary goal of this study was to examine age-related skeletal muscle macrophage responses following bed rest and rehabilitation and to determine if macrophages corresponded to changes in muscle and insulin sensitivity. We found robust age-related differences in muscle atrophy during short-term bed rest, yet older and younger adults were equally able to stimulate a muscle growth response during rehabilitation. Insulin sensitivity was reduced following bed rest, but was restored following rehabilitation and was not age-dependent. There were age-related differences in macrophage content but both young and older adults increased skeletal macrophages after rehabilitation. Muscle satellite cells during rehabilitation corresponded to increases in macrophage content. Finally, muscle tissue resident macrophages were not associated with change in insulin sensitivity following bed rest and rehabilitation.

4.1 Insulin sensitivity

Short-term bed rest reduced insulin sensitivity after bed rest and was similarly decreased in both younger (~36%) and older adults (~39%) and returned back to baseline following 2 months of exercise rehabilitation. Our data support the influence of short-term bed rest on impaired glucose utilization (Hamburg and others 2007; Stuart and others 1988). In contrast, Pisot et al found that young subjects (vs old) had greater reductions in insulin sensitivity (Matsuda Index) after 14 days of bed rest. However, the young subjects in that study had better insulin sensitivity (vs old) and were possibly more physically active at baseline and therefore were more sensitive to muscle disuse than the older adults. We too show that those with greater insulin sensitivity had larger reductions with bed rest. In the current study, older adults were primarily women, and had a similar physical activity level and insulin sensitivity as the younger adults (Tanner and others 2015). Physical activity promotes uptake of glucose into skeletal muscle, thus, regular physical activity is associated with improved glucose utilization and insulin sensitivity (Rosenthal and others 1983). Our data suggests that changes to insulin sensitivity during bed rest or rehabilitation are not age-related but are likely more influenced by physical activity status.

4.2 Muscle macrophages

Next we sought to examine if muscle resident-tissue macrophages were altered following bed rest and rehabilitation and if these changes were related to muscle mass and/or insulin sensitivity following bed rest and rehabilitation in an age-dependent manner. Interestingly, we found age related differences in some macrophage populations (CD68⁺CD11b⁺ and CD68⁺CD11b⁻ cells) but the muscle tissue resident macrophages, (with our quantification system (IHC)), were not associated with insulin sensitivity following bed rest or

rehabilitation. Previous reports have shown that macrophage muscle infiltration in obese and/or T2DM subjects were inversely correlated with insulin sensitivity (Fink and others 2014). Some, but not all, reports have demonstrated that long-term bed rest may result in impaired immunity and greater risk of infection/inflammation (Crucian and others 2014; Hoff and others 2015; Sonnenfeld and others 2007). It is unclear how soon changes in the immune system (leukocytes in particular) occur (Kelsen and others 2012) following bed rest and when or if they influence muscle-resident immune cells and inflammation in a manner sufficient to alter insulin sensitivity. Our previous reports suggested increased skeletal muscle, but not systemic inflammation following 5- (Kwon and others 2015a) and 7d (Drummond and others 2013) of bed rest in older adults. Yet, it is unknown if inactivity-induced inflammation was mediated through skeletal muscle resident tissue macrophages and their polarization states and if over a longer course of bed rest could influence insulin sensitivity. Indeed, the actions of anti-inflammatory macrophages in human skeletal muscle are positively related to improved glucose uptake following exercise training in humans (Fink and others 2013) and a single bout of exercise in mice (Ikeda and others 2013).

