THE EFFECTS OF INTERLEUKIN-10 ON SKELETAL MUSCLE INSULIN RESISTANCE AND MYOGENESIS

A Dissertation Presented

By

SEZIN DAGDEVIREN

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(November 30, 2016)

PHD PROGRAM

Molecular Medicine
THE EFFECTS OF INTERLEUKIN-10 ON SKELETAL MUSCLE INSULIN RESISTANCE AND MYOGENESIS

A Dissertation Presented
By
SEZIN DAGDEVIREN

Dissertation Defense Committee GSBS Members
Roger J. Davis
Interdisciplinary Graduate Program
Rita Bortell
Interdisciplinary Graduate Program
Michael P. Czech
Interdisciplinary Graduate Program

Chair of the Dissertation Committee
Laura C. Alonso
Interdisciplinary Graduate Program

External Dissertation Committee Member
Chih-Hao Lee
Harvard T.C. Chan School of Public Health, Professor of Genetics and Complex Diseases

Thesis Advisor
Jason K. Kim
Interdisciplinary Graduate Program

Student Program
Interdisciplinary Graduate Program

14 December 2016
Acknowledgements

Ph.D. is a long journey. I feel very lucky to be surrounded by such supportive mentors, lab members, colleagues and family during this journey. Most importantly, I would like to thank my advisor, Dr. Jason Kim; without him I would not have come this far. I thank him for encouraging me to improve myself further and further every day, teaching me how to be a good scientist, giving me the opportunity to learn state-of-the-art techniques, trusting me and challenging me when necessary. I am grateful for all of these. When I first joined the lab, I remember him saying that members of the lab are like your family. For the last 5 years, I was so lucky to have the most supportive lab family possible. I am deeply grateful to Randall Friedline, who supported me in every aspect of this journey. I also would like to thank our lab members Hye Lim Noh, Xiaodi Hu, Karen Kelly, Kunikazu Inashima, Duy Tran for all their help. I’d like to further thank our previous lab members; most importantly to Dae Young Jung for training me for the skills I used in this thesis, Payal Patel, Jong Hun Kim, Eun jung Lee, Nicholas Tsitsilianos, Taekyoon Kim, Hee Joon Kang, Jung Yeon Kwon, Suchaorn Saengnipanthkul, Yoshihiro Azuma, Hsunfan Wang and Socheata Ly for all of their contributions to this work, and my brilliant interns Marilia Loubato, Caitlyn Kearns and Cecilia Uong for their effort.

Thanks to the members of my thesis and dissertation committees, Dr. Roger Davis, Dr. Michael Czech, Dr. Rita Bortell, Dr. Laura Alonso for their valuable advice and feedback in developing these projects and their participation in this final step.
Thanks to my dear friends in this school, Elif Kamber Kaya, Zeynep Uyan, Ozgun Uyan, Yonca Karadeniz, Salih Topal, Ozlem Senol, Arda Konik, Fatih Bolukbasi, Ebru Kaymak, Aysegul Ozen, Barutcu’s, Hakan Guney, Aydemir’s, Yasin Bakis, Aslihan Dincer, Yvonne Chan, Adam Choi, Sungwook Choi, Cihan Aydin, Hulya Gundesli, Shruti Sharma and Gregory Orlowski for all of their valuable support during this process.

Finally, thanks to my family. Thanks to my parents, my brother, my grandmother, my aunt and Mostafa, my most valuable treasures, for their unconditional support and love.
ABSTRACT

Skeletal muscle insulin resistance is a major characteristic of obesity and type 2 diabetes. Although obesity-mediated inflammation is causally associated with insulin resistance, the underlying mechanism is unclear. Our lab and others have shown that a chronic low-grade inflammation takes place in skeletal muscles during diet-induced obesity, as evidenced by increased macrophage markers and pro-inflammatory cytokine levels. Interleukin (IL)-10 is a Th2-type cytokine that inhibits the synthesis and activity of pro-inflammatory cytokines and counteracts the Toll-like receptor-mediated inflammation. Our lab has previously demonstrated the preventive role of IL-10 against insulin resistance. Here, I have analyzed the effects of IL-10 on the skeletal muscle glucose metabolism and myogenesis in three different insulin resistant states (high fat diet-induced, leptin-deficiency-induced and aging-induced). The first model involved long-term (16 weeks) high-fat diet (HFD) feeding that resulted in markedly obese and hyperglycemic mice, representative of obese type 2 diabetic subjects. In mice overexpressing IL-10 specifically in the skeletal muscle (M\textsuperscript{IL10}), we observed improved whole-body and skeletal muscle insulin sensitivity as compared to wild-types after long-term high fat diet feeding. The improved insulin sensitivity in the skeletal muscle was due to increased Akt signaling and decreased muscle inflammation. Leptin is an important adipocyte-derived hormone that is elevated in obesity, and it regulates numerous physiological functions including the energy balance and inflammation. Thus, my second model examined the effects of muscle-specific overexpression of IL-10 on glucose metabolism in the hyperphagic, leptin-deficient ob/ob mice. We detected improved whole-body insulin sensitivity compared to the control mice.
My third model examined the effects of increased IL-10 expression using M^{IL10} mice during aging-induced insulin resistance. In 18-month old M^{IL10} mice, we found enhanced whole-body and skeletal muscle insulin sensitivity due to improved insulin signaling and decreased muscle inflammation as compared to wild-type mice. Last, to test whether direct signaling of IL-10 on skeletal muscle is responsible for the beneficial effects of IL-10 on muscle glucose metabolism, I generated mice lacking IL-10 receptor 1 type chain selectively in skeletal muscle (M-IL10R^{-/-}). We observed more prominent muscle inflammation and whole-body insulin resistance in HFD-fed M-IL10R^{-/-} mice as compared to wild-type mice. Interestingly, when studying insulin resistance in the IL-10 transgenic mouse models, we identified a consistent increased lean mass phenotype, and conversely decreased lean mass in the HFD-fed M-IL10R^{-/-} mice. Quantitative RT-PCR on HFD-fed M^{IL10} group muscles to measure myogenesis-related gene expression identified a correlation between lean mass and both IL-10 and MyoD mRNA expression levels. In support of this, I showed that IL-10 caused an increase in in vitro cultured myoblast proliferation rates. Together, these results highlight the potential benefits of IL-10 expression not only in muscle glucose metabolism but also in maintaining muscle mass during insulin resistant states. Overall, these results demonstrate that selective expression of IL-10 in skeletal muscle suppresses inflammation, improves glucose metabolism and muscle growth in obese and aging mice, and further establishes that these effects are at least partially mediated by direct activation of IL-10 signaling in skeletal muscle.
TABLE OF CONTENTS

Acknowledgements ........................................................................................................ iii
Abstract .......................................................................................................................... v
Table of Contents .......................................................................................................... vii
List of Figures and Tables ........................................................................................... ix
List of Common Abbreviations ..................................................................................... xii

Chapter I: Introduction ................................................................................................. 1
1.1 Insulin Resistance .................................................................................................... 2
1.1.1 Obesity, Type 2 Diabetes and Insulin Resistance ............................................. 2
1.1.2 Aging and Insulin Resistance ......................................................................... 4
1.1.3 Chronic Inflammation in Insulin Resistance .................................................... 5
1.2 Skeletal Muscle ...................................................................................................... 7
1.2.1 Skeletal Muscle as a Metabolic Tissue .............................................................. 7
1.2.2 Skeletal Muscle Insulin Resistance ................................................................. 10
1.2.3 Skeletal Muscle Inflammation ....................................................................... 13
1.2.4 Skeletal Muscle in Obesity and Aging ............................................................. 16
1.3 Inflammation .......................................................................................................... 18
1.3.1 HFD-related Impairment of Immune Function ................................................ 18
1.3.2 Aging-related Impairment of Immune Function .............................................. 19
1.3.3 Anti-inflammatory Approach for Diabetes and Insulin Resistance ............. 20
1.4 Interleukin-10 ....................................................................................................... 23
1.4.1 Interleukin-10 Function and Signaling ............................................................ 23
1.4.2 Interleukin-10 Receptor .................................................................................. 27
1.4.3 Interleukin-10’s Use as a Therapeutic Approach ........................................... 27
1.4.4 IL-10 Expression in Obesity and Old Age ...................................................... 30
1.4.5 Role of IL-10 in Whole Body and Muscle Glucose Metabolism ................... 32
1.5 IL-10 and Myogenesis ......................................................................................... 34
1.6 Mouse Models ....................................................................................................... 37
1.6.1 Long term HFD-fed M<sup>IL10</sup> mice ................................................................. 37
LIST OF FIGURES AND TABLES

CHAPTER I

Figure 1.1 Insulin signaling/glucose uptake pathway in skeletal muscle fibers .......... 12
Figure 1.2 IL-10 signaling ....................................................................................... 26

CHAPTER II

Table 2.1 List of primers .......................................................................................... 48
Figure 2.1 Metabolic profiles of muscle-specific IL-10-overexpressing mice (M^{IL-10}) and WT mice on standard chow and after 16 weeks of an HFD ...................................................... 50
Figure 2.2 A 72-hour metabolic cage measurement in M^{IL10} and WT mice on long term HFD feeding ................................................................................................................. 51
Figure 2.3 Hyperinsulinemic-euglycemic clamp in awake WT and M^{IL10} mice after 16 weeks of HFD or chow diet ........................................................................................................... 53
Figure 2.4 Insulin-stimulated Akt phosphorylation, plasma IL-10, IL-6 and IL-1α levels, liver CD68 and F4/80 macrophage marker mRNA levels in chow and HFD-fed WT and M^{IL10} mice ............................................................................................................... 55
Figure 2.5 Insulin signaling, muscle morphology and triglyceride and IL-10 levels in skeletal muscle ....................................................................................................................... 56
Figure 2.6 Local inflammation in skeletal muscles of M^{IL10} and WT mice after 16 weeks of chow or HFD ......................................................................................................................... 57
Figure 2.7 Insulin-stimulated glucose uptake and inflammation in adipose tissues ...... 59
Figure 2.8 Hepatic glucose metabolism and insulin signaling in WT and M^{IL10} mice fed chow or HFD for 16 weeks .............................................................................................................. 60
Figure 2.9 Metabolic effects of muscle-specific IL-10 overexpression in leptin-deficient ob/ob mice (MCK-IL10^{ob/ob}) ........................................................................................................... 62
Figure 2.10 Circulating free fatty acid levels of MCK-IL10^{ob/ob} mice, genotyping of M-IL10R^{−/−} mice, insulin-stimulated muscle glucose uptake levels and IL-10 levels in chow and HFD-fed WT and M-IL10R^{−/−} mice ................................................................................................................. 63
Figure 2.11 A 2-hour hyperinsulinemic-euglycemic clamp in awake MCK-IL10\textsuperscript{ob/ob} mice and \textit{ob/ob} mice ........................................................................................................................................64

Figure 2.12 Metabolic profile, insulin action, and skeletal muscle inflammation were assessed in M-IL10\textsuperscript{R\textsuperscript{-/-}} and WT mice after 6 weeks of HFD or chow diet feeding.........66

CHAPTER III

Table 3.1 List of primers ........................................................................................................................................80

Figure 3.1 Body weight and composition of M\textsuperscript{IL10} and wild-type (WT) mice between 4-18 months of age........................................................................................................................................82

