Short-term bed rest increases TLR4 and IL-6 expression in skeletal muscle of older adults

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Short Title: bed rest and muscle inflammation
Abstract

Bed rest induces significant loss of leg lean mass in older adults. Systemic and tissue inflammation also accelerates skeletal muscle loss, but it is unknown whether inflammation is associated to inactivity-induced muscle atrophy in healthy older adults. We determined if short-term bed rest increases toll-like receptor 4 (TLR4) signaling and pro-inflammatory markers in older adult skeletal muscle biopsy samples. Six healthy, older adults underwent seven consecutive days of bed rest. Muscle biopsies (vastus lateralis) were taken after an overnight fast before and at the end of bed rest. Serum cytokine expression was measured before and during bed rest. TLR4 signaling and cytokine mRNAs associated with pro- and anti-inflammation and anabolism were measured in muscle biopsy samples using western blotting and qPCR. Participants lost ~4% leg lean mass with bed rest. We found that after bed rest, muscle levels of TLR4 protein expression and Interleukin-6 (IL-6), Nuclear factor kappa B1, Interleukin-10, and -15 mRNA expression were increased after bed rest (P<0.05). Additionally, the cytokines, interferon gamma and macrophage inflammatory protein-1 beta were elevated in serum samples following bed rest (P<0.05). We conclude that short-term bed rest in older adults modestly increased some pro- and anti-inflammatory cytokines in muscle samples while systemic changes in pro-inflammatory cytokines were mostly absent. Upregulation of TLR4 protein content suggests that bed rest in older adults increases the capacity to mount an exaggerated and perhaps unnecessary, inflammatory response in the presence of specific TLR4 ligands, e.g. during acute illness.
Introduction

Acute hospitalization due to illness or injury can have devastating consequences on the functional capacity and performance of activities of daily living in older adults (3, 25). Profound inactivity characterizes acute hospitalization in older adults and can significantly and independently contribute to muscle and functional loss (11, 12). Several studies have shown that short-term bed rest leads to rapid deterioration of muscle mass and strength of the lower extremity even in healthy older subjects (7, 24). However, the precise mechanisms are still unclear. A better understanding of the cellular mechanisms leading to muscle atrophy with bed rest will allow us to identify specific targets for treatment to preserve physical function and independence in hospitalized older adults.

Muscle loss following bed rest is largely a result of reduced post absorptive and feeding-induced muscle protein synthesis rates (7, 9, 24). Protein breakdown may also play a role in muscle loss especially in circumstances of muscle wasting such as in critical ill (5, 22) and septic (41) patients and during cast immobilization (4, 16, 23). Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6), have catabolic effects on skeletal muscle (1, 15, 17-19). Moreover, pro-inflammatory cytokines are commonly elevated in the circulation of critical ill patients (5, 22, 41), during prolonged bed rest (2), and with exercise deconditioning (47), all circumstances associated with rapid muscle loss. Conversely, increased levels of physical activity are associated with a reduction in circulating inflammatory levels (10, 20). Moreover, non-immune cells, such as skeletal muscle, also express pro-inflammatory cytokines (15, 27, 38, 43), suggesting that cytokines may also have local biological effects on the muscle tissue. In support of this notion, Haddad and et al. (19)
reported decreased myofibrillar protein content in rodent limb muscle following 14 d of local IL-6 infusion. This effect was independent of changes in circulating IL-6 levels.

The biological mechanisms regulating skeletal muscle tissue inflammation are complex, but may originate partly through the toll-like receptor 4 (TLR4). TLR4 is most noted for a critical role in innate immunity as a first line of defense against specific pathogens. TLR4 has a number of endogenous ligands, including heat-shock protein 60 (HSP60). Specifically, HSP60 binds TLR4 in monocytes of diabetic patients and under conditions of cellular stress (6, 28, 30, 35). Activated TLR4 in turn initiates a signaling cascade that operates through the inhibitor of κB (IκB)/nuclear factor κB (NFκB) pathway. Upon activation, NFκB increases the expression of several inflammatory cytokines including IL-6, TNFα, and interleukin-1β (29, 31). TLR4 can be found in various cell types, including human skeletal muscle (14, 42). This raises the possibility that TLR4 may be involved in initiating events associated with the stimulation of pro-inflammatory cytokine transcription within skeletal muscle.