4.3 Muscle macrophages and insulin sensitivity

To further characterize muscle macrophages we examined proxies for inflammatory macrophages (M1) as CD68⁺CD11b⁺ cells and anti-inflammatory macrophages (M2) as CD206⁺ or CD68⁺CD163⁺ cells using immunohistochemistry. We then followed up with skeletal muscle mRNA analysis of known M1 (TNF α , IL-1 β , COX-2) and M2 (TGF β 1, IL-10 and IL-4R α) expressing markers alongside a pseudo confirmation of macrophage content with CD68 mRNA. Although we found that older adults had more CD68⁺ cells and CD68 mRNA, we found no clear relationship between insulin sensitivity and macrophage markers or mRNA expression of these targets. This finding coincides with data from Friedrichsen et al. demonstrating that skeletal muscle mRNA of macrophage markers and NF κ B activity are unchanged in younger adults following 9 days of bed rest and subsequent 6 weeks of cycle re-training in young healthy adults (Friedrichsen and others 2012). Similar to our findings, they and other unpublished findings (Groshong 2013) also showed that these muscle inflammation markers and macrophages were not correlated to insulin sensitivity (Friedrichsen and others 2012). However, other work from the same laboratory used microarray analysis in skeletal muscle and showed a prominent up-regulation of inflammatory pathways in skeletal muscle following insulin stimulation after bed rest (compared to pre) (Alibegovic and others 2010) suggesting a relationship between physical activity induced-inflammation and insulin action. Thus, it appears that basal (non-stimulated) assessment of inflammation via muscle macrophages and/or their markers is not related to insulin sensitivity. However, future studies are needed to examine the relationship between insulin sensitivity and muscle macrophages and/or their markers examined in the insulin or nutrient-stimulated condition. Another consideration is that inflammatory cytokines released from macrophages in adipose tissue could be working in a paracrine manner to alter skeletal muscle inflammation and insulin resistance (Bruun and others 2006; Di Gregorio and others 2005), however, this mechanism has yet to be demonstrated with inactivity-induced insulin resistance.

4.4 Muscle macrophages and muscle regrowth following inactivity

The role of skeletal muscle resident-tissue macrophages and their polarization states may likely have more bearing on skeletal muscle regeneration and growth rather than insulin sensitivity. It is clear that skeletal muscle macrophages are an essential component to skeletal muscle regeneration and regrowth (Tidball 2011) and may influence the magnitude of muscle hypertrophy (Dennis and others 2015; Dennis and others 2009). The age-related differences in macrophage numbers (Young > Old for CD68⁺CD11b⁺ cells; Old > Young for CD68⁺CD11b⁻ cells) in skeletal muscle at baseline may be related to the impaired skeletal muscle regrowth following injury and a slowed process of skeletal muscle regrowth in older adults, compared to younger adults, (Tanner and others 2015) following bed rest and immobilization (Suetta and others 2013b). M1 macrophages, generally characterized as pro-inflammatory, are recruited into skeletal muscle first after injury. Next, M2 macrophages repair skeletal muscle following M1 infiltration but also can contribute to fibrosis (Tidball and Wehling-Henricks 2007). Our data show that CD68⁺CD11b⁺ macrophages were significantly reduced in older adults compared to younger at every time point. However, we did not demonstrate changes in the M1 macrophage populations after bed rest and exercise rehabilitation. Previous research has demonstrated alterations of M2 macrophages with changes in physical activity and exercise training (Groshong 2013; Prokopchuk and others 2007; Przybyla and others 2006a). Indeed, phenotypic switching from M1 to M2 macrophages may be impaired with age, a process that could mediate the age-related impairment of skeletal muscle repair following short-term bed rest. Therefore, M2 (anti-inflammatory) macrophage counts may, theoretically, more closely parallel the insulin sensitivity changes seen following bed rest. Our future studies will involve more precise quantification and characterization of M2 macrophages (i.e., flow cytometry) in older versus young human skeletal muscle after bed rest and after exercise rehabilitation in order to improve clarity in the relationship between macrophages, muscle size and insulin sensitivity.

4.5 Muscle macrophage specificity limitations in human skeletal muscle

Research examining skeletal muscle immune cells in aging and exercise/regrowth in humans is sparse and there have been no reports, to our knowledge examining skeletal muscle macrophage content following bed rest and exercise rehabilitation. Our current knowledge of macrophages in skeletal muscle tissue in humans has been observed following damaging bouts of eccentric exercise (Deyhle and others 2015; Paulsen and others 2013; Saclier and others 2013) or down-hill running (Mackey and others 2007) and that they play important roles to promote skeletal muscle regeneration and growth through their positive interactions with satellite cells (Saclier and others 2013). Pyzabela et al. demonstrated that skeletal muscle macrophages are anti-inflammatory at rest and were increased following a single bout of resistance exercise in younger adults, but not older adults who were found to have less of these cell types at rest (Przybyla and others 2006b). After 8 weeks of eccentric exercise training we demonstrate that older and younger adults increased skeletal muscle macrophage (CD68⁺) content to a similar degree, which corresponded to a remarkably similar and equivalent increase in satellite cells (Pax7⁺) in older and younger adults. In support of the interaction between muscle resident macrophages and satellite cells during human muscle growth and regeneration we demonstrated a modest correlation between Pax7⁺ satellite cell content and macrophages (CD68 and CD206) content ($r=0.500-0.515$, $p<0.001$). However,