Figure 3.2 Longitudinal assessment of energy balance using metabolic cages in aging M\textsuperscript{IL10} and wild-type (WT) mice ........................................................................................................................................83

Figure 3.3 VO\textsubscript{2} rate normalized to lean mass, VCO\textsubscript{2} rate normalized to lean mass, and VO\textsubscript{2} rate normalized to fat mass in male M\textsuperscript{IL10} and WT mice at 12, 14, 16, and 18 months of age........................................................................................................................................84

Figure 3.4 Skeletal muscle mRNA levels of uncoupling protein (UCP) 3, succinate dehydrogenase, and citrate synthase in young (4 months of age) and aging (18 months of age) M\textsuperscript{IL10} and WT mice ........................................................................................................................................86

Table 3.2 Metabolic profile in aging WT vs. M\textsuperscript{IL10} mice ........................................................................................................................................87

Figure 3.5 Total distance run on treadmill until exhaustion and muscle hGH levels in M\textsuperscript{IL10} and WT mice ........................................................................................................................................89

Figure 3.6 A 2-hour Hyperinsulinemic-euglycemic clamp to assess insulin action and glucose metabolism in awake M\textsuperscript{IL10} and wild-type (WT) mice at 18 months of age........90

Figure 3.7 Insulin signaling and glucose/lipid metabolism in skeletal muscle of M\textsuperscript{IL10} and wild-type (WT) mice at 18 months of age ........................................................................................................................................91

Figure 3.8 Skeletal muscle mRNA and protein levels if inflammatory cytokines, macrophages, and chemokines in M\textsuperscript{IL10} and wild-type (WT) mice ........................................................................................................................................93

Figure 3.9 Glucose metabolism and inflammation in adipose tissue and heart of aging M\textsuperscript{IL10} and wild-type (WT) mice at 18 months of age ........................................................................................................................................94
Figure 3.10 Hepatic glucose production (HGP) and hepatic insulin action during hyperinsulinemic-euglycemic clamp in awake M^{IL10} and wild-type mice at 18 months of age .......................................................... 95

CHAPTER IV
Table 4.1 List of primers ........................................................................................................... 109
Figure 4.1 Lean mass phenotypes of IL-10 transgenic and M-IL10R^{−/−} mice .............. 111
Figure 4.2 Muscle: fat ratios calculated by X-ray computed tomography of WT and M^{IL10} mice.................................................................................................................. 112
Figure 4.3 Muscle weights of WT and M^{IL10} mice fed with HFD for 16 week ........ 113
Figure 4.4 IL-10 increased proliferation in myoblasts ..................................................... 115
Figure 4.5 IL-10 does not affect myoblast differentiation ............................................... 116
Figure 4.6 mRNA levels of muscle growth genes in WT and M^{IL10} mice gastrocnemius muscles .................................................................................................................. 118
Figure 4.7 Flow cytometry analysis of skeletal muscle immune cell population in quadriiceps muscles of of WT and M^{IL10} mice fed with HFD for 16 weeks .......... 119
Figure 4.8 Muscle MyoD and Myf5 mRNA expressions are correlated with lean mass in HFD fed M^{IL10} mice ........................................................................................................... 121
Figure 4.9 Muscle IL-10 mRNA expressions are correlated with lean mass in HFD fed M^{IL10} mice .................................................................................................................. 122
Figure 4.10 IL-10 overexpression in skeletal muscles of HFD fed mice is protecting mice against muscle growth cessation due to insulin resistance in late-stage obesity .......... 127

CHAPTER V
Figure 5.1 Schematic representation of IL-10’s potential effects on skeletal muscle glucose metabolism ................................................................................................................ 131
Figure 5.2 The schematic of effects of IL-10 on skeletal muscle ...................................... 135
LIST OF FREQUENTLY USED ABBREVIATIONS

BAT- Brown adipose tissue
DAG- Diacylglycerol
DG- Deoxy glucose
ER- Endoplasmic reticulum
FFA- Free fatty acid
GLUT- Glucose transporter
GTP- Guanosine triphosphate
HFD- High fat diet
HGP- Hepatic glucose production
IFN- Interferon
IGF- Insulin-like growth factor
IL- Interleukin
IRS- Insulin receptor substrate
JNK- c-Jun N-terminal kinase
KO- Knock out
MAFbx- Muscle atrophy F-box
MCK- Muscle creatine kinase
MCP- Monocyte chemoattractant protein
MURF- Muscle RING-finger protein
NIDGU- Non-insulin dependent glucose uptake
NF-κB- Nuclear factor kappa B
ROS- Reactive oxygen species
SOCS- Suppressor of cytokine signaling
STAT- Signal transducer and activator of transcription
TNF- Tumor necrosis factor
WAT- White adipose tissue
Chapter I: INTRODUCTION
1.1 Insulin Resistance:

1.1.1 Obesity, Type 2 Diabetes and Insulin Resistance

Excess body weight poses a risk for developing serious metabolic diseases such as type 2 diabetes, cardiovascular disease and cancer [1]. Unfortunately, the obesity prevalence in the United States has increased by 2-fold in the last 30 years since the 1980s, with the USA being the country with the highest body mass index average among developed countries [1]. Western diet, physical inactivity and irregular eating habits due to the modern lifestyle are fueling the spread of obesity across the nation [2]. Although many precautions are taken to promote healthy lifestyle changes, obesity still stands out as the most prevalent health problem in the population affecting one out of every three people [1].

Type 2 diabetes is a metabolic disease created by the failure of a highly-regulated multi-organ network to sustain circulating glucose at euglycemic levels. It is characterized by obesity, impaired insulin action, insulin secretory dysfunction, and increased endogenous glucose production [3]. Some studies suggest the decreased insulin action is the initial step in type 2 diabetes development [4-6], whereas others propose that β cell dysfunction as the primary factor [7-9]. Both are important for the early pathogenesis of type 2 diabetes [3, 10]. Genetic susceptibility and family history are also important factors in developing type 2 diabetes [11-13].

The most noticeable problems leading to type 2 diabetes are pancreatic β cell failure and liver, adipose tissue and muscle insulin resistance [14]. β cells are responsible for the
release of insulin that stimulates glucose uptake into metabolic organs, and the loss of β cell functionality is a major factor in the pathogenesis of type 2 diabetes [15]. Insulin resistance, defined as decreased response to insulin-stimulated glucose uptake in tissues [16], develops prior to type 2 diabetes. Insulin induces glucose uptake into skeletal muscle and adipose tissue [17] and acts on the liver to suppress hepatic glucose production [18]. Adipose tissue, skeletal muscle and liver sensitivity to insulin diminishes as insulin resistance develops. Since glucose uptake into muscle and fat decreases and liver glucose output increases due to insufficient suppression of glycogen breakdown and gluconeogenesis, glucose levels rise in the circulation [17].

β cells can respond to the decreased sensitivity of insulin in metabolic tissues by increasing β cell mass to raise insulin levels [19, 20]. β cell proliferation was also detected in mice even before the onset of insulin resistance [21], and researchers showed that obesity can result in a 50% increase in β cell mass [22]. Hyperinsulinemia due to the β cell mass increase can maintain physiologic glucose levels initially, however prediabetes or type 2 diabetes eventually develops in part due to the inability to maintain compensatory insulin levels because of insufficient pancreatic β cell mass and activity [23, 24].

Obesity is a main factor initiating insulin resistance and type 2 diabetes [25]. Increased circulating free fatty acids (FFA), adipose tissue dysfunction and inflammation, and impaired insulin signaling in muscle during obesity all lead to type 2 diabetes [26]. Abdominal fat deposition is a strong hallmark of these diseases [27] along with the low-grade chronic inflammation linked to insulin resistance and type 2 diabetes [28-30]. Stress kinase pathways like Jun N-terminal kinase (JNK), nuclear factor kappa B (NFκB), IκB β
kinase (IKKβ) play important roles in inflammation-mediated suppression of insulin signaling, mainly by serine phosphorylation of Akt [31-33]. Other stress factors such as endoplasmic reticulum (ER) stress [34-36], induction of inducible nitric oxide synthase (iNOS) [37], and reactive oxygen species (ROS) are also associated with type 2 diabetes [38, 39].

It is projected that by 2030, 5 out of every 100 people will develop type 2 diabetes [40]. It is indisputable that diabetes is standing as an urgent priority, and growing like an epidemic costing almost $250 billion yearly in the USA [16, 41]. Increasing prevalence of diabetes is also a global threat accounting for 2 million deaths every year and, it is the 7th leading cause of disability in the world [42, 43]. Unless necessary actions are taken to prevent and treat it, worldwide morbidity and mortality rates of diabetes will become a great burden in the near future.

1.1.2 Aging and Insulin Resistance

Aging is related to peripheral insulin resistance as evident from higher type 2 diabetes rates in the elderly [44-47]. This insulin resistance in the periphery is correlated with a gradual increase in total adiposity with age [48]. Aging is associated with abdominal increased adipose tissue and decreased lean mass phenotype [49]. Abdominal fat accumulation and physical inactivity coming with old age are thought to be the main reasons causing insulin resistance in old age [45, 50-52]. Research results confirmed that obese, sarcopenic old subjects were found to develop more profound insulin resistance [49]. Moreover, in a study where visceral fat was removed from aging rats, they found that
insulin resistance was improved [53]. However, there are studies supporting that insulin resistance during aging is independent of total obesity [54].

Insulin resistance in the elderly was also found to be associated with reduced mitochondrial function due ectopic fat accumulation in muscle and liver [47]. Yet, increased obesity and inflammation during aging were still suggested to be the most important factors promoting the insulin resistance [55].

Since insulin resistance and type 2 diabetes are more frequent in elders and are associated with frailty, they pose a risk for the healthy course of life. As lifespan is increasing with recent advancements in healthcare and science, it is essential that we direct resources to the welfare of the old population.

### 1.1.3 Chronic Inflammation in Insulin Resistance

The preventive effects of anti-inflammatory agents against insulin resistance was reported many times in history, yet the mechanism was unclear until recently [56-58]. In the early 90’s researchers found the link between adipose tissue inflammation, obesity, and insulin resistance [59]. Hotamisligil et. al showed that inflammatory tumor necrosis factor alpha (TNFα) is synthesized from adipose tissues of obese subjects resulting in insulin resistance [60]. Not long after, the relationship between inflammation and insulin resistance was widely accepted in the field as a trending topic [61-65].

Systemic inflammation was tied to insulin resistance in large population studies [66-68]. Increased inflammatory markers from type 2 diabetic and insulin resistant patients were among the typical findings. Also, disruption of the IKKβ, JNK pathways, that are
essential for mediating the inflammatory response in tissues, increased insulin sensitivity in mice and human [33, 69-73]. Cellular stresses like ER stress, ROS induction were all found to be associated with inflammation, triggering the insulin resistance [36, 74]. However, inflammation occurring during obesity is different from the traditional large-scale inflammatory response with highly and acutely elevated inflammatory cytokines upon stimulation. Obesity-induced inflammation is a low-scale chronic response referred to as ‘metaflammation’ with constant and modest amounts of released cytokines activating different mechanisms [65, 66, 75, 76]. This subclinical inflammation can precede type 2 diabetes [67, 68].