The purpose of the current investigation was to characterize the mechanisms of bed rest-induced muscle wasting by examining the regulatory pathways of muscle inflammation through TLR4 signaling in the post-absorptive state. A secondary aim was to determine the effect of bed rest on muscle anti-inflammatory mediators (suppressor of cytokine signaling-3, Interleukin-10) (44, 50) and cytokines associated with anabolism (Interleukin-15) (34, 40). We hypothesized that a 7-day bed rest protocol, mimicking an acute hospitalization, would increase systemic and local levels of pro-inflammatory cytokines, including TLR4 signaling in skeletal muscle biopsy samples of older adults.
Further, we hypothesized that anti-inflammatory and anabolic cytokines would be reduced after short-term bed rest in skeletal muscle samples of these older adults.
Methods

Participants. Six healthy older adults (5 men, 1 woman; age 67±2 y; height 174±2 cm; weight 75±4 kg) were recruited from the University of Texas Medical Branch Pepper Center Volunteer Registry. Subjects were community-dwelling, overall healthy, independent older adults as determined by clinical history, Mini Mental State Exam, physical examination and laboratory tests. All subjects read and signed a written informed consent before participating in the study. The informed consent and protocol were approved by the Institutional Review Board of the University of Texas Medical Branch. Body composition and protein turnover data from this study have been previously published (7).

Experimental Design. Research participants underwent a 7-day bed rest experiment in the University of Texas Medical Branch Institute for Translational Sciences- Clinical Research Center. Bed rest was preceded by a 3-day in-hospital run in (maintaining the subject’s habitual diet and physical activity) and followed by a 3-day in-hospital rehabilitation period (7). Over the bed rest period, non-pharmacological deep venous thrombosis prevention was performed which consisted of intermittent lower leg compression devices, compression stockings, and daily passive range of motion by a physical therapist. Bathing and hygiene activities were performed in the bed with nursing help, while toilet privileges were limited to a bedside commode. Adherence to bed rest was continuously monitored via closed-circuit cameras and reinforced daily by nursing staff and study personnel. A DXA scan to measure body tissue composition was conducted before and after bed rest as reported previously (7). DXA scan measurements were carefully controlled between measurements which entailed
measurements taken at the same time of day (8AM) after an overnight fast, participants wearing the same clothing and controlling for fluid shifts with participants laying supine prior to scans.

After an inpatient stay and an overnight fast, percutaneous muscle biopsies were collected in the morning of the initiation of bed rest day 1 (pre-bed rest) and at the same time in the morning of bed rest day 7 (post-bed rest). Muscle biopsies were taken from the *vastus lateralis* muscle of one leg (pre-bed rest) and the opposite leg (post-bed rest) using aseptic technique, local anesthesia (1% lidocaine), and a 5 mm Bergström biopsy needle with suction. All muscle tissue was immediately blotted and dissected free of visible non-muscle tissue, flash-frozen in liquid nitrogen, and stored at -80°C for protein and mRNA analysis.

