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Overload-Induced Skeletal Muscle Extracellular Matrix Remodeling And Myofiber Growth in Mice Lacking IL-6

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Abstract

Aim—Overloading healthy skeletal muscle produces myofiber hypertrophy and extracellular matrix remodeling, and these processes are thought to be interdependent for producing muscle growth. Inflammatory cytokine interleukin-6 (IL-6) gene expression is induced in overloaded skeletal muscle, and the loss of this IL-6 induction can attenuate the hypertrophic response to overload. Although the overload induction of IL-6 in skeletal muscle may be an important regulator of inflammatory processes and satellite cell proliferation, less is known about its role in the regulation of extracellular matrix remodeling. The purpose of the current study was to examine if overload-induced extracellular matrix remodeling, muscle growth, and associated gene expression were altered in mice that lack IL-6, when compared to wild-type mice.

Methods—Male C57/BL6 (WT) and C57/BL6 × IL-6^{-/-} (IL-6^{-/-}) mice (10 wks of age) were assigned to either a sham control or synergist ablation overload (OV) treatments for 3 or 21 days.

Results—Plantaris muscle mass increased 59% in WT and 116% in IL-6^{-/-} mice after 21d OV. Myofiber CSA was also increased by 21d OV in both WT and IL-6^{-/-} mice. Overload induced a 2-fold greater increase in the volume of non-contractile tissue in IL-6^{-/-} muscle as compared to WT. Overload also induced a significantly greater accumulation of hydroxyproline and procollagen-1 mRNA in IL-6^{-/-} muscle, when compared to WT muscle after 21d OV. TGF-β and IGF-1 mRNA expression were also induced to a greater extent in IL-6^{-/-} muscle when compared to WT muscle after 21d OV. There was no effect of IL-6 loss on the induction of myogenin, and cyclin D1 mRNA expression after 3d OV. However, MyoD mRNA expression in 3d OV IL-6^{-/-} muscle was attenuated when compared to WT overload mice.

Conclusion—IL-6 appears to be necessary for the normal regulation of extracellular matrix remodeling during overload-induced growth.

Keywords

functional overload; extracellular matrix; inflammation; transforming growth factor beta

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Introduction

Overloading skeletal muscle induces a rapid growth response that includes the induction of skeletal muscle mass, myofiber cross-sectional area, protein synthesis, and collagen synthesis (1,6,7,28,29). Many genes activated at the onset of overload are involved in the regulation of cell cycle progression, cell differentiation, extracellular matrix remodeling, and the inflammatory response (10). The increased expression of myogenic regulatory factors, cell cycle regulator cyclin D1, and growth factor IGF-1 coincide with the initiation of overload-induced muscle growth (1,35). Satellite cells, fibroblast cells, and infiltrating immune cells all play important roles in the muscle growth process (11,44). These various cells are both targets of growth factor and cytokine action, as well as, the site of synthesis of important hypertrophic signaling molecules. The sequential coordination of immune, fibroblast, and satellite cell activities in relation to the overloaded myofiber is likely critical for optimal muscle growth.

Cytokines are low molecular weight signaling proteins that have a variety of functions such as B- and T-cell differentiation, but they can also mediate the inflammatory response through stimulation of acute-phase proteins (17). Acute inflammation is a critical component for successful muscle regeneration to occur (11,44). Although interleukin-6 (IL-6) is classified as a pro-inflammatory cytokine, it has a variety of biological functions. IL-6 can also act in an anti-inflammatory manner to inhibit the inflammatory response (51). Chronically elevated circulating IL-6 is associated with heart failure (21), cancer (36), insulin insensitivity, and obesity (24). Chronic exposure to IL-6 reduces hepatic insulin sensitivity in mice (26), and can reduce autophosphorylation of the insulin receptor as well as tyrosine phosphorylation of insulin substrates-1 and -2 (26). Chronically elevated IL-6 is associated with muscle wasting in mice with intestinal tumors (3,4). Direct administration of IL-6 to skeletal muscle induces muscle catabolism (16), and impaired muscle growth in young growing rats (5). Transgenic mice over-expressing IL-6 also exhibit skeletal muscle atrophy (45), which can be reversed with administration of an IL-6 receptor antibody (46). In addition, the knockout of IL-6 inhibits muscle wasting in mice with intestinal and colon tumors (3). However, the actions of systemic inflammation induced circulating IL-6 may be distinct from the tissue level autocrine/paracrine regulation initiated by IL-6 for the regulation of remodeling after injury.