The link between inflammation and insulin resistance was first discovered in adipose tissue, therefore, most of the research was based on adipose tissue inflammation. Researchers reported increased macrophage infiltration and proliferation, cytokine and chemokine expression in adipose tissues of insulin-resistant mice [59, 60, 77-80]. Macrophages were defined as key players in inflammation-induced insulin resistance in obesity [81]. Other immune cells like natural killer cells and mast cells were also found to be elevated in obese patients [82, 83]. Inflammation became a much-studied link between obesity and insulin resistance that was under the spotlight for quite a while. Yet, obesity does not induce insulin resistance only in adipose tissue. Other metabolic tissues such as skeletal muscle, liver and heart also become insulin resistant during obesity [84]. Even though some researchers believe that insulin resistance in these metabolic tissues is triggered indirectly by adipokines released to the circulation and ectopic fat surrounding these tissues [64, 85-88], there is evidence that skeletal muscle and liver themselves
develop increased inflammation when mice are fed with HFD [89-94]. The latest view is
the whole organism including adipose tissue, skeletal muscle, liver and even intestines
cross-talk with each other during this chronic inflammation [95]. Overall, obesity seems to
generate a mild inflammation in all metabolic tissues resulting in pronounced insulin
resistance in the end.

Considering inflammation is defined to be the link between obesity and insulin
resistance, anti-inflammatory therapies in the treatment of these conditions gain
importance. Generating monoclonal antibodies against inflammatory cytokines, or
working on natural anti-inflammatory cytokines to restore the balance between pro-
inflammatory and anti-inflammatory mediators are becoming common strategies. More
studies are revealing the mechanisms connecting the immune system to insulin resistance
every day, that will render it possible to develop strategies for severe outcomes in the near
future.

1.2 Skeletal Muscle

1.2.1 Skeletal Muscle as a Metabolic Tissue

Approximately 40% of our body mass consists of skeletal muscle [96]. Skeletal
muscle is the largest metabolic tissue and is mainly responsible for glucose homeostasis in
the body. Skeletal muscle by itself is the major site for glucose uptake after a meal, taking
up ~80% of the insulin-stimulated glucose uptake after eating [97]. Skeletal muscle
maintains glucose uptake by both insulin-dependent and insulin-independent mechanisms,
and requires a significant proportion of energy, being responsible for physical activity [98].
In the post-absorptive state, insulin signaling stimulates the glucose transporters for glucose uptake, whereas during exercise, contractions increase glucose uptake by facilitated diffusion [99]. Intense exercise can also increase muscle blood flow up to 20-fold, promoting the glucose delivery for tissue uptake [100]. The glucose uptake during contractions, and the increased muscle glucose metabolism and insulin sensitivity after exercise do not require insulin [101]. Under euglycemic conditions, majority of non-insulin dependent glucose uptake (NIDGU) occurs in non-muscle tissues [98]. On the other hand, researchers stated that during hyperglycemia, half of the total glucose uptake in the skeletal muscle is non-insulin dependent [98] and hyperglycemia can increase GLUT4 translocation to the membrane [102]. Therefore, both insulin-dependent and insulin-independent glucose uptake are important for skeletal muscle metabolism.

Blood flow into skeletal muscle in resting state is only ~0.04 ml/min per gram tissue, therefore blood flow into skeletal muscle is a rate-determining state for the delivery of insulin and glucose [103]. Insulin, by interacting with endothelium, can vasodilate skeletal muscle vasculature and recruit capillaries to increase blood flow and increase glucose uptake [103, 104]. Thus, insulin can control its own delivery to the muscle by acting on the vasculature [105]. This vasodilating action which provides more blood flow is impaired during insulin resistance. Insulin passes across the endothelial cells via insulin receptor signaling, and thereby promotes its own transfer to reach to the muscle fibers [106, 107]. Insulin is concentrated at endothelial cells for its trans-endothelial transport [108]. Transport of insulin across the vascular wall to muscle interstitium is suggested to be a rate limiting step [109], and only 50% of the plasma insulin was measured to reach muscle
interstitium [110]. Insulin reaches the muscle fibers after passing through the endothelial cell barrier.

Obesity results from excess calorie intake and lack of physical activity. When we provide more fuel into muscles than it needs and muscles cannot consume it; the energy balance is disturbed resulting in obesity and diabetes. Skeletal muscle regulates euglycemia by cross-talking with liver and adipose tissue. Therefore, for keeping healthy metabolic state, maintaining homeostasis in insulin-responsive tissues is crucial.

Skeletal muscle uses glucose and FFA as energy fuels. During the fasting state FFA is the primary fuel, whereas glucose uptake is the main source in the post-absorptive state [111]. Glucose uptake in tissues is facilitated mainly by GLUT4 and GLUT1 [112]. GLUT4 is the most dominant form in the skeletal muscle and is responsible for transferring glucose inside the fibers to be metabolized [113, 114]. Upon food intake insulin is secreted from β cells and travels through the circulation to reach skeletal muscles. Insulin then binds to insulin receptors on the fiber surface and activates intrinsic tyrosine kinase to auto-phosphorylate insulin receptor [113]. Then tyrosines are recognized by phosphotyrosine binding domains of insulin receptor substrate (IRS) proteins [115]. When insulin receptor phosphorylates tyrosine residues on IRS-I, it generates docking sites for the Src homology 2 (SH2) domains of the phosphatidylinositol 3-kinase (PI3K) [116]. Then PI3K converts phosphatidylinositol bisphosphate (PIP$_2$) to phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$) which activates PIP$_3$ dependent kinase (PDK1), the serine kinase that activates Akt [117]. Activated Akt inactivates glycogen synthase kinase 3 (GSK3). Inactivation of GSK3 promotes glycogen synthesis. Akt substrate of 160 kDa (AS160) has binding sites for Rab-
GTPase activating proteins which load Rab proteins with guanosine triphosphate (GTP) [118]. GLUT4 glucose transporters are usually found inside the cytoplasm attached to vesicles. Rab proteins are responsible for membrane trafficking [119]. They help to fuse these vesicles with cell membrane translocating more GLUT4 to the membrane [118]. Increased GLUT4 numbers in the muscle membrane in return increases glucose uptake in the skeletal muscle (Fig. 1.1). After uptake into muscles by GLUT4, glucose is phosphorylated by hexokinase, and it is either turned into glycogen to be stored or enters the glycolytic pathway. Therefore, insulin signaling pathway is a fundamental and complex process for providing glucose supply to the resting muscles.

1.2.2 Skeletal Muscle Insulin Resistance

Skeletal muscle is a very large insulin-responsive tissue. Along with the liver and adipose tissue, the glucose metabolism in skeletal muscle is important for the whole-body glucose homeostasis. Researchers define skeletal muscle insulin resistance as one of the major defects in type 2 diabetes, along with the insulin deficiency due to β cell failure [120, 121]. Studies have revealed that it can precede hyperglycemia [122, 123] and impairment of muscle glycogen synthesis pathway was found to be an important step during diabetes [124]. Researchers analyzed glucose metabolism of normoglycemic subjects with diabetic parents and found that hyperinsulinemia due to β cell activity, muscle insulin resistance, and impaired glucose oxidation were already present before the onset of diabetes [66]. In another study, researchers found that in the offspring of diabetic parents there was a 50% reduction in total glucose metabolism, a 70% reduction in muscle glycogen synthesis and a 40% reduction in muscle glucose-6-phosphate (G6P) concentration like their diabetic
parents [125]. These results suggested that muscle glucose transport, metabolism, and glycogen synthesis impairments are some of the fundamental steps for the onset of type 2 diabetes along with other factors.

Skeletal muscle insulin resistance can occur due to impairment of the insulin signaling pathway. When Insulin-IRS-Akt cascade is impaired, the muscle becomes insulin resistant [126, 127]. An 80% reduction in IRS-I phosphorylation and 90% reduction in PI3K activation was detected in obese, diabetic mouse muscles [128]. Tyrosine phosphorylation of IRS-I is one of the central switches controlling insulin signaling. Stress kinases like IKKβ, NF-κB and JNK are known to cause serine phosphorylation of IRS-I which inhibits its phosphorylation at tyrosine residues [129, 130]. These kinases can be activated following metabolic stress or inflammation [131]. Researchers in collaboration with our lab showed that inhibition of JNK signaling in skeletal muscle improves insulin sensitivity of skeletal muscle without affecting obesity [132].

Skeletal muscle insulin resistance can be caused by obesity, since weight loss can restore muscle insulin sensitivity in mice [133, 134]. Elevated fatty acids or lipid intermediates during obesity were also found to be linked to muscle insulin resistance [135], mostly through protein kinase C (PKC) which inhibits IRS-I, PI3K and Akt signaling [136-138]. Especially when uptaken fatty acids are not metabolized by β oxidation, they accumulate as diacylglycerol (DAG) and ceramides which can inhibit insulin signaling [139, 140]. There is also in vitro evidence that fatty acid treated macrophages induce insulin resistance in myocytes [141]. High plasma FFA concentrations due to HFD are also
Figure 1.1 Insulin signaling/glucose uptake pathway in skeletal muscle fibers.
suggested to cause toxic intramyocellular lipid accumulation, activation of inflammatory pathways, increase in ROS, inducible nitric oxide synthase (iNOS) and mitochondrial dysfunction which may result in insulin resistance [142]. However, abundant intramyocellular lipid levels in insulin sensitive endurance athletes suggest that increased fatty acids are not the major factor contributing to muscle insulin resistance [143, 144]. Also, systemic effects of obesity like inflammation or fatty acids seem to be necessary for skeletal muscle insulin resistance since when isolated myocytes from obese patients are cultured without stimulation they don’t show insulin resistance [145]. In the bigger picture, excess energy intake seems to result in elevated fatty acids, oxidative stress and inflammatory cytokines which synergistically increase lipid accumulation and insulin resistance in skeletal muscle. Identifying the exact molecular mechanisms resulting in skeletal muscle insulin resistance in obesity is not accomplished yet and requires further investigation.

### 1.2.3 Skeletal Muscle Inflammation

Chronic inflammation in metabolic tissues during insulin resistance was reported numerous times in the field. Either through increased immune cells and inflammatory cytokines in muscle or, through FFAs in circulation [146]; inflammation is a major pathway resulting in skeletal muscle insulin resistance [147].

When researchers are investigating the relationship between inflammation and insulin resistance, the attention is usually focused on the adipose tissue inflammation [86]. However, there is also convincing data about the role of skeletal muscle inflammation
during diabetes. Skeletal muscle is a secretory organ that can release cytokines called myokines [148, 149]. TNFα, IL-6 and IL-10 were shown to have all direct effects on skeletal muscle [147]. In addition to the increased circulating levels of inflammatory cytokines released from adipose tissue during obesity, the muscle itself can also release inflammatory cytokines in response to stress [150-152].