**Serum Cytokines.** Venous blood samples from participants were collected before bed rest, and on the 2^{rd}, 3^{rd}, 4^{th} and 7^{th} day of bed rest. Samples were collected at the same time in the morning, after an overnight fast. Blood samples were allowed to clot at room temperature for 20 min in a serum separator tube, which were then centrifuged. Supernatant serum was collected and stored at -80°C until analysis. Cytokines (IL-1β, -2, -4, -5, -6, -7, -8, -10, -12, -13, -17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β and TNFα) were measured in serum samples using a Bio-Plex Pro Human Cytokine 17-plex Assay (M50-00031YV; Bio-Rad, Hercules, CA) on a Bio-Plex 200 Instrument (Bio-Rad). For each analyte, a standard curve was generated using recombinant proteins to estimate protein concentration in the unknown sample (BioPlex Array Manager).
**SDS-PAGE & Immunoblotting.** Muscle tissue was homogenized using a glass pestle and pre-chilled tube in a buffer cocktail with protease and phosphatase inhibitors (50 mM Tris-HCL, 250 mM mannitol, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1mM benzamidine, 0.1mM phenylmethylsulfonyl fluoride, 5µg/ml soybean trypsin inhibitor). Whole muscle homogenates were centrifuged and the supernatant was collected. Total protein concentration for each sample was determined on a SmartSpec (Bio-Rad) using a colorimetric protein assay (Bio-Rad; Bradford) and an albumin standard curve. Whole muscle homogenates were diluted 1:1 in a 2X sample buffer. Homogenates (50µg of total protein) were loaded on a 7.5% and 15% polyacrylamide gel (Criterion; Bio-Rad), depending on the molecular weight of the protein, and subjected to SDS-PAGE (150V) for 1h in running buffer. Each gel contained alternating pre and post-bed rest samples loaded in duplicate, and a molecular weight ladder. An internal control (rodent muscle homogenate) was loaded in duplicate on each gel for band normalization and comparisons across blots. Protein was transferred (50V; 1h) to a polyvinylidene flouride membrane in transfer buffer then blocked for 1h at room temperature with 5% non-fat dry milk (NFDM) in Tris-buffered saline in 0.1% Tween-20 (TBST). Membranes were incubated overnight in primary antibody diluted in 5% NFDM or bovine serum albumin in TBST. The next morning, blots were rinsed in TBST for 5 minutes, rocked in secondary antibody for 1h at room temperature in 5% NFDM in TBST then serially washed (15 min, 3 x 5 min) in TBST. Chemiluminescence reagent (ECL Plus, GE Healthcare) was applied to each blot for 5 min. Optical density measurements were obtained with a digital imager (ChemiDoc XRS, Bio-Rad).
Membranes containing phospho-specific proteins were stripped (25mM glycine, pH 2.0 and 1% SDS) of primary and secondary antibodies then re-probed for the total protein of specific target. Densitometric analysis was performed using Quantity One 4.5.2 software (Bio-Rad). After subtracting out background, all western blot data were normalized to the internal control and replicate samples were averaged. Alpha-tubulin was used to verify equal loading across lanes.

**Antibodies.** The following antibodies were used in this experiment: Toll-like receptor 4 (Cat# sc-10741) from Santa Cruz Biotechnology (Santa Cruz, CA), while the following antibodies were purchased from Cell Signaling Technology (Boston, MA): heat shock protein 60 (Cat# 4870), phosphorylated IκBα (S32; Cat# 2859) and total IκBα (Cat# 9242), phosphorylated NFκB p65 (S536; Cat# 3033) and total NFκB p65 (Cat# 8242). Alpha tubulin was purchased from Sigma-Aldrich (Cat# F2168). Donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from GE Healthcare Amersham while a mouse secondary antibody was purchased from Santa Cruz Biotechnology.

**RNA extraction, cDNA synthesis and semi-quantitative qPCR.** Total RNA, cDNA synthesis and real-time qPCR were conducted as previously reported (8). Total RNA was isolated by homogenizing 15-20mg tissue with a hand-held homogenizing dispenser (T10 Basic Ultra Turrax, IKA, Wilmington, NC) in a solution containing 1ml of Tri reagent. The RNA was separated into an aqueous phase using 0.2ml of chloroform and precipitated from the aqueous phase using 0.5ml of isopropanol. Extracted RNA was washed with 1ml of 75% ethanol, dried, and then suspended in a known amount of nuclease-free water. RNA integrity for these samples (RIN: ~9) have been published.
previously (7). RNA was DNase-treated using a commercially available kit (DNA-free, Ambion, Austin, TX). Afterwards, 1µg of total RNA was reverse transcribed into cDNA (iScript, Bio-Rad). All isolated RNA and cDNA samples were stored at -80°C until analyzed with qPCR. Real-time qPCR was carried out with an iQ5 Multicolor Real Time PCR cycler (Bio-Rad). Taqman pre-designed primers were purchased from Applied Biosystems (Carlsbad, CA). The thermal cycling conditions were the following: a 10min initial denaturing step at 95°C followed by 40 cycles consisting of denaturation for 15s at 95°C and anneal and elongation for 1min at 60°C. Values were normalized to beta 2-microglobulin (since Ct values remained stable following bed rest) then fold change from pre-bed rest values was calculated (32).