Skeletal muscle is a major source of IL-6 production during physiological and pathophysiological stress (14). Functional overload induces skeletal muscle IL-6 gene expression during the initial hypertrophic process (10,43). Muscle contraction also induces IL-6 gene expression shortly following a bout of exercise (23). Investigation of IL-6 function in many tissues and disease states has been analyzed through the use of homozygote knockout of IL-6, which are able to generate pups that develop normally (27). However, following injury IL-6 knockout mice do not increase gene expression of liver haptoglobin, alpha-1 acid glycoprotein, and serum amyloid A, and represents an impaired acute-phase immune response (27). IL-6 knockout mice have been shown to develop type 2 diabetes after 9 months of age, and corroborates the importance of IL-6 in glucose homeostasis (49).

The effect of functional overload induced muscle hypertrophy has also been examined in IL-6 and Leukemia inhibitory factor (LIF) knockout mice (38,39). The knockout of LIF, a member of the IL-6 family of cytokines, inhibited overload-induced growth of the fast-type plantaris muscle (39), and this response was related to decreased satellite cell proliferation (40). IL-6 knockout mice also demonstrate an attenuation of overload-induced muscle growth, which was thought to be related to inhibition STAT3 mediated satellite cell activity (38,39). The effect of IL-6 loss on functional overload-induced muscle extracellular matrix

remodeling is not well understood. The purpose of the current study was to examine if overload-induced extracellular matrix remodeling, myofiber growth, and associated gene expression were altered in mice lacking IL-6, when compared to wild-type mice. We hypothesized that the loss of IL-6 during overload would induce extracellular matrix remodeling and coincide with decreased ability of myofibers to hypertrophy. To test this hypothesis, plantaris muscles from wild-type and IL-6 null mice were examined after 3 and 21 days of functional overload. An additional set of IL-6 null mice were examined after 56 days of functional overload.

Methods

Animals

Approximately 10 week old male C57/BL6 (WT) and C57 $\times$ IL-6^{-/-} (IL-6^{-/-}) mice were acquired from the animal resources facility at the University of South Carolina as previously described (3,4). Original IL-6^{-/-} mating pairs were purchased from Jackson Laboratories (Bar Harbor, Maine). IL-6^{-/-} mice were compared to their WT littermates. Animals were housed individually, kept on a 12:12-h light-dark cycle, and given ad libitum access to normal rodent chow and water for the duration of the study at the fully accredited animal care facilities at the University of South Carolina, Columbia. Mice were randomly assigned to either a sham-control group or a functional overload group. All procedures were approved by the University of South Carolina Animal Care and Use Committee.

Skeletal Muscle Overload

The hindlimb plantaris muscles were functionally overloaded for 3, 21 or 56 days by a modified bilateral surgical ablation method as previously described (50). Body weights were obtained on the day of the sacrifice. Briefly, mice were anesthetized with an intramuscular injection of ketamine hydrochloride (45mg/kg body weight), xylazine (7mg/kg body weight), and acepromazine (1mg/kg body weight). In a sterile environment, the dorsal surface of the hindlimb was shaved and cleaned, and the gastrocnemius muscles was exposed by a posterior longitudinal incision through the skin and bicep femoris muscle of each lower limb and the distal third of each gastrocnemius head was excised. The mice that were assigned to the sham-control group received a sham surgery that consists of the same surgical procedure as the OV group, except the gastrocnemius tendon was not cut.