The link between inflammation and skeletal muscle insulin resistance is reflected in a series of observations: infiltration of inflammatory cells (macrophages, natural killer cells, dendritic cell, T cells) into skeletal muscles of type 2 diabetes patients and insulin resistant-obese mice [93, 153-158]; inflammatory cytokines overproduced by insulin resistant muscles from obese humans [94, 153]; inflammatory cytokines released from myocyte cultures of diabetic patients [159]; ability of muscles to generate and release inflammatory cytokines [160, 161]; ability of myocytes to recruit leukocytes from the blood stream [162]; expression of immune system components like Toll-like receptors (TLR) by muscle fibers [150, 163]; inflammatory chemokines triggering insulin resistance in cultured myoblasts [164]; and induction of skeletal muscle insulin resistance by cytokine infusion into humans [165, 166]. Pro-inflammatory cytokines TNFα, IL-6, IL-1 and monocyte chemoattractant protein-1 (MCP-1), were all shown to exert adverse effects on skeletal muscle insulin signaling [166]. Furthermore, pro-inflammatory cytokine TNFα resulted in insulin resistance in myocytes [167] and, was shown to be directly synthesized by the muscle [94]. It was also synthesized 4-fold higher in insulin resistant subjects’ muscles than controls. Macrophage numbers were also reported to be higher in skeletal muscles of obese patients [168]; and inflammatory receptor TLR4 and JNK proteins were
shown to be more abundant in the skeletal muscles of type 2 diabetic subjects [169, 170]. Another study investigating the muscle inflammation-insulin resistance relationship showed that when M1 activated macrophages were knocked out, inflammatory cytokine expressions in muscle dropped and insulin sensitivity was regain [156]; and when an anti-inflammatory protein is knocked out in macrophages, muscle inflammation and insulin resistance increased [171]. Skeletal muscles of HFD-fed mice were shown to contain CD11c+ macrophages and when the macrophages are depleted insulin resistance and inflammation seems to be prevented [155, 156]. Our own lab has shown in several studies that HFD-fed mice displayed increased levels of CD68, F4/80 macrophage markers, and TNFα, IL-6, C-C motif chemokine ligand 2 (Ccl2) cytokines in skeletal muscle [93, 133, 172].

Some researchers believe that the source of inflammation in muscle is intramyocellular adipose depot and adipocytes recruit macrophages [78, 155, 157, 173]. However, a recent study by our lab showed exercise-mediated weight loss improved insulin sensitivity in obese mice without affecting adipose tissue inflammation, but by reducing local inflammation in muscle. This finding supported the role that muscle inflammation is playing in obesity-induced insulin resistance [133]. Elevated fatty acids in circulation during obesity can be considered as another factor that induces inflammation in skeletal muscle through NF-κB [146, 174]. When muscle cells are co-cultured with macrophages, fatty acid treatment caused inflammatory cytokine TNFα, IL-6 release, JNK/IKKβ activation, and insulin resistance in muscle cells directly, which suggests adipokines are not necessary to start inflammation and insulin resistance in muscle [141, 168].
Anti-inflammatory agents like aspirin were shown to attenuate muscle insulin resistance in obese rats by inhibiting NOS production, IKKβ/JNK activation and restoring Akt signaling, which shows the importance of inflammation in muscle insulin resistance [175]. This study also shows the direct effect of aspirin on cultured muscle cells suggesting that it is targeting directly muscle, not the adipose tissue. Other anti-inflammatory agents like anti-TNFα antibody also result in improved insulin signaling in muscle [176]. Endoplasmic reticulum (ER) stress also recently became prominent in the obesity-inflammation research field [177]. ER stress in obese pregnant women, mouse, and myocyte cultures was also found to contribute to skeletal muscle inflammation and insulin resistance [178, 179].

All these findings support the important role of obesity-induced skeletal muscle inflammation in the pathogenesis of type 2 diabetes, and further investigation is clearly needed to clarify the mechanisms associating obesity-muscle inflammation-insulin resistance for reaching an in-depth understanding of the metabolic syndrome.

**1.2.4 Skeletal Muscle in Obesity and Aging**

Aging results in decline in skeletal muscle mass termed ‘sarcopenia’ since 1989 [180] as a combination of the words ‘sarco’ (flesh) and penae (deficiency) in Greek. It is described as ‘muscle mass below two standard deviations of a young control group’ [181]. Muscle mass and function decrease yearly 2% up to 40-50% after the 50’s [182-185]. This decrease poses a threat to healthy aging.
Sarcopenia is associated with type 2 diabetes and they both display an increased inflammatory phenotype [186, 187]. Inflammation in skeletal muscle during aging is shown to cause degenerated muscle fibers and proteins [188-190]. Pro-inflammatory cytokines are found to be higher in skeletal muscles of the elderly, and in return, they contribute to sarcopenia [128-131]. As an example, researchers found that skeletal muscles in elderly contained higher IL-1β levels compared to young subjects, and macrophage response due to physical activity in aging muscles was impaired [191]. Increased levels of circulating IL-6 and TNFα and decreased levels of circulating IL-10 concentrations were again correlated with lower muscle mass and strength in healthy elderly [84, 192-194]. Apart from inflammation, skeletal muscle protein synthesis is also resistant to the anabolic action of insulin in older subjects resulting in muscle loss in the old population [195].

In elderly, sarcopenia and obesity can progress synergistically making the condition even worse and leading to insulin resistance [49, 196]. Obesity is another factor that can impair the muscle mass balance. With increasing overload, anti-gravity muscles of obese people initially grow larger for a while [197], but the muscle performance does not improve. Muscle regeneration is another factor impacted by obesity. Satellite cells were shown to be impaired with increasing lipid accumulation at later stages of obesity [198, 199]. Skeletal muscle wasting during high-fat feeding might be related to impaired satellite cell function, since the wasting could be reversed by mesenchymal stem cell administration [200]. Moreover, infiltrated intramyocellular fat in skeletal muscle during obesity can cause loss of muscle function and strength in people [201]. Aging muscle also faces a decrease in satellite cell number, regeneration, and activity [202]. Taken together, during
both aging and obesity muscle growth, repair, strength, and function are impaired, decreasing the life quality of old and overweight people.

1.3 Inflammation

1.3.1 HFD-related Impairment of Immune Function

The detrimental effects of HFD on the immune system were known as early as 1980’s. T cell, B cell responses, macrophage and natural killer (NK) cell activities were all altered in HFD-fed animals [203]. Studies report that HFD results in a decrease in lymphocyte proliferation, and an increase in phagocytic activities is causing a more pro-inflammatory response type [204]. Adipose tissue-resident macrophages are suggested to be the main source of inflammation in type 2 diabetes and obesity [205]. Researchers found that HFD increases CD11c+ CD11b+ subset of dendritic cells in the adipose tissue resulting in M1 polarization in macrophages [155, 206]. Macrophages are not the only immune cell types that are triggered in HFD-induced inflammatory response. T lymphocyte activation even precedes the macrophages in HFD-fed mice increasing the macrophage activation and recruitment [207]. Our lab has shown that genetic ablation of lymphocytes protects against diet-induced obesity in mice [208]. B cells also accumulate in adipose tissue in HFD-fed mice, increasing inflammatory immunoglobulin and cytokine concentrations and polarizing macrophages and lymphocytes to induce insulin resistance [209]. Taken together, these findings imply that HFD-feeding is causing changes in multiple components of the immune system altering its working balance completely.
1.3.2 Aging-related Impairment of Immune Function

Inflammatory markers increase with age creating a pro-inflammatory state [186, 210, 211], popularly referred to as ‘inflamm-aging’ nowadays [212-215]. This constant inflammatory state is associated with multiple metabolic disorders and makes it hard to maintain a healthy lifespan in elderly. Like obesity-mediated inflammation, aging-associated inflammation is a persistent low-grade inflammation unlike the canonical inflammatory response. It might be caused by accumulated cell debris due to insufficient recycling and toxic mediators released from them, pathogenic organism debris resulting from impaired immune system during aging, increased cell senescence or immunosenescence of the immune system [212]. ROS in circulation is another factor going up during aging that can contribute to inflammation [216]. As some components of the immune response diminish with age, other components start to show a subtle hyper-activation disturbing the balance. As the traditional response to pathogens worsens, unnecessary chronic activation of inflammatory agents takes place.

Aging-related changes in immune cells is called ‘immunosenescence’ [217]. Aging can result in thymic atrophy that impairs T cell function, and while memory T cells are increasing with age, naïve T cells diminish, changing the T cell environment [218, 219]. Organ-specific B cell proliferation and diversity can also decrease with age [220]. Macrophages, on the other hand, are constantly activated which results in a constant pro-inflammatory state. These imbalances in the immune cell repertoire prevent the immune system from responding appropriately to stimuli.
Inflammaging is shown to be associated with increased instances of diabetes, cardiovascular disease, obesity and neurological disorders in elderly. Aging increases expression of inflammatory mediators in the cardiovascular system that may be linked to atherosclerosis [221]. Elevated circulating levels of IL-6 and [222] and TNFα [223] detected in elderly were found to be associated with mortality, and even used as risk markers in elderly [224, 225]. The exaggerated inflammatory response in aging also may be caused by declining anti-inflammatory IL-10 levels with age [226]. Even though a significant amount of studies focuses on inflammaging, the complete picture is not revealed yet. As more detailed studies are conducted on aging-induced inflammation, preventive methods can be developed to increase healthspan in late life.

1.3.3 Anti-inflammatory Approach for Diabetes and Insulin Resistance

Since chronic low-grade inflammation is correlated with serious health risks including diabetes, obesity, sarcopenia and cardiovascular disease [215], strategies to prevent this basal inflammation gain importance. Therapeutic interventions targeting inflammation are therefore being investigated to improve life quality in diabetic patients.

Salicylates, commonly used painkillers that block IKKβ pathway, were shown to prevent insulin resistance in lipid-induced, type 1 diabetic and obese rodents [33, 227, 228]. Salsalate (a prodrug dimer of salicylates) was in clinical trials for cardiovascular diseases and diabetes. The initial results of clinical studies for salsalate showed variance according to the patient characteristics. However, 7 independent clinical trials have shown some improvement of glycemia with salsalate [229-235]. One clinical trial suggested salsalate
consistently showed clinical efficacy in the treatment of type 2 diabetes in different patient groups [233]. Likewise, in another study salsalate-treated type 2 diabetic patients had lower insulin, c-peptide and glycemia levels [236]. A more recent clinical trial that assessed the long-term efficacy and safety of salsalate in patients who were being treated for type 2 diabetes showed that salsalate improved glycemia and decreased inflammation in type 2 diabetic patients [232]. However, body weight, low density lipoprotein cholesterol levels and urinary albumin levels were higher in the salsalate-treated group, suggesting possible adverse effects of salsalates in cardiovascular and renal metabolism. Also, a limitation of this study was ~300 patients were tested during one year, so a longer-duration trial was suggested as a next step. The effect of salsalates on cardiovascular disease is currently being investigated in clinical trials and has not been concluded yet (*Clinicaltrials.gov; Trial #NCT00624923 Targeting Inflammation Using Salsalate in CardioVascular Disease (TINSAL-CVD)). More studies are required to investigate the renal and cardiovascular effects of long-term salsalate treatment.

In obese patients, salsalate did not reduce coronary plaque formation during a recent clinical trial [237]. However, it reduced total white blood cell, lymphocyte, monocyte, and neutrophil counts and increased adiponectin levels without changing C-reactive protein levels. Moreover, fasting glucose and triglycerides levels in this study were lower in the salsalate treatment group compared with the controls suggesting a positive role for salsalate in metabolism [237].

In clinical trials with prediabetic patients, the results were less promising. Salsalate was reported to reduce fasting plasma glucose and HbA1c levels in the trials [230, 231,
However, while the first study suggested that salsalate treatment reduced insulin resistance [235], subsequent studies did not report any increase in peripheral insulin sensitivity and suggested additional mechanisms for metabolic improvement [230]. An intriguing finding in the prediabetic and non-diabetic obese patient trials was that salsalate reduced insulin clearance, which may be beneficial for diabetes treatment [230, 238, 239].