it is unknown if this association is due to a close interaction between the two cell types or similar immunoreactivity (Paulsen and others 2013; Saclier and others 2013; Tidball and Villalta 2010). Although, we cannot preclude age-related responses of macrophages and satellite cells during the initiation of the eccentric exercise training our data indicate that after 8 weeks older and younger adults have similar cell abundances. One important consideration is that the immunohistochemical macrophage markers we and others employ may not be fully specific to macrophages (Paulsen and others 2013; Tidball and Villalta 2010) and/or able to delineate anti- or pro-inflammatory polarization (Groshong 2013). It has been reported that that CD68 (Paulsen and others 2013) or CD206 (Saclier and others 2013; Tidball and Villalta 2010) surface antigens might be immunoreactive with satellite cells. Co-labeling as we have done here and others have done (Saclier and others 2013; Tidball and Villalta 2010) may help improve the specificity, but is cumbersome and time consuming. In comparison to rodent research, we know very little regarding specificity, polarization and functions of human skeletal muscle-resident or infiltrating immune cells. Thus development of a feasible flow cytometry protocol for human skeletal muscle stands to greatly benefit the field in resolving these many unknowns concerning the role of these immune cells in human skeletal muscle.

4.6 Eccentric exercise rehabilitation following inactivity

Rehabilitation was sufficient to return the loss in muscle mass and strength in both young and older adults. Previous research has suggested that older adults have an impaired ability to recover muscle mass and strength following disuse (Pišot and others 2016; Suetta and others 2013a). These disuse-induced deficits, are seen after 2 weeks of concurrent training, 4 weeks of resistance exercise rehabilitation (Hvid and others 2010; Hvid and others 2017; Hvid and others 2014; Suetta and others 2013a; Suetta and others 2009) or 6 weeks of aerobic exercise (Gram and others 2015; Vigelsø A 2015). Although our study was not designed to determine the exact countermeasure that facilitated this recovery, we speculate that the longer (8 week) duration, protein supplementation or more likely, the rehabilitation exercise mode (eccentric contractions) were the contributing factors. Hortobágyi et al. reported that eccentric exercise training was superior to other contraction modes in recovering muscle mass and strength lost following 3 weeks of immobilization, in young adults (Hortobágyi and others 2000). Also, we have shown the enormous potential for eccentric exercise training to improve muscle mass, strength, and mobility in elderly patients and various clinical populations (LaStayo and others 2003) as other have also suggested (Gault and Willems 2013).

4.7 Muscle size

Here we demonstrate that older adults atrophy the leg muscles (via whole muscle CSA, myofiber CSA and DXA leg lean mass) more than younger adults over a short period of inactivity (5 d of bed rest). The younger adults did not experience muscle atrophy during the short 5 d period, which coincides with previous reports (Brocca and others 2012; Ringholm and others 2011) also demonstrating lack of muscle atrophy at the myofiber level over short-term bed rest (7 and 8 d) in young adults. We speculate that younger adults do not demonstrate muscle atrophy over shorter durations of inactivity (bed rest) because of a pseudo-tapering response (Murach and Bagley 2015) and/or variability and lack of

sensitivity in the muscle size measurements. The older adults however, atrophied about 2–3% (via MRI), which compared to the younger adults, suggests faster atrophy with disuse in aging as has been suggested with cross-sectional comparisons (English and Paddon-Jones 2010). The atrophy in older adults in response to bed rest could be related to the reduction in CD68 and IL-10 mRNA, which was restored and increased above baseline expression levels following rehabilitation. The only other bed rest study comparing younger and older adults used a longer bed rest period (14 d) and similar to our findings, demonstrated greater atrophy in the old (Pišot and others 2016). Collectively, these findings demonstrate that bed rest causes greater leg atrophy in older compared younger adults. This finding is particularly concerning given that a large majority of older adults are expected to experience repeated periods of hospitalization as they age.