Muscle extraction

Mice were anesthetized with an intramuscular injection of ketamine hydrochloride (90mg/kg body weight), xylazine (7mg/kg body weight), and acepromazine (1mg/kg body weight). The plantaris muscles from overloaded mice were excised 3, 21 or 56 days after surgery. One side was snap frozen in liquid nitrogen and stored at -80°C for gene expression analysis and the other was mounted in OCT for morphological analysis.

Total protein and hydroxyproline analysis

Total muscle protein was quantified as previously described (30). Approximately 10 mg of plantaris muscle was hydrolyzed in 6N hydrochloric acid (90 μ L per 1mg of tissue) for 17 hr at 105°C. Samples were cooled on ice and assayed for total protein and hydroxyproline concentration as described by (42) with the following modifications. Sample volumes were modified to be run in 400 μ L vol/well microplates. Briefly, standards and samples were diluted 1:5 and 50 μ L of each were incubated with 100 μ L Buffer A [350 mg% Cholramine-T; 10% (v/v) n-Propanol; 80% (v/v) Hydroxyproline Buffer] for 20 min. 100 μ L of Buffer B [15% (w/v) 4-dimethylaminobenzaldehyde; 71.4% (v/v) n-Propanol; 26% (v/v) 60% HClO₄] were added and incubated at 65°C for 25 min. Hydroxyproline assay buffer

consisted of 3.1% (w/v) Citric acid; 0.75% (v/v) Glacial acetic acid; 4.5% (w/v) Sodium Acetate; 2.1% (w/v) sodium hydroxide; 18.75% (v/v) n-Propanol; 0.1% (v/v) toluene at pH 6.0. Microplates were cooled to room temperature and read at 570nm (Tecan, Crailsheim, Germany). A standard curve was generated by simple linear regression using known amounts of hydroxyproline. Samples were quantified for hydroxyproline from the standard curve and corrected for the amount of tissue hydrolyzed.

Total RNA isolation and cDNA synthesis

RNA was extracted with Trizol reagent (Life Technologies, Grand Island, NY) as previously described (50). Briefly, plantaris muscles were homogenized in Trizol. Total RNA was isolated, DNase treated, and its concentration and purity was determined by UV spectrophotometry. RNA with a 260/280 ratio of ≥ 1.6 was used for cDNA synthesis. cDNA was reverse transcribed from 3 μ g of total RNA with 1 μ l of random hexamers and 50 units of Superscript III RT (Invitrogen) in a final volume of 20 μ l at 25°C for 10 min, followed by 42°C for 50 min, and 70°C for 10 min.

Real Time Polymerase Chain Reaction

Real-time PCR was performed, and results were analyzed using the ABI 7300 thermocycler Real-Time detection system (Sequence Detection Systems, model 7300; Applied Biosystems, Foster City, CA, USA) as previously described (34). cDNA was amplified in a 25 μ l reaction that contained appropriate primer pairs and ABI SYBR Green or TaqMan Universal Mastermix (Applied Biosystems, Foster City, CA, USA). Samples were incubated at 95°C for 4 min, followed by 40 cycles of denaturation, annealing, and extension at 95°C, 55°C, and 72°C, respectively. SYBR Green or TaqMan fluorescence was measured at the end of the extension step following each cycle. Fluorescence labeled probes for procollagen-1 (COL1A1, FAM dye), MyoD (FAM dye), myogenin (FAM dye), cyclin D1 (FAM dye), TGF- β (TGF- β 1, FAM dye) and the ribosomal RNA 18s (VIC dye) were purchased from Applied Biosystems and quantified with TaqMan Universal mastermix. Insulin like growth factor-1 (IGF-1) primer sequence was synthesized by Integrated DNA Technologies (IDT) (Carlsville, IA, USA) and quantified with SYBR Green mastermix. The primer sequences for IGF-1 were: forward, 5'-TGGATGCTCTTCAGTTCGTG-3'; reverse, 5'-GTCTTGGGCATGTCAGTGTG-3'. Cycle threshold (Ct) was determined, and the Δ Ct value calculated as the difference between the Ct value and the 18s Ct value. Final quantification of gene expression was calculated using the $\Delta\Delta$ CT method {Ct = [Δ Ct(calibrator) - Δ Ct(sample)]}. Relative quantification was then calculated as $2^{-\Delta\Delta$ Ct}. Melt curve analysis was performed following SYBR Green PCR analysis to verify no primer dimers were formed.