Circulating inflammatory cytokines are elevated in obesity, insulin resistance, and sarcopenia. TNFα antagonists like ‘etanercept’ and ‘infliximab’ that are used commonly for the treatment of rheumatoid arthritis and inflammatory bowel disease were shown to have preventive effects on insulin resistance and sarcopenia [240-244]. However, other studies did not report a significant improvement in glucose levels of type 2 diabetic patients treated with TNFα antagonists [245-248]. Anakinra, the antagonist for the major pro-inflammatory cytokine IL-1β, was also shown to improve euglycemia and β cell function, and decrease inflammation in type 2 diabetic patients [249-252]. This effect was not observed in nondiabetic obese subjects [253]. Another anti-inflammatory approach is to use natural endogenous anti-inflammatory cytokines. Studies in our lab previously showed that the major anti-inflammatory cytokine IL-10 prevented IL-6-mediated inflammation and insulin resistance in mice [172]. Additional studies by our lab found that IL-10 infusion and transgenic IL-10 expression in muscle improves insulin sensitivity in short-term HFD-fed mice [93]. However, the clinical trials of recombinant IL-10 on diabetes are halted for now (see below for a more detailed review of IL-10 treatment).

Although numerous clinical trials have shown that IL-1 antagonists and salsalate enhance glucose metabolism [254], the findings of clinical trials for anti-inflammatory
mediators are not completely conclusive up to now and a better understanding of the inflammatory basis for diabetes and further investigation are needed to develop anti-inflammatory therapies for inflammation-related pathologies.

1.4 Interleukin-10

1.4.1 Interleukin-10 Function and Signaling

IL-10 is a 40kD homodimer T₇₂ cytokine previously named as ‘cytokine synthesis inhibitory factor’ when it was first defined [255]. It is positioned on chromosome 1 at 1q31-32 and is very polymorphic [256]. Multiple single nucleotide polymorphism are found in the IL-10 5’ flanking region, and they form haplotypes that are associated with different IL-10 production levels. The IL-10 gene contains the code for a 178 amino acid length polypeptide [257]. IL-10 production is genetically controlled by IL-10 promoter, which is also highly polymorphic [256]. IL-10 is the most broadly characterized anti-inflammatory cytokine to date. As we can understand from the previous name, it acts to suppress pro-inflammatory response by blocking the synthesis and release of inflammatory cytokines and macrophage recruitment [226].

IL-10 is synthesized by several cell types including helper T (T₇) cells, B cells and macrophages [226]. While in acute inflammation the main source is macrophages, in chronic inflammatory phenotypes IL-10 is also secreted from T cells [258]. It is a pleiotropic cytokine that is shown to inhibit the production of pro-inflammatory cytokines IL-1, IL-6, interferon (IFN)‐γ, granulocyte-colony stimulating factor (G-CSF) and TNFα by monocytes [259, 260]. It also increases the release of anti-inflammatory soluble
antibodies for these cytokines [261, 262], suppresses major histocompatibility complex II (MHCII) expression [259], selectively inhibits transcription of inflammatory genes [263] and acts to suppress the expression of the chemokines that recruit macrophages [264]. Mice that are overexpressing IL-10 in their macrophages were shown to synthesize less pro-inflammatory cytokines [265]. Vice versa, when IL-10 is decreased inflammatory cytokine release increases [266]. IL-10 has an essential function in maintaining a healthy balance between pro- and anti-inflammatory responses. Deletion of IL-10 can result in colorectal cancer [267], inflammatory bowel disease [268], osteopenia [269], motor deficits [270], vessel stiffness, increased heart size and impaired heart function [271]. IL-10 overexpression was shown to suppress atherosclerosis in vivo [272]. Moreover, IL-10 promoter polymorphisms are linked to type 1 diabetes onset age [273].

IL-10 relays its signal through the IL-10 receptor. In most of the cells bearing IL-10 receptor, IL-10 induces both signal transducer and activator of transcription (STAT)1 and STAT3, but STAT3 is the major player [258, 265, 274, 275]. Our lab showed that IL-10 induces STAT3 phosphorylation also in skeletal muscle [93]. IL-10 binds to IL-10 receptor activating Jak proteins by trans-phosphorylation, and they in return, phosphorylate IL-10 receptor generating a docking site for STAT3 [274] (Fig. 1.2). STAT3 gets phosphorylated at the receptor, and then translocates to the nucleus to induce STAT3 regulated genes like suppressor of cytokine signaling (SOCS)3; which in return block activation of inflammatory genes [258]. IL-10 itself is immune to blocking by SOCS3 because it lacks SOCS3 recruitment motifs in IL-10 receptor. However, SOCS3 can block inflammatory cytokines like IL-6 and TNFα [276]. SOCS3 can also inhibit JNK
phosphorylation by binding to TNF-receptor associated factor 6 [277]. Moreover, STAT3 controls IL-10 promoter resulting in a positive feedback mechanism for increased IL-10 expression [278]. IL-10 can block NF-κB activity through suppression of IKK and DNA binding capability of NF-κB, or activation of STAT3 [279, 280].

Along with its suppressive roles, IL-10 also has the ability to convert M1 macrophages to M2 phenotype [281]. IL-10 was shown to polarize the adipose tissue macrophages recruited during obesity and skeletal muscle macrophages in dystrophic mice towards the M2 phenotype [206, 282]. M2 polarization of macrophages by Th2 cytokines in obesity is important because it was shown to boost insulin sensitivity [283]. It is important that IL-10 can signal directly on skeletal muscle. Although the main source for IL-10 production is immune cells, IL-10 expression was detected in mouse and human skeletal muscles [93, 191, 284] and myoblast cultures [285]. Detecting IL-10 in sole myoblast cultures suggests that muscle fibers are able to secrete IL-10 themselves. This finding is also supported by IL-10 release into circulation after exercise [286-288].

Our lab and others also showed that myoblasts express IL-10 receptor [93, 289], which suggests that IL-10 can actively signal through the skeletal muscle. The absence of IL-10 in the IL-10 knock out mice exacerbates response to inflammatory stimuli in the skeletal muscle [290]. Another finding suggesting the direct effect of IL-10 on skeletal muscle cells is when myoblasts are pretreated with IL-10 they can resist JNK phosphorylation induced by TNFα [289]. Moreover, IL-10 treatment on myoblasts can
Figure 1.2 IL-10 signaling
prevent TNFα-induced IL-6 expression and inflammatory effects of TNFα and IL-1β. Moreover, when IL-10 is knocked out IL-6 response upon inflammatory stimuli is elevated [277, 289, 291].

Overall, these data support the notion that IL-10 is an essential cytokine in controlling the immune response triggered by obesity in skeletal muscle and other tissues.

1.4.2 Interleukin-10 Receptor

The IL-10 receptor is a transmembrane glycoprotein of class II cytokine receptor family [292] with a docking site for STAT3 downstream signaling [293]. The IL-10 receptor has two chains: IL-10R1 required for STAT3 docking and IL-10R2 for class II cytokine signaling [294]. IL-10R1 has two tyrosine residues that when phosphorylated by Jak1 become docking sites for STAT3 [258]. IL-10R2 is an accessory part to bring tyrosine kinase 2 to the receptor complex to catalyze the trans-phosphorylation of IL-10R for mediation of IL-10 signaling [295].

IL-10R1, although expressed primarily in monocytes, is expressed in various tissue types including skeletal muscle, bone marrow, thymus, spleen, salivary gland, colon, liver, pancreas and lung [296]. The IL-10 receptor is detectable in myoblasts and muscles as shown by our lab and others [93, 282, 289]. Deficiency of IL-10 receptor causes severe defects in regulatory T cell and macrophage function and generation [297, 298]. Also, loss of IL-10 signaling due to IL-10 receptor ablation inhibits IL-10 release from intestine and bone marrow-derived macrophages, suggesting IL-10 needs to be stimulated by itself to increase its production [297].
1.4.3 Interleukin-10’s Use as a Therapeutic Approach

IL-10 was tested as a possible therapeutic agent in autoimmune diseases due to its anti-inflammatory properties. The current literature in the field involves mostly negative results as well as a few positive results. Even though the results in rodent models look promising, human application of recombinant IL-10 was not effective in most cases.

**Animal models:** Some studies conducted in rodent models suggest a positive effect of IL-10 in obesity and diabetes, depending on the method of delivery. Intramuscular injection of IL-10 containing plasmid to non-obese diabetic (NOD) mouse model reduced the incidence of diabetes [299]. Also, daily administration of human IL-10 reduced the severity of insulitis and promoted normal insulin production by β cells in NOD mice [300]. Likewise, our lab showed IL-10 co-treatment reduced the IL-6 and lipid-induced skeletal muscle and hepatic insulin resistance in mice [172]. However, the positive effects of IL-10 in diabetes have not been tested in humans and its disadvantages would possibly outweigh the benefits.

Very recently, Toita and colleagues synthesized and used IL-10-containing phosphatidylerine-liposomes that generate ‘eat me’ signals for macrophages to prevent the effects of obesity-induced chronic inflammation in mice [301]. This delivery method worked efficiently resulting in decreased cholesterol levels, inflammatory cytokine secretion from adipocytes, adipocyte size, and liver injury in mice. Another group tried to increase IL-10 levels by hydrodynamic gene delivery in mice resulting in rapid hepatic expression and increased levels in the circulation [302]. Oral gene delivery was also
successful for inflammatory bowel disease in mice [303]. Even though site-specific delivery of IL-10 is being studied widely in animal models, it is a big challenge to adapt it to humans without gene therapy. Application of recombinant IL-10 as a targeted therapeutic agent does not seem promising at the moment and still needs further improvements for managing site-specific delivery.

**Human Clinical Trials:** Recombinant IL-10 termed as ‘ilodecakin’ was produced by drug companies (mainly Schering-Plough, NJ, USA; Tenovil®) to be tested for autoimmune diseases. In the last 25 years in some diseases, recombinant IL-10 showed mildly improved results, whereas in others such as Crohn’s disease it was ineffective and had possible side effects [304-308]. Most current clinical data arise from trials in Crohn’s disease and psoriasis patients. In Crohn’s disease, the clinical trials did not reach significance [309, 310], even though clinical and endoscopic improvement was observed in some patients [304]. Researchers suggested the level of improvement may vary due to IL-10 levels of the patient himself or disease stage of the patient [305, 311]. When it is used at low doses it was usually well-tolerated [306, 312]. However, when used at high doses it showed serious side effects [308, 313]. In psoriasis, only temporary effectiveness was reported in some phase II trials [314], even though others suggested it decreased the relapse effectively [315]. Recombinant IL-10 human clinical trials to treat acute pancreatitis also failed and they were terminated last year. Renal transplant patients could tolerate low doses of IL-10, whereas high doses caused sensitization to immunosuppressant drugs [316]. No clinical improvement was observed in rheumatoid arthritis patients [317]. However, PEGylated IL-10 has been shown to induce the expansion of tumor-reactive T
cells and is currently being tested for anti-tumor activity in several phase I cancer trials [318].

Targeting persistent IL-10 expression to the desired tissue is an important challenge since the half-life of IL-10 is relatively short (~2 hours) [319] and it is an anti-inflammatory substance and a growth factor which could have potentially dangerous side-effects during long-term delivery.