5. Conclusions

Older adults displayed a significant decrease in macrophage numbers in skeletal muscle at baseline, after bed rest, and following exercise compared to young. Inflammatory cell populations have been shown to play an important role in modulating muscle regeneration, and thus reduced numbers of macrophages may be an underlying mechanism contributing to impaired muscle regrowth with age following recovery from disuse. Although some literature suggests a link between muscle-tissue resident macrophages and insulin resistance, the methods we employed did not reveal that a relationship following bed rest and rehabilitation in younger or older adults. We are hopeful that more precise methods (i.e., flow cytometry) and alternate designs (insulin-stimulated conditions) could help examine this relationship further. Furthermore, it is unfortunate that all the previous investigations examining age-related differences in muscle atrophy and regrowth have exclusively examined men. Our study is not powered to examine age and sex differences, but we included both men and women in our study and show these unique individual responses in Supplemental Figure 3 with the hope that future studies will examine sex differences in aging (Smith and Mittendorfer 2015).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AFM	absolute fat mass
ALM	arm lean mass
AUC	area under the curve
β2M	beta-2-microglobulin
BCAA	branch chained amino acids
CCTS	center for clinical and translational science
CD11b	cluster of differentiation 11b
CD68	cluster of differentiation 68
CD163	cluster of differentiation 163
CD206	cluster of differentiation 206
CLIX-β	clamp-like index-beta
CLIX-IR	clamp-like index-insulin resistance
COX-2	cyclooxygenase 2
CSA	cross sectional area
DAPI	4',6-diamidino-2-phenylindole
DXA	dual-energy X-ray absorptiometry
IL-1 β	interleukin 1 beta
IL4Ra	interleukin 4 receptor alpha
IL-10	interleukin 10
LLM	leg lean mass
M1	pro-inflammatory macrophages
M2	anti-inflammatory macrophages
MHC I	myosin heavy chain 1
MHC II	myosin heavy chain 2
MRI	magnetic resonance imaging
NHS	normal horse serum

NOS3	nitric oxide synthase 3
OCT	optimal cutting temperature
OGTT	oral glucose tolerance test
Pax7	paired box protein 7
PBS	phosphate buffered saline
RFM	relative fat mass
RT	room temperature
TGFβ1	transforming growth factor beta 1
TLM	total lean mass
TNFα	tumor necrosis factor alpha
WBLM	whole body lean mass
WGA	wheat germ agglutinin

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Highlights

- Older adults (vs young) experienced atrophy during bed rest, but both young and old gained lean mass similarly following exercise rehabilitation.
- Insulin sensitivity decreased after bed rest and returned to baseline levels after rehabilitation, independent of age.
- There were age-related differences in skeletal muscle macrophages, but both young and old increased macrophage content after exercise rehabilitation.
- Changes in muscle macrophages corresponded to changes in satellite cells, but not insulin sensitivity during rehabilitation.

Figure 1.

Changes (PRE TO BED REST, BED REST TO REHAB or PRE TO REHAB) in quadriceps muscle CSA (assessed by MRI) (A,B), leg lean mass (assessed by DXA) (C,D), *vastus lateralis* mean myofiber CSA (E,F), knee extension power (G,H) and trunk lean mass (assessed by DXA) (I,J) as mean $\pm$ SEM bar graphs between older and younger adults after BED REST and following rehabilitation (REHAB). A,C,E,G and I depict mean responses while B,D,F,H, J depicts the individual responses.

Figure 2.

Clamp-like index as CLIX-IR (A) and CLIX- β (B) as a proxy of insulin sensitivity in older and younger adults before (PRE) and following BED REST and rehabilitation (REHAB). Data are mean $\pm$ SEM. * vs PRE, # vs BED REST as $p < 0.05$.

Figure 3.

Vastus lateralis myofiber-type specific satellite cell content in older and younger adults at PRE and following BED REST and rehabilitation (REHAB). Data are mean $\pm$ SEM. ^ for age difference, * vs PRE, # vs BED REST and & vs MHC II as $p < 0.05$ unless indicated otherwise.

Figure 4.

Vastus lateralis macrophage marker (CD68, CD11b, CD206, and CD163) immunofluorescence in older and younger adults at PRE and following BED REST and rehabilitation (REHAB). Data are mean $\pm$ SEM. ^ for age difference, * vs PRE, # vs BED REST as $p < 0.05$ unless indicated otherwise.

Figure 5.

Vastus lateralis mRNA expression of macrophage markers CD68 (A), IL-10 (B), TNF α (C), IL-4R α (D), NOS3 (E), TGF β 1 (F), COX-2 (G) and IL-1 β (H) in older and younger adults at PRE and following BED REST and rehabilitation (REHAB). Data are mean $\pm$ SEM. * vs PRE, # vs BED REST as $p < 0.05$ unless indicated otherwise.