Morphological Analysis

Cross-sectional analysis and percentage of non-contractile tissue was determined as previously described (34). For cross-sectional area analysis approximately 8-distinct digital images from H&E stained muscle sections (10 μ m) from the mid-belly of the plantaris at a 40 $\times$ objective magnification were taken and analyzed for fiber cross-sectional area using NIH imaging software (Image J). Each fiber was traced with a handheld mouse and the number of pixels traced was calibrated to a defined area in μ m². The researcher was blinded and approximately 150 fibers were traced per sample. All fibers in the cross-section images were quantified unless the sarcolemma was not intact. For non-contractile tissue percentages (%NCT), approximately 8 digital images of H&E stained sections of plantaris muscle were analyzed. An 18 $\times$ 14 grid overlaid the digital images. Each dot was counted if it was not on a muscle fiber by a blinded investigator. Dots at least 75% in the extracellular matrix were counted. Dots that were not clearly distinguishable were omitted from the count.

Statistical Analysis

Data is reported as means $\pm$ SE. At 3 days of overload, muscle weights and gene expression were analyzed using a two-way ANOVA. At 21 days of overload, body weights, muscle weights, total protein, mean cross sectional area, volume of non-contractile tissue, hydroxyproline concentration and gene expression were also analyzed using a two-way ANOVA. At both time points, post hoc analysis was conducted using Student-Newman-Keuls method. Chi-square analysis was used to determine shifts in plantaris fiber frequency distribution at 21 days of overload. At 56 days of overload, body weight and muscle weight, and total protein values were analyzed using independent t-tests. Significance was set at $p \leq 0.05$.

Results

Muscle growth

Since a previous study had demonstrated an attenuation of myofiber hypertrophy in IL-6 null animals after synergist ablation several variables were examined related to overload-induced muscle growth (38). Muscle wet weight was not reported in the previous study of overloading in IL-6 null mice (38). The main effect of functional overload increased plantaris muscle wet weight after 3 days of overload by 26% and 13% in wild-type and IL-6^{-/-} mice, respectively. After 21 days of overload, IL-6^{-/-} mice had a significantly greater increase in muscle wet weight compared to wild-type mice (Table 1). Wild-type plantaris muscle wet weight significantly increased 59% after 21-days of overload, while muscle weight increased 116% in IL-6^{-/-} mice. There was a main effect of 21 days of overload for increasing plantaris total protein across both wild type and IL-6^{-/-} mice ($p < 0.001$, Table 1) when compared to the sham mice. An additional group of IL-6^{-/-} mice were subjected to 56 days of synergist ablation-induced overload (n=5 OV; n=5 sham) and plantaris wet weight increased 80% compared to sham mice (Table 1). Total protein content was increased 68% in IL-6^{-/-} mice after 56 days of overload.