1.4.4 IL-10 Expression in Obesity and Old Age

IL-10 is produced by many cell types and its expression during different metabolic states like obesity or aging is altered [226]. Hyperinsulinemia during obesity was shown to suppress IL-10 production by regulatory T cells [320]. Also, IL-10 levels measured from serum are inversely correlated with body weight, glucose intolerance and type 2 diabetes during hyperinsulinemic-euglycemic clamps [321]. In a study, IL-10 levels were shown to be elevated in obese women, yet low IL-10 levels in circulation were found to be associated with metabolic syndrome in this population [322]. However, in another study IL-10 promoter polymorphisms were linked to obesity and insulin resistance [323]. IL-10 levels are not only altered in circulation during obesity. Previous studies by our lab found that muscle IL-10 levels are decreased as well in HFD-fed and obese mouse models [93]. Even though there are a few studies suggesting otherwise, IL-10 levels were also decreased in adolescent obesity models. Studies performed on young adolescents showed decreased circulating IL-10 levels associated with obesity [324]. IL-10 was also found to have
receptors in the hypothalamus and prevent hyperphagia-related obesity due to increased insulin and leptin sensitivity in the hypothalamus [325].

IL-10 levels are altered during aging as well. There is contradicting data describing both elevated and decreased expression of IL-10 during senescence. Nevertheless, the majority of the studies support declining IL-10 levels with age. IL-10 expression was found to be decreased in muscles of elderly after exercise compared to young subjects [191]. Although IL-10 levels were decreased in elderly, certain genotypes associated with high IL-10 production were found to be related to longevity and successful aging in men [326]. Researchers have found that low IL-10 production capacity was correlated with high plasma glucose, high HbA1c, type 2 diabetes, and dyslipidemia. Also, the high production capacity of IL-10 was associated with muscle strength in aging people [194]. This effect was observed in both genders, since high IL-10 and low IL-6 levels due to single nucleotide polymorphisms in aging females were also associated with improved physical performance [327]. This was also true for animal models. Aging IL-10 knock out (KO) mice were shown to develop inflammatory pathway activation and have decreased muscle strength [328]. Moreover, the longer average lifespan of Dark Agouti rats was attributed to their ability to retain macrophage IL-10 production during aging [329]. IL-10 levels are not just decreased in circulation in elderly. Intracellular organ-specific content of IL-10 were also shown to be lower in skeletal muscles of aging mice [330]. In this study researchers measured IL-10 and IL-6 content in aging and young mice organ lysates (addressing the intracellular compartment) and organ-conditioned media. They found that intracellular organ expression of IL-10 in old vs young mice was lower as detected by ELISA and
immunohistochemistry. However, organ-conditioned media IL-10 levels were higher in the aging group. Lowered IL-10 levels in old age was also found to be associated with the insulin resistance. Leiden 85-Plus study that was performed with 599 elderly subjects has revealed that IL-10 production capacity is linked to metabolic syndrome and type 2 diabetes [331]. IL-10 decrease with age can also generate an elevated inflammatory phenotype. IL-6 expressions were highly elevated in skeletal muscles of old but not young IL-10 knock out mice in response to inflammatory stimuli [290].

Overall, the current literature mostly defines a decrease in IL-10 expression in obese and aging subjects which may result in an increased pro-inflammatory state; and polymorphisms that are linked to the higher expression of IL-10 are associated with a general healthier metabolic state.

1.4.5 Role of IL-10 in Whole Body and Muscle Glucose Metabolism

Since obesity-mediated insulin resistance is associated with chronic inflammation, the major anti-inflammatory cytokine IL-10 was a potential target in metabolism research for our lab and others. The Leiden study carried out in large populations revealed that low production capacity of IL-10 was associated with increased type 2 diabetes and metabolic syndrome occurrence [331]. Our lab was the first to show that IL-10 co-treatment prevents IL-6 and lipid-mediated insulin resistance in mice, especially in the skeletal muscle [172]. A 3-day IL-10 infusion also improved whole-body, hepatic and muscle insulin sensitivity [93]. Since our observation for insulin-sensitizing effect of IL-10 was mainly in muscle, our lab generated a transgenic mouse model that overexpresses IL-10 selectively in skeletal
muscle [93]. Short term HFD-fed ‘MIL10’ mice were protected from whole-body and skeletal muscle insulin resistance, and skeletal muscle inflammation. These experiments defined IL-10 as a potential anti-inflammatory agent to prevent inflammation-mediated insulin resistance in muscle.

Other labs have also shown that when LPS triggers a pro-inflammatory response in macrophages IL-10 is elevated, and muscle cells become more insulin sensitive. However, when fatty acids trigger pro-inflammatory response in macrophages inflammatory cytokines are released instead which impair insulin signaling in skeletal muscle [141]. In this study, IL-10 treatment of muscle cells improved insulin action and GLUT4 translocation in skeletal muscle fibers and decreased the actions of MCP-1 to impair insulin-stimulated glucose uptake in skeletal muscle [164].

The positive effects of IL-10 are not limited to muscle insulin resistance. IL-10 was also shown to ameliorate endothelial dysfunction during type 2 diabetes [332]. Furthermore, intermuscular injection of IL-10-containing vector ameliorated insulitis in type I diabetic mice [299]. In another study, intramuscular IL-10 coding-adeno-associated virus injection raised serum IL-10 levels 12-fold in obese Zucker rats and ameliorated renal function and insulin sensitivity [333].

A recent study showed that hydrodynamic i.v. delivery of IL-10 gene to HFD-mice prevented weight gain, insulin resistance, adipose tissue inflammation and ectopic fat accumulation in the liver [302]. The biggest limitation of this study according to us was although the whole-body insulin sensitivity was largely improved, the researchers did not
look at any parameters in skeletal muscle. In another study that has been very recently published, researchers injected an adeno-associated virus that is expressing IL-10 into ob/ob mice and raised plasma IL-10 levels 60-fold [334]. This resulted in decreased hyperphagia, obesity, insulin resistance and adipose tissue inflammation in ob/ob mice. The scientists proposed hypothalamus and white adipose tissue (WAT) as target tissues for IL-10 where STAT3 phosphorylation was increased. However, interestingly the researchers did not look at the effects in skeletal muscle where they injected the virus.

All these studies define IL-10 as a possible mediator in metabolism research and suggest further studies should follow [299-302, 325, 334].

1.5 IL-10 and Myogenesis

Interestingly IL-10’s positive effects on muscle are not confined to glucose metabolism. We have serendipitously discovered constructive effects of IL-10 on muscle mass during our studies. IL-10’s ability to prevent pro-inflammatory cytokine release is promising for preventing inflammation-mediated muscle loss. Several other studies that have been published recently also defined a constructive role for IL-10 in skeletal muscle growth. As an example, when the intestines of the IL-10 whole-body knock out mice (model for inflammatory bowel disease) were inoculated with a mixture of bacteria the mice showed decreased skeletal muscle growth and growth-related proteins and ribosome biogenesis [335]. In another study, IL-10 ablation in dystrophic mice decreased muscle structure and function; and IL-10 re-treatment reduced M1 activation, polarized muscle macrophages to M2 phenotype and increased muscle regeneration in dystrophic mice.
IL-10 was also shown to reduce myoblast necrosis in newborn rodents that had hypoxia-induced skeletal muscle injury [336].

One of the reasons that IL-10 can prevent muscle loss is, its abilities to suppress pro-inflammatory cytokines. Pro-inflammatory cytokines that are released during obesity-induced low-grade chronic inflammation such as TNFα and IL-6 can interfere with skeletal muscle growth. For example, it is shown that IL-6 infusion into skeletal muscles lowered muscle protein content and blocked muscle growth signaling and insulin’s anabolic effects [337]. TNFα, IL-1β, and IL-6 can be secreted by skeletal muscle cells as well as immune cells upon inflammatory stimuli, and they can decrease insulin-like growth factor-I (IGF-I) content in skeletal muscle [150, 338-340]. Also as a feedback loop, TNFα and IL-1 can increase IL-6 expression in myoblasts [152]. TNFα as an inflammatory cytokine was shown to block muscle protein synthesis, decrease protein content and myogenin expression which is essential for muscle differentiation [341-343]. Another inflammatory cytokine IL-1β was found to activate NF-κB, and the major muscle atrophy proteins muscle ring-finger protein 1 (MURF1) and muscle atrophy f-box (MAFbx) in mouse myoblasts [344, 345]. Expression of IGF-I in skeletal muscle, which is also an essential mediator of skeletal muscle growth, is also decreased with TNFα and IL-1β [338, 340, 346]. Research on myoblasts shows that IL-10 can completely suppress TNFα’s JNK-mediated negative effects to inhibit myogenin-induced myogenesis [289]. Inflammatory cytokines can also impair muscle growth by decreasing Akt activation. Akt activation is essential for muscle growth, and even acute conditional Akt activation in mice can induce rapid hypertrophy in skeletal muscles [347]. IGF-I also stimulates the muscle growth due to muscle load through
PI3K-Akt pathway [348, 349]. Akt/mTOR (mammalian target of rapamycin) signaling is especially important for muscles that undergo compensatory hypertrophy due to weight-bearing, as in the case of obesity [350].

Muscle wasting is associated with insulin resistant and insulin-deficient conditions and was observed in diabetic rodents [351, 352]. Insulin itself is a growth hormone [353]. Therefore, IL-10 by its insulin sensitizing effect may also exert a positive effect on muscle mass maintenance. IL-10 itself is also a potent growth factor for B lymphocytes [260], and skeletal muscle has receptors for IL-10 [93]. So, it is possible that it may act as a growth factor on muscles. More interestingly, IL-10 was found to have insulin-like effects in adipose cells that may explain IL-10 associated growth. It is observed that IL-10 treated adipocytes have increased IRS-I and PI3K signaling and show immediate phosphorylation of Akt [206, 354].

Although other researchers have not found a direct proliferative effect of IL-10 on myoblast cultures like we did; they have demonstrated that IL-10 treated myocyte-macrophage co-cultures show increased proliferation [355]. After muscle atrophy, IL-10 depletion impaired M1 to M2 conversion and decreased muscle growth [355]. M1 polarization can have adverse effects on muscle growth. Hence when myotubes are cultured with M1 polarized macrophages, the expression of atrophy genes like MAFbx or MURF1 were shown to increase [168]. Also, IL-10 was shown to restore IGF-I induced muscle growth that is suppressed by inflammation [277]. Specifically, researchers demonstrated that IGF-I induced-myogenin expression was inhibited by TNFα and IL-1β, and IL-10 could prevent this by intervening TNFα- and IL-1β-induced JNK
phosphorylation [277, 289]. In those studies, IL-10 was found to enhance differentiation and protect myoblasts when IGF-I and TNFα are present [289].

In the light of all these data, we can conclude that IL-10 has potential to prevent muscle wasting during insulin resistant conditions. Since we observed IL-10’s positive effect on multiple insulin resistant states it stands as a promising target. Therefore, more detailed studies should be conducted to define the mechanism of this effect.