Statistical analysis. A linear model (SAS v9.2 Cary, NC) with subject as a blocking factor was used to test differences across time (Day 2, 3, 4, 7) for serum cytokine samples. Cytokine measurements were corrected for multiple hypotheses testing using the Tukey-Kramer method. For ease of readership, actual means are reported in Table 1 but data was adjusted for statistical analysis. Correlations were determined between TLR4 and IL-6 and changes in leg lean mass following bed rest using a linear regression model. Analysis of baseline protein content and mRNA expression between pre- and post-bed rest was conducted using a paired t-test (SigmaPlot; Version 12.0). Significance was set at P<0.05. All values are presented as mean ± SE.
Results

Lean Mass. Following 7-days of bed rest, older adults lost ~1.6 kg of total body lean mass (PRE: 50.4±2.9; POST: 48.8±2.5 kg; P=0.03) while approximately 50% (0.8 kg) of this loss came from leg (right + left) lean mass (PRE: 18.3±1.1; POST: 17.5±1.0 kg; P=0.01).

Serum cytokines (Table 1). We found no changes across time in 14 out of the 17 cytokines. IFN-γ (P=0.05) and MIP-1β (P=0.02) increased over time. Serum IL-2 was undetectable throughout the experimental period and IL-17, G-CSF, and GM-CSF were detectable only in n=4. All detectable serum cytokines were within range of the standard curve.

Skeletal muscle toll-like receptor 4 and NFκB signaling. We found that the 7-day bed rest increased the abundance of TLR4 in skeletal muscle by ~70% compared to pre-bed rest values (Figure 1A; P=0.03). However, HSP60, a ligand for TLR4, was unaltered following bed rest (Figure 1B; P=0.12). To evaluate the effect of increased TLR4 protein expression, we measured NFκB signaling. We found no differences when NFκB phosphorylation (S536) was expressed relative to total NFκB p65 protein abundance (Figure 2A; P=0.37) following 7-days of bed rest in skeletal muscle of healthy older subjects. Similarly, bed rest did not affect the phosphorylation of NFκB at S536 (PRE: 3.53±0.58; POST: 2.77±0.31 AU; P=0.26) or NFκB p65 protein levels (PRE: 1.18±0.21; POST: 1.27±0.25 AU; P=0.42). Further, there were no changes in IκBα phosphorylation (S32) relative to its total protein levels (Figure 2B; P=0.50) or the
independent phosphorylation of IκBα at S32 (PRE: 0.99±0.26; POST: 1.03±0.25 AU; 
P=0.50) and total IκBα protein levels (PRE: 1.78±0.77; POST: 2.14±0.93 AU; P=0.13).

*Pro-inflammatory mRNA expression.* We found that NFκB1 mRNA expression in 
skeletal muscle biopsy samples was increased ~40% compared to pre-bed rest values 
(Figure 3A, P=0.03) but independent of changes in total protein (Figure 3B). 
Additionally, IL-6 mRNA expression increased ~130% after 7-days of bed rest in older 
adult skeletal muscle (Figure 3B; P=0.04). Bed rest did not affect TNFα(Figure 3C; 
P=0.64), TNFR1SA1 (Figure 3D; P=0.42), or IL1β mRNA expression (Figure 3E; 
P=0.89). CD45 mRNA expression, a cell surface marker of immune cells, did not 
change as a result of bed rest (PRE: 1.11±0.20; POST: 1.29±0.19 fold change; P= 
0.56).

*Cytokines associated with anti-inflammation and anabolism.* There were no 
differences in SOCS3 mRNA expression after bed rest in older adult skeletal muscle 
samples (Figure 4A; P=0.68). However, we found that IL-10 (Figure 4B; P=0.03) and 
IL-15 (Figure 4C; P=0.02) mRNA expression increased as a result of bed rest.

*Relationship between bed rest-induced changes in leg lean mass and TLR4 and 
IL-6 expression.* There was not a statistically significant correlation between changes in 
TLR4 protein and IL-6 mRNA expression and leg lean mass other than a tendency: 
TLR4 protein expression and leg (right + left) lean mass (Figure 5A; R²= 0.31, P=0.25) 
and IL-6 mRNA expression and leg lean mass (Figure 5B; R²= 0.42, P=0.16).
Discussion

The primary finding of this study was that 7-days of controlled bed rest, mimicking the inactivity of an acute hospital stay, markedly increased TLR4 protein abundance and IL-6 mRNA expression in the skeletal muscle biopsy samples of healthy older adults. Conversely, we did not find significant changes in muscle NFκB signaling while changes in circulating inflammatory levels were modest and mostly absent. We also found that some, but not all, pro-inflammatory and anabolic cytokine mRNA expression in muscle biopsy samples was increased after bed rest. These data provide preliminary evidence that short-term physical inactivity can locally induce a small, but likely important, pro-inflammatory response in skeletal muscle biopsy samples of healthy older adults in the absence of an obvious increase in systemic inflammation. The elevation in TLR4 with bed rest may suggest an increased muscle capacity to mount a rapid and possibly uncontrolled inflammatory response in the presence of TLR4 ligands, such as during an acute illness or infection), in older adults.