Myofiber cross-sectional area was measured in histological cross-sections of plantaris muscle taken at the mid-belly of the muscle after 21 days of overload (Figure 1). There was a main effect of overload, which resulted in an increased mean cross-sectional area in both wild-type and IL-6^{-/-} plantaris muscle. Myofiber mean cross-sectional area from wild-type mice increased 27% ($1,484 \pm 61 \mu\text{m}^2$ vs. $1,882 \pm 75 \mu\text{m}^2$). The overloaded muscle from IL-6^{-/-} mice demonstrated a similar increase (27%) in mean myofiber cross sectional area ($1,548 \pm 82 \mu\text{m}^2$ vs. $1,965 \pm 126 \mu\text{m}^2$) following 21 days of functional overload. Both wild-type and IL-6^{-/-} animals had similar shifts in fiber size distribution after 21 days of functional overload (Figure 1A & B). In wild-type mice small diameter myofibers ($<800 \mu\text{m}^2$, Figure 1A) decreased 63% after 21 days of overload (19% sham vs. 7% OV), while large diameter fibers ($>2400 \mu\text{m}^2$) increased 41% with overload (19% sham vs. 30% OV). IL-6^{-/-} mice demonstrated a 44% decrease in small diameter fibers ($<800 \mu\text{m}^2$, Figure 1B) after 21 days of overload (16% sham vs. 9% OV), and a 71% increase in large diameter fibers ($>2400 \mu\text{m}^2$) with overload (21% sham vs. 36% OV). When the myofiber size distributions of overloaded WT and overloaded IL-6^{-/-} mice were directly compared, IL-6^{-/-} muscle demonstrated a larger percentage of large diameter fibers (30% WT vs. 36% IL-6^{-/-}; data not shown), while there were no differences in the distribution of small diameter fibers.

Non-contractile tissue

Because muscle from IL-6^{-/-} mice demonstrated a larger increase in muscle wet weight, but only a similar increase in mean myofiber cross sectional area following 21 days of functional overload, we evaluated the volume percent of non-contractile tissue in overloaded muscle by examination of histological cross-sections of plantaris muscle taken at the mid-

belly (Figure 2A & 2B). Twenty-one days of functional overload induced a significant increase in the volume of non-contractile tissue in wild-type animals (Figure 2B). The IL-6^{-/-} mice exhibited an approximate 2.5-fold increase in the volume percent of non-contractile tissue, which was significantly greater than the wild-type mice. Hydroxyproline concentration in muscle is a quantitative indicator of collagen content and fibrosis (48). Wild-type mice overloaded for 21 days increased muscle hydroxyproline concentration approximately 250% ($p < 0.001$; Figure 2C), when compared to sham mice. Overload increased IL-6^{-/-} muscle hydroxyproline concentration approximately 6-fold when compared to their sham group. The concentration of hydroxyproline was significantly greater in overload IL-6^{-/-} muscle ($p < 0.001$), when compared to overloaded wild type mice.

Procollagen-1, TGF- β and IGF-1 gene expression

Gene expression related to extracellular matrix remodeling and muscle growth was examined in 21 day overloaded muscle (Figure 3). There was a significant interaction between mouse genotype and overload for muscle procollagen-1, TGF- β , and IGF-1 mRNA expression at 21 days. Although overload induced a 6-fold increase in WT muscle procollagen-1 mRNA expression ($p = 0.01$), there was a significantly larger 14-fold induction ($p = 0.001$) in IL-6^{-/-} muscle (Figure 3A). Overload induced a 4-fold increase in WT muscle IGF-I mRNA concentration ($p = 0.006$) after 21 days of overload, and a significantly larger 7-fold induction ($p = 0.001$) in IL-6^{-/-} muscle (Figure 3B). TGF- β mRNA expression was induced by overload approximately 2-fold in wild-type plantaris, and a significantly larger 3.6-fold in IL-6^{-/-} muscle (Figure 3C).

Cell Cycle regulator gene expression

Gene expression related to general and myogenic cell cycle regulation was only quantified at 3 days of overload (Figure 4). Muscle cyclin D1 ($p < 0.001$; Figure 4A) and myogenin ($p < 0.001$; Figure 4C) mRNA expression were significantly induced by overload, regardless of genotype. Muscle MyoD mRNA expression was significantly increased by 3 days of overload (Figure 4B) in both WT (350%) and IL-6^{-/-} (80%) mice. However, overloaded muscle from IL-6^{-/-} overload mice had a significantly attenuated induction of MyoD mRNA when compared to wild-type muscle ($p = 0.001$). Muscle IL-6 mRNA expression (Figure 4D) was increased approximately 3.5-fold after 3 days of overload in wild-type muscle, while IL-6 mRNA expression was not detectable in sham or overloaded muscles of IL-6^{-/-} mice.