1.6 Mouse Models

1.6.1 Long-term HFD-fed M<sup>IL-10</sup> Mice

M<sup>IL-10</sup> mice: M<sup>IL-10</sup> mouse was designed to generate a model that elevates IL-10 specifically in skeletal muscles within a physiological range [93]. In C57/BL6J mice IL-10 gene was placed under a 1.3 kb segment of a modified muscle creatine kinase promoter. Transgene expression was restricted to skeletal muscle to avoid any potential consequences of expression in cardiac muscle by using a promoter in which 3 E-boxes responsible for cardiac expression are mutated [93, 356, 357]. An untranscribed sequence of human growth hormone was also added after IL-10 sequence to increase transcription efficiency and make genotyping easier. IL-10 levels in skeletal muscle were only elevated 2-fold under chow and HFD to stay at a physiological level and avoid cytotoxicity. IL-10 levels were not increased in other metabolic organs such as liver, WAT or heart under chow diet [93]. Also under chow-fed conditions, M<sup>IL-10</sup> mice were similar to wild-type counterparts in terms of body weight, body composition, muscle structure, metabolic activity and physical appearance [93]. M<sup>IL-10</sup> mice fed with 3-week short-term diet were less hyperglycemic, less
hyperinsulinemic and more insulin sensitive than wild-type mice especially in the skeletal muscle [93]. This was due to the decreased macrophage numbers and pro-inflammatory cytokine levels in the skeletal muscles of short-term HFD-fed M\(^{IL10}\) mice.

1.6.2 MCK-IL10\(^{ob/ob}\) Mice

**Leptin-deficient ob/ob model:** Obesity is not just triggered in response to high-fat nutrition content. Obesity can also develop due to genetic mutations in the genes responsible for satiety mechanism. Adipocytes secrete a 16 kD weight hormone called leptin which is essential for transmitting a satiety signal to the hypothalamus [358]. It is synthesized by the \(ob\) gene where single point mutations result in morbid obesity when homozygous. This mutation was spontaneously discovered in Jackson Labs back in the 1950’s where they observed mice that were weighing as twice of their littermates [359]. Mice that have the homozygous mutation for \(ob\) gene are hyperphagic, hyperglycemic and hyperinsulinemic [360]. They fit in a model similar to type 2 diabetes symptoms, therefore are suitable models for studying genetic obesity-induced insulin resistance. Leptin is known to have a role in regulating immune response, since most immune cells express leptin receptors. It can protect against T cell and macrophage proliferation and death [361].

To study the effects of IL-10 in a non-high-fat diet-mediated obesity model we crossed M\(^{IL10}\) mice with \(ob/+\) mice (both on C57BL/6J background) to generate muscle creatine kinase (MCK)-IL10\(^{ob/ob}\) mice. The mice were back-crossed for several generations to obtain genotypically heterogeneous MCK-IL10\(^{ob/ob}\) mice that lack leptin and overexpress IL-10 specifically in skeletal muscle.
1.6.3 Aging M$^{IL10}$ Mice

Aging itself was shown to be associated with insulin resistance in human and mouse models [44, 362]. M$^{IL10}$ mice were generated on C57/BL6J background, and C57/B16 mice enter aging period by 18 months [363]. Therefore, for our aging model we maintained M$^{IL10}$ mice until they reach 18 months on standard chow diet. M$^{IL10}$ mice and WT siblings were then used in our metabolic experiments.

1.6.4 HFD-fed IL10R KO Mice

**MCK-Cre IL10R1 KO model:** Both IL-10 receptor 1 (R1) and 2 (R2) are necessary for IL-10 signaling [364, 365]. Binding of dimeric IL-10 crosslinks R1 and R2 chains and relays the signal [260]. Therefore, in our mouse model lacking the main R1 chain, no IL-10 signal is conducted in skeletal muscle fibers. To generate the muscle IL-10 receptor knock out model we crossed muscle creatine kinase-Cre mice with IL-10 Receptor 1 fl/fl mice kindly donated by Dr. Werner Muller from University of Manchester, UK. Since muscle creatine kinase was abundantly expressed in skeletal muscles, IL-10 receptor was excised from floxed sites by Cre-lox recombination [366]
Chapter II: Altered Interleukin-10 Signaling in Skeletal Muscle Regulates Obesity-Mediated Inflammation and Insulin Resistance

2.1 Abstract

Skeletal muscle insulin resistance is a major characteristic of obesity and type 2 diabetes. Although obesity-mediated inflammation is causally associated with insulin resistance, the underlying mechanism is unclear. To investigate the role of skeletal muscle inflammation in chronic obesity-mediated muscle insulin resistance, we examined the effects of chronic obesity in mice with muscle-specific overexpression of interleukin-10 (M\textsuperscript{IL-10}). After 16 weeks of a HFD, M\textsuperscript{IL-10} mice became markedly obese but showed improved insulin action compared to that of wild-type mice, which was largely due to increased glucose metabolism and reduced inflammation in skeletal muscle. Since leptin regulates inflammation, the beneficial effects of IL-10 were further examined in leptin-deficient ob/ob mice. Muscle-specific overexpression of IL-10 in ob/ob mice (MCK-IL-10\textsuperscript{ob/ob}) did not affect spontaneous obesity, but MCK-IL-10\textsuperscript{ob/ob} mice showed increased glucose turnover compared to that in ob/ob mice. Lastly, mice with muscle-specific ablation of IL-10 receptor (M-IL10R\textsuperscript{-/-}) were generated to determine whether IL-10 signaling in skeletal muscle is involved in IL-10 effects on glucose metabolism. After an HFD, M-IL10R\textsuperscript{-/-} mice developed insulin resistance with reduced glucose metabolism compared to that in wild-type mice. Overall, these results demonstrate IL-10 effects to attenuate obesity-mediated inflammation and improve insulin sensitivity in skeletal muscle, and our findings implicate a potential therapeutic role of anti-inflammatory cytokines in treating insulin resistance and type 2 diabetes.
2.2 Introduction

Obesity has emerged as a global problem in recent decades and is associated with numerous human diseases including insulin resistance, type 2 diabetes, and cardiovascular diseases [367, 368]. The underlying mechanism by which obesity induces numerous health problems remains poorly understood. In that regard, increasing evidence suggests an important role of dysregulated immune system in obesity-mediated insulin resistance [30, 369]. Obesity is characterized by altered levels of circulating cytokines, and adipose tissue macrophage and inflammation have been causally associated with insulin resistance [78, 370]. However, recent studies have challenged this earlier notion on the causal role of adipose tissue macrophages and inflammation in the development of insulin resistance [133, 371].

While adipose tissue is widely viewed as the epicenter of obesity-mediated inflammation, it is not the only organ shown to develop macrophage infiltration and inflammation in obesity. In fact, recent studies indicate that obesity-mediated inflammation and macrophage accumulation develop in multiple organs, including skeletal muscle, liver, pancreas, heart, and brain [89, 93, 372-374]. In that regard, our recent study found that exercise-mediated weight loss improved insulin action without affecting adipose tissue inflammation in diet-induced obese mice [133]. Additionally, improved insulin action following weight loss was associated with reduced local inflammation in skeletal muscle, suggesting an important role of muscle inflammation in obesity-mediated insulin resistance [133]. These findings clearly contest the “adipocentric” view of insulin resistance.
Therefore, we aimed to investigate the relationship of muscle inflammation with insulin resistance.

IL-10 is a Th2-type cytokine that inhibits the synthesis and activity of pro-inflammatory cytokines and counteracts Toll-like receptor-mediated inflammation [375-377]. We have previously shown that transgenic expression of IL-10 selectively in skeletal muscle improved glucose metabolism in mice after 3 weeks of an HFD [93]. While our previous data suggested a potential therapeutic role of IL-10 in type 2 diabetes, our interpretation was limited due to a short-term feeding of HFD not resulting in mice having developed type 2 diabetes phenotypes (i.e., hyperglycemia). We hypothesized that anti-inflammatory IL-10 expression in skeletal muscle would suppress local inflammation and insulin resistance during chronic obesity. Therefore, the current study was designed to specifically examine the role of IL-10 in markedly obese and diabetic mice after 16 weeks of an HFD (chronic HFD), a better representation of obese type 2 diabetic subjects. Additionally, leptin is an important adipocyte-derived hormone that is elevated in obesity and regulates numerous physiological functions including energy balance and inflammation [361]. Thus, the present study also examined the effects of muscle-specific transgenic expression of IL-10 on glucose metabolism in leptin-deficient ob/ob mice. Lastly, we examined whether IL-10 signaling in skeletal muscle is directly responsible for IL-10 effects on muscle glucose metabolism using a newly generated mouse model lacking IL-10 receptor 1 type chain selectively in skeletal muscle. Our findings indicate that selective targeting of IL-10 signaling in skeletal muscle improves glucose metabolism in obese and diabetic mice following chronic HFD or deficient in leptin and further
demonstrate that these effects are mediated by direct activation of IL-10 signaling in skeletal muscle.

2.3 Materials and Methods

**Chronic HFD in M^{IL10} mice** Male transgenic mice with muscle-specific overexpression of IL-10 (M^{IL10}) and wild-type (WT) littermates were fed with HFD (55% fat by calories; Harlan Teklad TD93075, Madison, WI, USA) or standard chow diet (Labdiet Prolab Isopro RMH 3000 5P75, St. Louis, MO, USA) *ad libitum* for 16 weeks (n=6/group). During chronic high-fat feeding, we performed a weekly measurement of body composition to determine the changes in whole-body fat and lean mass.

**Generation of MCK-IL10^{ob/ob} mice** We generated leptin-deficient mice with muscle-specific overexpression of IL-10 (MCK-IL10^{ob/ob}) by cross-breeding M^{IL10} mice with ob/+ heterozygous mice (purchased from the Jackson Laboratory, Bar Harbor, ME, USA). The F1 female MCK-IL10^{ob/+} mice were then intercrossed with male ob/+ mice to generate MCK-IL10^{ob/ob} mice. The metabolic studies were conducted in MCK-IL10^{ob/ob} mice that have been backcrossed for more than 5 generations (ob/ob, n=7; MCK-IL10^{ob/ob}, n=12).

**Generation of M-IL10R^{−/−} mice** Mice lacking IL-10 signaling in skeletal muscle (M-IL10R^{−/−}) were generated by cross-breeding MCK-Cre expressing mice (kindly donated by Dr. Roger J. Davis) and floxed IL10R1^{−/−} mice (kindly donated by Werner Muller). The metabolic studies were conducted in M-IL10R^{−/−} mice that have been backcrossed for several generations. M-IL10R^{−/−} mice and MCK-Cre mice (referred to as WT) serving as controls were fed with HFD or chow diet for 6 weeks (n=6-11/group). All mice were housed under controlled temperature (23°C) and light/dark cycle with free access to food.
and water. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

**Body composition and energy balance** Whole body fat and lean mass were non-invasively measured using $^1$H-magnetic resonance spectroscopy (MRS) (Echo Medical Systems, Houston, TX, USA). Indirect calorimetry and energy balance parameters including food/water intake, energy expenditure, respiratory exchange ratio, and physical activity were non-invasively assessed for 3 days using metabolic cages (TSE-Systems Inc., Bad Homburg, Germany). We used the TSE-Systems LabMaster platform with easy-to-use calorimetry featuring fully-automated monitoring for food and water and activity in x, y, z planes. Labmaster cages that are most similar to facility home cages were used, thereby allowing the use of bedding in the cage and minimizing any animal anxiety during the experimental period. The system provides intuitive software with flexibility for experimental setup and data utilization.