This study is the first demonstrating an elevation in TLR4 expression in skeletal muscle biopsy samples following 7-days of controlled bed rest in healthy older subjects. Previous work has shown that increased physical activity and exercise training can reduce TLR4 mRNA in skeletal muscle of healthy (51) and diabetic rats (36) and in frail obese older adults (26). When interpreted in light of those results, our novel data support the notion that TLR4 expression is highly sensitive and inversely correlated to the level physical activity in human skeletal muscle.

A signature response of increased TLR4 activity is an upregulation of pro-inflammatory cytokines including TNFα, IL-6, and IL-1β (27). We found that bed rest
increased the skeletal muscle levels of IL-6 mRNA but TNFα, the mRNA associated with TNFα receptor (TNFR1SA1), and IL-1β mRNA were unchanged. Our findings are consistent with those reported by Reyna and colleagues (42) who found elevated skeletal muscle levels of TLR4 mRNA and protein and IL-6 mRNA in obese adults with type 2 diabetes as compared to lean non-diabetic controls. Other human models of muscle wasting (critically ill) have also been associated with elevated muscle IL-6 mRNA levels whereas changes in TNFα have been inconsistent (5, 22). To the best of our knowledge, until now no one had examined muscle tissue levels of inflammatory cytokines as a result of controlled bed rest. While we cannot exclude that macrophages and other immune cells could have contributed to the increased inflammatory state as noted by detectable levels of CD-45 mRNA levels in our muscle homogenate samples, the finding that 15 of the 17 serum cytokines were unaltered following bed rest strongly suggests the lack of a systemic pro-inflammatory response with bed rest. In addition, even a systemic pro-inflammatory condition, such as type 2 diabetes in obese subjects, is not necessarily associated with immune cell infiltration of the skeletal muscle tissue, as reported by Reyna et al. (42). Thus, the likelihood that our findings were confounded by activated immune cells migrating into the muscle tissue is low. Regardless, an increase in cytokine secreting immune cells in skeletal muscle would have a significant impact on muscle metabolism and homeostasis. Collectively, these data support that as little as 7-days of physical inactivity in healthy older adults increases some markers of local inflammation, including TLR4 protein and IL-6 mRNA expression.

We did not detect an increase in the TLR4 ligand, HSP60, within skeletal muscle homogenate samples after bed rest other than a trend (P=0.12) suggesting that HSP60
may not be involved during the early stages of bed rest. More importantly, other than a subtle increase in NFκB1 mRNA, we did not find any significant changes in downstream TLR4 signaling (IkBα/NFκB) in skeletal muscle biopsy samples after bed rest. It is possible that, due to the limited amount of human muscle sample available, the use of whole muscle homogenates instead of isolated nuclear and cellular fractions might have reduced our sensitivity to detect changes. Alternatively, TLR4 signaling may occur through another pathway (i.e., p38, JNK) (13), or increased expression of IL-6 mRNA may be mediated by an alternate (37) or unknown mechanism independent of TLR4 and NFκB. A more likely explanation is that bed rest, in the absence of disease, increased TLR4 protein levels without activating NFκB because of lack of TLR4 ligands (i.e., HSP60). If this is the case, then inactivity per se in older adults increases the capacity to mount an elevated, yet abnormal, muscle inflammatory response to acute noxious stimuli (e.g. an infection or a cardiovascular event requiring hospitalization). At best, changes in TLR4 and IL-6 showed a tendency to be related to muscle loss following bed rest but interpretation must be taken with caution with a small sample size and making correlations with DXA measurements. Perhaps, with longer periods of bed rest-induced inactivity, an over sensitization to infection (i.e., TLR4) may lead to a much larger and potentially catastrophic loss of skeletal muscle mass.