Discussion

The results from the current study extend our understanding of the physiological importance of IL-6 expression for overload-induced growth in mouse plantaris muscle. To the best of our knowledge, this is the first study to demonstrate that mice lacking IL-6 have accentuated extracellular matrix remodeling after synergist ablation-induced overload. In the absence of IL-6, myofiber growth could not account for the accretion of muscle mass over 21-days of overload, and our results indicate that an increase in fibrosis is a major contributor to overall muscle mass. Coinciding with the histological evidence for an increase in non-contractile tissue after overload, IL-6 null mice demonstrated a larger induction of muscle hydroxyproline content and increased expression of procollagen-1 mRNA expression as compared to wild-type mice. Previous reports have demonstrated that impaired satellite cell activation occurs during overload in mice lacking IL-6 (38), and the current study demonstrates that the overload induction of MyoD mRNA was attenuated in mice that lack IL-6. However, muscle cyclinD1 mRNA expression was significantly elevated in the IL-6 null animals after 3 days of overload, which indicates that proliferation of other cell types

was likely enhanced. Taken together, IL-6 appears to be a critical regulator of overload-induced ECM remodeling in the mouse plantaris muscle.

Interleukin-6 has the potential to regulate many critical processes related to skeletal muscle adaptation to overload. These processes include the regulation of cell proliferation, cell differentiation, and the overall tissue regeneration response (2,9,22). Interleukin-6 initiates intracellular signaling by binding to its membrane bound receptor and inducing Janus Kinases (JAKs) phosphorylation, which recruits and phosphorylates signal transducers and activator of transcription (STATs) proteins (17). Increased STAT3 and JAK2 levels have been observed during functional overload and during regrowth of atrophied skeletal muscle (13). Interleukin-6 can also activate the mitogen activated protein kinase pathway (47) and has been shown to be involved in increased protein synthesis during skeletal muscle growth or regeneration (12,33). Interleukin-6 gene expression is up-regulated as early as 3 days following functional overload (10), coinciding with a period of high satellite cell activity.

Considerable effort has been placed on understanding cytokine regulation of satellite cell activity in overloaded skeletal muscle (38,39). We demonstrate that in the absence of IL-6, functional overload-induced myogenic gene expression related to myoblast proliferation is disrupted. The previously reported attenuation of hypertrophy in IL-6 knockout mice was reported to be related to inhibition of STAT3 mediated satellite cell activity (38). Leukemia inhibitory factor (LIF) is another member of this family that can regulate satellite cell proliferation (40). In addition, LIF knockout mice do not display a hypertrophic response in the plantaris muscle due to functional overload (39). However, the current study demonstrates that in the absence of IL-6, myofibers were still able to enlarge in response to overload. The increase in myofiber area regardless of IL-6 expression indicates that other signaling pathways are functioning independent of IL-6, which may be important for individual myofiber growth. There is the possibility that overlapping or compensatory cytokine signaling in the IL-6 null animals may have occurred during overload, and include the exaggerated induction of IGF-1 mRNA at 3 days of overload in the IL-6 null animals.

The discrepancy in findings related to myofiber growth between the current study and previous work is not easy to reconcile, and may be related to the different sources of the knockout animals, their breeding, and the surgical overload methods employed in the studies. The researchers in the current study are very experienced with the synergist ablation overload model. The IL-6 knockout mice used in the current study originated from commercially available mice from Jackson Laboratories (Bar Harbor Maine) and are extensively bred and used in research studies at the University of South Carolina. Additionally, the IL-6^{-/-} mice in our current study had no detectable levels of IL-6 mRNA in control muscles and no overload induction of IL-6 mRNA as in wild-type mice. An additional difference was that Serrano et al used IL-6^{-/-} mice on a 129SvJ background; whereas, the mice used in the current study were on a C57/BL6 background (38). Thus, it is possible that genetic background in which IL-6 has been knockout may influence the ultimate phenotype observed in work overload experiments.