Table 1

Baseline subject characteristics

All	Young	Old	vs Old
Sex	7F, 7M	7F, 2M	—
Age (y)	23.14 ± 0.84	66 ± 1.09	<i>p</i> <0.001
Height (m)	1.74 ± 0.02	1.71 ± 0.03	<i>p</i> =0.381
Weight (kg)	66.52 ± 3.55	71.59 ± 3.93	<i>p</i> =0.362
BMI (kg·m⁻²)	21.97 ± 0.89	24.49 ± 0.80	<i>p</i> =0.064
RFM (%)	26.7 ± 1.58	36.64 ± 1.94	<i>p</i> <0.001
WBLM (kg)	46.33 ± 2.58	43.24 ± 2.90	<i>p</i> =0.444
LLM (kg)	15.73 ± 0.91	13.35 ± 0.97	<i>p</i> =0.100
Quad CSA (cm²)	120.48 ± 9.92	97.63 ± 7.32	<i>p</i> =0.082
KE Power (Watts)	164.74 ± 4.75	150.22 ± 5.41	<i>p</i> =0.579

Data are mean ± SEM. Body mass index, BMI; cross-sectional area, CSA; knee extension, KE; leg lean mass, LLM; relative fat mass, RFM; whole body lean mass, WBLM. QUAD CSA was completed in only 9 young subjects (7F, 2M) due to a late amendment of including MRI into the protocol.

Table 2

Body Composition in older and younger adults at baseline and following bed rest and rehabilitation.

(kg)		PRE	BED REST	REHAB
WBLM	Young	46.33 ± 2.58	46.01 ± 2.48	46.76 ± 2.54 [#]
	Old	43.24 ± 2.9	41.35 ± 2.76 [*]	42.83 ± 2.89 [#]
ALM	Young	4.91 ± 0.40	4.96 ± 0.40	4.97 ± 0.40
	Old	4.35 ± 0.41	4.30 ± 0.40	4.34 ± 0.41
TLM	Young	22.69 ± 1.21	22.35 ± 1.16	22.75 ± 1.21
	Old	22.73 ± 1.45	21.38 ± 1.36 [*]	22.29 ± 1.41 [#]
LLM	Young	15.73 ± 0.91	15.65 ± 0.87	16.04 ± 0.88 [#]
	Old	13.35 ± 0.97	12.84 ± 0.95 [*]	13.34 ± 1.01 [#]
AFM	Young	17.85 ± 1.51	17.99 ± 1.52	18.1 ± 1.57
	Old	26.17 ± 1.9 [^]	26.34 ± 1.91 [^]	26.63 ± 2.07 [^]
RFM	Young	26.7 ± 1.58	26.94 ± 1.61	26.72 ± 1.60
	Old	36.64 ± 1.94 [^]	37.68 ± 1.85 ^{*^}	37.14 ± 1.90 [^]

Data are mean ± SEM. Arm lean mass, ALM; absolute fat mass, AFM; leg lean mass, LLM; trunk lean mass, TLM; relative fat mass, RLM; whole body lean mass, WBLM.

^{*} p<0.05 vs PRE for that age group,

[#] p<0.05 vs BED REST,

[^] P<0.05 vs young.

Table 3

Vastus Lateralis myofiber CSA in older and younger adults at pre and following bed rest and rehabilitation.

	Age	PRE	BED REST	REHAB
Mean	Young	3111 ± 160	3316 ± 181	3298 ± 268
	Old	2912 ± 369	2425 ± 213 ^{*^}	3445 ± 338 [#]
MHC I	Young	3026 ± 162	3226 ± 205	3191 ± 253
	Old	3142 ± 360	2573 ± 233	3634 ± 307 [#]
MHC II	Young	3209 ± 207	3366 ± 191	3468 ± 330
	Old	2715 ± 400	2308 ± 232 [^]	3181 ± 393 [#]

Data are mean ± SEM. Myosin heavy chain, MHC.

^{*}
p=0.07 vs PRE

[#]
p<0.05 vs BEDREST,

[^]
P<0.05 vs young.

Table 4

Baseline mRNA expression between old and young adults

	TNF α	IL-10	IL-1 β	NOS3	TGF β 1	COX-2	IL-4Ra	CD68
Young	1.11 $\pm$ 0.21	1.04 $\pm$ 0.14	1.77 $\pm$ 0.66	1.55 $\pm$ 0.36	1.39 $\pm$ 0.47	1.84 $\pm$ 0.59	1.14 $\pm$ 0.16	1.20 $\pm$ 0.20
Old	1.95 $\pm$ 0.56	3.05 $\pm$ 0.74	1.13 $\pm$ 0.37	2.53 $\pm$ 0.81	2.44 $\pm$ 0.55	1.54 $\pm$ 0.49	2.82 $\pm$ 0.74	2.60 $\pm$ 0.30
p-value	0.119	0.003	0.465	0.227	0.166	0.716	0.013	<0.001

Data are mean $\pm$ SEM.