We also found that skeletal muscle IL-10 mRNA expression was upregulated with bed rest in older adults, while there were no observed changes in SOCS3 mRNA. IL-10 is well known for its function to suppress inflammatory immune responses by targeting the transcription of pro-inflammatory mediators (33). Since IL-10 mRNA was elevated with bed rest, it is possible that this might be a mechanism to counteract a pro-
inflammatory response (21). In fact, IL-6 is known to stimulate IL-10 production, thereby providing a physiological brake on the overall pro-inflammatory status (45). We also found that the anabolic cytokine IL-15 mRNA (34, 40) was increased after bed rest as previously reported in animals (39). Taken together, the increased expression of local anti-inflammatory and anabolic cytokines following acute bed rest in older adults may be a mechanism to counter heightened levels of IL-6.

An interesting observation was that bed rest largely did not affect serum inflammatory markers even in the early period of bed rest (Day 2-4). In a previous report, 14-days of bed rest in young volunteers resulted in increased circulating IL-6 levels (2). Perhaps we did not observe changes in serum inflammatory cytokines because cellular sources of inflammatory mediators require a longer period of bed rest to be distributed into the circulation. Although a majority of circulating cytokines were unaltered during (and after) 7-days of bed rest even with a comprehensive cytokine profiling, we did find elevated serum levels of MIP-1β and a tendency for IFN-γ to be increased. An increase in MIP-1β may represent an immune response to regenerate new tissue during bed rest (46, 49) while elevated IFN-γ is consistent with an increased pro-inflammatory response as observed following muscle injury in old rodents (48).

We conclude that short-term bed rest in healthy older adults increased some, but not all, local pro-inflammatory mediators, namely TLR4 protein and IL-6 mRNA expression, as well as anti-inflammatory (IL-10) and anabolic (IL-15) cytokine mRNA expression. These changes were largely independent of alterations in local NFκB signaling and serum markers of inflammation. Increased expression of TLR4 may be an important predisposing factor for catastrophic inflammatory and muscle catabolic
responses triggered by a concomitant acute disease and bed rest inactivity in older adults (e.g. inactivity during hospitalization for an acute illness).

Acknowledgements

We would like to thank the research volunteers for their time and effort completing this study. Additionally, we are grateful to the nursing staff of the Institute for Translational Sciences Clinical Research Center and the Claude D. Pepper Older Americans Independence Center Volunteer Registry study coordinators. We also would like to thank Junfang Hao for technical assistance.

This study was supported by National Institutes of Health Grants: UL1 TR000071, R01 AG018311, P30 AG024832, R01 AR04987, K01 AG038556.
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36. **Oliveira AG, Carvalho BM, Tobar N, Ropelle ER, Pauli JR, Bagarolli RA, Guadagnini D, Carvalheira JB, and Saad MJ.** Physical exercise reduces circulating
lipopolysaccharide and TLR4 activation and improves insulin signaling in tissues of DIO rats. *Diabetes* 60: 784-796, 2011.


Figure Legends

**Figure 1. Effect of bed rest on TLR4 and HSP60 protein expression.** Data represent protein expression for: A) TLR4 and B) HSP60 before (light grey) and after (dark grey) 7-days of bed rest in *vastus lateralis* skeletal muscle biopsy samples of healthy older adults (n=6). Insets are representative immunoblot images. Equal protein loading was confirmed with α-tubulin. Line plots within figures represent individual means for males (M1-5) and female (F1). P-values are indicated on figures. Values are in arbitrary units and presented as mean ± SE.

**Figure 2. Effect of bed rest on NFκB signaling.** Data represent: A) NFκB phosphorylation (S536) relative to NFκB total protein expression and B) IκBα phosphorylation (S32) relative to IκBα total protein expression before (light grey) and after (dark grey) 7-days of bed rest in *vastus lateralis* skeletal muscle of healthy older adults (n=6). Insets are representative immunoblot images. Line plots below figures represent individual means for males (M1-5) and female (F1). P-values are indicated on figures. Values are presented as mean ± SE.

**Figure 3. Effect of bed rest on pro-inflammatory muscle mRNA markers.** Data represent: A) NFκB1, B) IL-6, C) TNFα, D) TNFR1SA1, and E) IL-1β mRNA expression before (light grey) and after (dark grey) 7-days of bed rest in *vastus lateralis* skeletal muscle of healthy older adults (n=6). Line plots below figures represent individual means for males (M1-5) and female (F1). Values are reported as fold change from baseline. P-values are indicated on figures. Values are presented as mean ± SE.
Figure 4. Effect of bed rest on the mRNA expression of anti-inflammatory and anabolic cytokines. Data represent: A) SOCS3, B) IL-10, and C) IL-15 mRNA expression before (light grey) and after (dark grey) 7-days of bed rest in vastus lateralis skeletal muscle of healthy older adults (n=6). Line plots below figures represent individual means for males (M1-5) and female (F1). Values are reported as fold change from baseline. P-values are indicated on figures. Values are presented as mean ± SE.