The biological role of IL-6 in overloaded muscle may also be important for regulating processes in cells other than myofibers, since IL-6 regulates cell cycle activity in a variety of cell types (15,47). We demonstrated a large increase in cyclin D1 gene expression at the onset of functional overload in IL-6 null mice, which is indicative of cell proliferation. However, these proliferating cells could consist of myoblasts, fibroblasts, as well as, other cell types found in muscle. The overload induction of MyoD expression, a marker of myoblast proliferation, was attenuated in the absence of IL-6, and may indicate that cells other than satellite cells are the source of this increased proliferation at the onset of overload. This finding is in contrast to Serrano et. al., who demonstrated a decrease in cyclin

D1 with overload in IL-6^{-/-} muscle (38), and this difference in cyclinD1 expression may account for some of the differences seen between our studies. In our current study, IL-6 loss combined with overload induced a larger increase in non-contractile tissue when compared to wild-type mice. Changes in non-contractile tissue or muscle weight were not reported in the previous study examining overload adaptation in muscle from IL-6 null mice (38).

The extracellular matrix accounts for 1-10% of skeletal muscle (25). The extracellular matrix can act as a scaffold for other proteins as well as a reservoir of growth factors and cytokines. It also has a role as a mechanotransducer, which can activate intracellular signaling related to load sensing. Fibroblast cells maintain the extracellular matrix in skeletal muscle by secreting collagen protein. Collagen is an abundant component of the extracellular matrix, and during muscle hypertrophy total collagen content increases (41). We demonstrate a 6-fold increase in procollagen-1 mRNA abundance in wild-type mice; whereas, IL-6^{-/-} mice had a 14-fold increase in procollagen-1 mRNA abundance. In addition, IL-6^{-/-} mice had a larger increase in hydroxy proline content in the plantaris after 21 days of overload when compared to wild-type mice. Furthermore, we noted a larger increase in IGF-1 mRNA following 21 days of functional overload in the IL-6^{-/-} mouse. Myofibers are a well known source of local IGF-1 production (1). IGF-1 can mediate the effects of increased loading of fibroblasts to produce collagen (8). However, fibroblasts are also a source of IGF-1 production (19), which could account for the exaggerated response observed in the IL-6 knockout mouse. An important function of IL-6 signaling during overload-induced growth may be to regulate collagen and IGF-1 gene expression, which could impact overall adaptation to the stimulus. Overproduction of fibrotic tissue can inhibit normal physiological growth and healing in injured skeletal muscle (37). Further work is required to determine if IL-6 mediated signaling can serve to repress extracellular matrix expansion in overloaded muscle through the regulation of collagen gene expression.

Transforming growth factor beta (TGF- β) is an important fibroblastic stimulus. TGF- β stimulates fibroblast cells to proliferate and synthesize collagen (18) and can also induce collagen and fibronectin synthesis in skeletal muscle cells (20). We have demonstrated that IL-6 loss accentuated the overload induction of TGF- β mRNA expression in plantaris muscle when compared to WT mice. Normal TGF- β expression is critical for extracellular matrix synthesis, which is an important component of regeneration. TGF- β can act in a paracrine/autocrine fashion thereby triggering muscle cells to differentiate into fibrotic cells (31). TGF- β can also decrease the expression of myogenic proteins such as MyoD and desmin (31). This overproduction of collagen could prevent the proliferation of satellite cells. Inhibition of TGF- β has been shown to decrease fiber scar formation and improve muscle healing (32), and muscle healing is enhanced when fibrosis is prevented (37). It remains to be determined if an important signaling function of IL-6 during overload-induced hypertrophy is to repress TGF- β expression, thereby attenuating extracellular matrix expansion, and ultimately improving myofiber regeneration and growth.

In summary, IL-6 appears to be critical for normal physiological growth induced by functional overload. Overload induced a dysregulation of genes related to ECM remodeling and growth in the absence of IL-6. The large increase in non-contractile tissue and procollagen-1 mRNA expression implies that IL-6 is a critical factor regulating the fibrotic response of skeletal muscle during overload-induced hypertrophy.

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Figure 1.

The effect of 21 days of functional overload on plantaris muscle myofiber size distribution after 21 days of overload. A) Wild-type plantaris muscle. B) IL-6^{-/-} plantaris muscle. Values are means $\pm$ SE, n = 5 for each group. *Difference in the percentage of small or large fibers.

A)

B)

C)

Figure 2.

The effect of 21 days of functional overload on plantaris non-contractile tissue. A)) H&E staining of muscle cross sections of *a*, wild-type sham, *b*, wild-type overload, *c*, IL-6^{-/-} sham and *d*, IL-6^{-/-} overload. **B)** The effect of 21 days of functional overload on volume percentage of noncontractile tissue in the plantaris muscle. **C)** The effect of 21 days of functional overload on hydroxyproline concentration. Values are means $\pm$ SE, $n = 4-6$ animals for each group. *Significantly different from same genotype control ($p < 0.05$). †Significantly different from wild-type overload ($p < 0.05$).

Figure 3.

The effect of 21 days of functional overload on plantaris muscle procollagen-1, IGF-1, and TGF- β , and mRNA expression. A) Procollagen-1 mRNA expression. B) IGF-1 mRNA expression. C) TGF- β mRNA expression. Target gene expression corrected with 18s expression. Values are means $\pm$ SE, $n = 4-6$ animals per group. *Overload significantly different from same genotype control ($p < 0.05$). †Significantly different from wild-type overload ($p < 0.05$).

Figure 4.

Cell cycle regulatory factors and IL-6 gene expression after 3 days of functional overload. A) Cyclin D1 mRNA expression. B) MyoD mRNA expression. C) Myogenin mRNA expression. D) IL-6 expression. Target gene expression corrected with 18s expression. Values are means $\pm$ SE, $n = 4-6$ for each group. *Overload significantly different from same genotype control. #Main effect of overload. ($p < 0.05$). ND, not detectable.

Table 1
Plantaris muscle weight and muscle weight relative to body weight in overloaded mice

Functional overload was accomplished by bilateral surgical ablation at 21 and 56 days. Total protein was run with n = 5-6 for each group. Values are means $\pm$ SE.

Treatment	N	Body Wt (g)	Muscle Wt (mg)	Muscle:BW (mg/g)	Total Muscle Protein (mg)
21 day					
Wild Type					
21d Sham	11	27.1 $\pm$ 1.0	18.6 $\pm$ 1.3	0.69 $\pm$ 0.05	2.8 $\pm$ 0.2
21d OV	13	28.1 $\pm$ 0.8	29.5 $\pm$ 1.5*	1.06 $\pm$ 0.05*	5.1 $\pm$ 0.7 [#]
IL-6 ^{-/-}					
21d Sham	10	24.7 $\pm$ 0.5	18.1 $\pm$ 1.2	0.73 $\pm$ 0.05	2.3 $\pm$ 0.4
21d OV	10	26.5 $\pm$ 0.8	39.1 $\pm$ 3.1* [†]	1.53 $\pm$ 0.16* [†]	5.5 $\pm$ 0.1 [#]
56 day					
IL-6 ^{-/-}					
56d Sham	5	27.7 $\pm$ 0.6	21.8 $\pm$ 2.1	0.78 $\pm$ 0.07	3.4 $\pm$ 0.5
56d OV	5	27.6 $\pm$ 1.0	39.3 $\pm$ 3.0*	1.43 $\pm$ 0.13*	5.6 $\pm$ 0.7*

* Significantly different from aged matched control animals (p < 0.05).

[†] Significantly different from aged-matched wild type overload animal (p < 0.05).

[#] Main effect of overload (p < 0.05).