Figure 5. Relationship between the changes in leg lean mass and TLR4 and IL-6 expression. Data represent the delta change (pre – post bed rest) correlation for the following dependent variables in skeletal muscle of healthy older adults (n=6): A) TLR4 protein expression vs leg (right + left) lean mass loss and B) IL-6 mRNA expression vs leg (right + left) lean mass loss.
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<td>IFN-γ</td>
<td>58.4 ± 10.0</td>
<td>60.4 ± 4.6</td>
<td>76.2 ± 18.2</td>
<td>61.8 ± 12.2</td>
<td>72.9 ± 16.7</td>
<td>0.05</td>
</tr>
<tr>
<td>MCP-1</td>
<td>19.7 ± 3.9</td>
<td>17.2 ± 4.3</td>
<td>17.9 ± 4.7</td>
<td>16.4 ± 3.1</td>
<td>21.9 ± 5.9</td>
<td>0.50</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>49.5 ± 25.2</td>
<td>52.1 ± 24.8</td>
<td>51.8 ± 23.1</td>
<td>52.9 ± 24.0</td>
<td>62.0 ± 30.2</td>
<td>0.02</td>
</tr>
<tr>
<td>TNFα</td>
<td>14.0 ± 3.5</td>
<td>15.8 ± 2.7</td>
<td>18.1 ± 4.4</td>
<td>15.1 ± 3.0</td>
<td>15.1 ± 2.0</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Values are pg/ml and are mean ± SE. P-value signifies main effect for time (days of bed rest). Values for IL-17, G-CSF, and GM-CSF are n=4. IL-1β: Interleukin-1 beta; IL-4: Interleukin-4; IL-5: Interleukin-5; IL-6: Interleukin-6; IL-7: Interleukin-7; IL-8: Interleukin-8; IL-10: Interleukin-10; IL-12: Interleukin-12; IL-13: Interleukin-13; IL-17: Interleukin-17; G-CSF: Granulocyte-colony stimulating factor; GM-CSF: Granulocyte macrophage-colony stimulating factor; IFN-γ: Interferon-gamma; MCP-1: monocyte chemoattractant protein-1; MIP-1β: Macrophage inflammatory protein-1 beta; TNFα: Tumor necrosis factor alpha.
Figure 1. Effect of bed rest on TLR4 and HSP60 protein expression

A) TLR4 Protein Expression (Arbitrary Units)
P = 0.03

B) HSP60 Protein Expression (Arbitrary Units)
P = 0.12

- Pre-Bed Rest
- Post-Bed Rest

TLR4

HSP60

α-tubulin
Figure 2. Effect of bed rest on NFkB signaling

A) NFkB p65 (S536) and NFkB p65

B) IkBα (S32) and IkBα

Pre-Bed Rest
Post-Bed Rest

P=0.37

P=0.50
Figure 3. Effect of bed rest on pro-inflammatory muscle mRNA markers

A) NFκB1 mRNA

B) IL-6 mRNA

C) TNFα mRNA

D) TNFR1SA1 mRNA

E) IL-1β mRNA

Pre-Bed Rest
Post-Bed Rest
Figure 4. Effect of bed rest on the mRNA expression of anti-inflammatory and anabolic cytokines.

A) SOCS3 mRNA

B) IL-10 mRNA

C) IL-15 mRNA

Effect of bed rest on the mRNA expression of anti-inflammatory and anabolic cytokines.
Figure 5. Relationship between the change in leg lean mass and TLR4 and IL-6 expression

A) Loss in Leg Lean Mass (kg, Pre-Post) vs. Change in TLR4 protein (AU, Pre - Post)

- $R^2 = 0.30$
- $P = 0.25$

B) Loss in Leg Lean Mass (kg, Pre-Post) vs. Change in IL-6 mRNA (Fold Change, Pre - Post)

- $R^2 = 0.42$
- $P = 0.16$