**Hyperinsulinemic-euglycemic clamp** Following chow or HFD, a survival surgery was performed at 5~6 days before clamp experiments to establish an indwelling catheter in jugular vein. On the day of clamp experiment, mice were fasted overnight (~17 hrs), and a 2-hr hyperinsulinemic-euglycemic clamp was conducted in conscious mice with a primed and continuous infusion of human insulin (150 mU/kg body weight priming followed by 2.5 mU/kg/min; Humulin, Eli Lilly, IN, USA) [172]. To maintain euglycemia, 20% glucose was infused at variable rates during clamps. Whole body glucose turnover was assessed with a continuous infusion of $[3^-3]$H]glucose (PerkinElmer, Waltham, MA, USA), and 2-deoxy-D-[1-$^{14}$C]glucose (2-[14C]DG) (PerkinElmer, Waltham, MA USA) was
administered as a bolus (10 μCi) at 75 min after the start of clamps to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamps, mice were anesthetized, and tissues were taken for biochemical analysis.

**Biochemical analysis and calculation** Glucose concentrations during clamps were analyzed using 10 μl plasma by a glucose oxidase method on Analox GM9 Analyser (Analox Instruments Ltd., London, UK). Plasma concentrations of [3-3H]glucose, 2-[14C]DG, and 3H2O were determined following deproteinization of plasma samples as previously described [172]. For the determination of tissue 2-[14C]DG-6-Phosphate content, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate 2-[14C]DG-6-P from 2-[14C]DG. Plasma insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Diagnostics, Salem, NH, USA). Intramuscular triglyceride concentrations were determined by homogenizing muscle samples (quadriceps) in chloroform-methanol and using a triglyceride assay kit (Sigma, St. Louis, MO, USA).

Rates of basal hepatic glucose production (HGP) and insulin-stimulated whole-body glucose turnover were determined as previously described [172]. Insulin-stimulated rate of HGP was determined by subtracting the glucose infusion rate from whole-body glucose turnover. Whole body glycolysis and glycogen plus lipid synthesis from glucose were calculated as previously described [172]. Insulin-stimulated glucose uptake in individual tissues was assessed by determining the tissue (e.g., skeletal muscle) content of 2-[14C]DG-6-phosphate and plasma 2-[14C]DG profile. The level of plasma non-esterified
fatty acids is measured by NEFA kit (Zenbio, Durham, NC, USA) according to manufacturer’s protocol.

**Molecular analysis for insulin signaling and inflammation** Skeletal muscle (quadriceps) and liver samples were collected at the end of clamp experiments to assess insulin signaling by immunoblotting with rabbit monoclonal antibodies against Akt and p-Akt-Ser\(^{473}\) (Cell signaling, Danvers, MA, USA). Muscle samples were homogenized, and plasma and homogenized muscle samples are used to measure levels of interleukin IL-6, IL-10, IFN-\(\gamma\), and IL-1\(\alpha\) by Luminex 200 Multiplex ELISA platform (Millipore, Darmstadt, Germany). Plasma IL-10 levels were measured using an ELISA kit (Abcam, Cambridge, UK). For quantitative RT-PCR (qRT-PCR), RNA isolation was performed with homogenized muscle (gastrocnemius and quadriceps), liver and WAT samples using TRIzol (Life Sciences, Carlsbad, CA, USA) according to manufacturer’s protocols. cDNA was synthesized from 2 \(\mu\)g of total RNA by use of Omniscript cDNA synthesis kit (Qiagen, Venlo, Netherlands). cDNA and SYBRgreen supermix (Bio-rad, Hercules, CA, USA) was run in Biorad-CFX96 real time system with the primers (Table 2.1). Relative gene expression was calculated compared to the expression of the housekeeping gene.

**Histologic analysis** Muscle samples (gastrocnemius) were collected, fixed in 10% neutral formalin for 48 hours, and embedded in paraffin blocks. 5 \(\mu\)m sections were taken and stained with hematoxylin-eosin (H&E). Images were taken under 20X magnification.

**Statistical analysis** Data are expressed as mean \(\pm\) standard error (SE) values. The significance of difference in mean values was determined using two-way analysis of
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68-Forward</td>
<td>TGTCTGATCTTGCTAGGACCG</td>
</tr>
<tr>
<td>CD68-Reverse</td>
<td>GAGAGTAACGGCCTTTTTGTG</td>
</tr>
<tr>
<td>F4/80 (Emr1)-Forward</td>
<td>TGACTCACCTTGTTGTCCTAA</td>
</tr>
<tr>
<td>F4/80 (Emr1)-Reverse</td>
<td>CTTCCCAGAACCCAGTCTTTTCC</td>
</tr>
<tr>
<td>G6Pase-Forward</td>
<td>CGACTCGCTATCTCAGAAGTGA</td>
</tr>
<tr>
<td>G6Pase-Reverse</td>
<td>GTTGAACCAGTCTCCGACCA</td>
</tr>
<tr>
<td>PEPCK-Forward</td>
<td>CTGCATAACGGTCTGGACTTC</td>
</tr>
<tr>
<td>PEPCK-Reverse</td>
<td>CAGCAACTGCCCCGTACTCC</td>
</tr>
<tr>
<td>IL-1β-Forward</td>
<td>GAAATGCCACCTTTTTGACAGTG</td>
</tr>
<tr>
<td>IL-1β-Reverse</td>
<td>CTGGATGCTCCTCATCAGGACA</td>
</tr>
<tr>
<td>IL-6-Forward</td>
<td>AGTTGCCTTCTGGGACTGA</td>
</tr>
<tr>
<td>IL-6-Reverse</td>
<td>TCCACGATTTCGCCAGAGAAC</td>
</tr>
<tr>
<td>IL-10-Forward</td>
<td>TGAATCCCTGGGTGAGAAG</td>
</tr>
<tr>
<td>IL-10-Reverse</td>
<td>GCTCCACTGCCTTGCTCTTA</td>
</tr>
<tr>
<td>TNF-α-Forward</td>
<td>CAGCCGGTGTCATTGTCTC</td>
</tr>
<tr>
<td>TNF-α-Reverse</td>
<td>CGATCACCCCCGAGTTAGTAG</td>
</tr>
</tbody>
</table>

Table 2.1. List of primers
variability analyzed using one-way analysis of variance (ANOVA) with Newman-Keuls and Games-Howell tests for post hoc analysis and Student’s t test where applicable. The statistical significance was set at $P < 0.05$ value.

2.4 Results

**Chronic feeding of HFD in $\text{M}^{\text{IL10}}$ mice** Starting at ~6-7 weeks of age, male $\text{M}^{\text{IL10}}$ and WT mice were fed with chow diet or HFD *ad libitum* for 16 weeks. At the end of the feeding period, all mice were age-matched at the time of metabolic studies. $\text{M}^{\text{IL10}}$ and WT mice showed similar body weights on chow diet, and after 16 weeks of HFD, both groups of mice became obese with comparable increases in body weights (Fig. 2.1 A). Consistent with this, whole-body fat mass, measured using $^{1}$H-MRS, was not different between chow-fed $\text{M}^{\text{IL10}}$ and WT mice, and fat mass increased by 3–4-fold after HFD in both groups of mice, being lower in $\text{M}^{\text{IL10}}$ mice (Fig. 2.1 B). Although whole-body lean mass was statistically different between the groups on chow diet (25.1±0.3 in WT mice vs. 23.5±0.6 in $\text{M}^{\text{IL10}}$ mice; $P=0.03$), this difference of 1.5 g of lean mass is within the range of variability for the lean mass of C57BL/6 mice. Whole body lean mass measurements after HFD did not differ between groups (Fig. 2.1 C). Metabolic cage analysis showed no significant difference in daily food intake, $\text{VO}_{2}$ consumption, and physical activity values between $\text{M}^{\text{IL10}}$ and WT mice after 16 weeks of HFD (Fig. 2.2).

$\text{M}^{\text{IL10}}$ mice are protected from HFD-induced insulin resistance Basal glucose levels increased after 16 weeks of HFD in both groups of mice, and plasma insulin levels were also elevated by more than 6-fold in both groups of mice after HFD (Fig. 2.1 D&E). To determine the effects on whole-body glucose metabolism, a 2-hr hyperinsulinemic-euglycemic clamp was conducted in awake mice. During clamp, plasma glucose levels
Figure 2.1 Metabolic profiles of mice with muscle-specific IL-10-overexpression (M^{IL10}) and WT mice on standard chow and after 16 weeks of an HFD. A: Body weight. B: Whole body fat and C: Whole body lean mass measured using $^1$H-MRS. D, E: Basal plasma glucose and insulin levels following overnight fast (~17 hrs). Values are means ± SE for 6 mice in each group. *$P$<0.05.
Fig 2.2 A 72-hour metabolic cage measurement in M<sup>IL10</sup> and WT mice on long term HFD feeding. A: Oxygen consumption rates B: Daily food intake and C: Hourly physical activity of M<sup>IL10</sup> and WT mice on 16 weeks of HFD. Values are means ± SE for 6 mice in each group.
were maintained at euglycemia (~7 mM), and plasma insulin levels were raised to ~130 pM and ~230 pM in both groups of chow and HFD-fed mice, respectively (Fig. 2.3 A&B).

The WT mice developed insulin resistance after 16 weeks of HFD as shown by a ~60% decrease in glucose infusion rates during clamp compared to the level in chow-fed WT mice (Fig. 2.3 C). Although glucose infusion rates also decreased in the \( M^{IL_{10}} \) mice after HFD, the glucose infusion rates in the HFD-fed \( M^{IL_{10}} \) mice were significantly higher than HFD-fed WT mice (Fig. 2.3 C). Radioactive isotope labeling data during the clamp showed significantly increased whole-body glucose turnover in the HFD-fed \( M^{IL_{10}} \) mice compared to the HFD-fed WT mice (Fig. 2.3 D), indicating that the \( M^{IL_{10}} \) mice were more insulin sensitive than WT mice after 16 weeks of HFD.

Increased insulin sensitivity in HFD-fed \( M^{IL_{10}} \) mice was largely due to a 30% increase in glucose uptake in skeletal muscle (quadriceps) (Fig. 2.3 E). Insulin-stimulated glucose uptake in gastrocnemius muscle also increased in HFD-fed \( M^{IL_{10}} \) mice compared to that in HFD-fed WT mice, this difference did not reach a statistical significance (Fig. 2.3 F). To further examine muscle insulin action, we performed Western blotting using skeletal muscle to assess insulin signaling and found that Ser-473 phosphorylation of Akt did not differ between WT and \( M^{IL_{10}} \) mice on chow diet, consistent with comparable muscle glucose uptake in these mice (Fig. 2.4 A). After 16 weeks of an HFD, muscle Akt phosphorylation was decreased by ~70% in WT mice (\( P=0.1 \)), and muscle Akt phosphorylation tended to be higher in HFD-fed \( M^{IL_{10}} \) mice than in HFD-fed WT mice (Fig. 2.5 A). H&E stained sections of skeletal muscle from WT and \( M^{IL_{10}} \) mice showed no obvious anomaly in the overall structure (Fig. 2.5 B). Additionally, intramuscular
Figure 2.3 A 2-hour hyperinsulinemic-euglycemic clamp in awake WT and $M^{IL10}$ mice after 16 weeks of HFD or chow diet to assess insulin sensitivity. A, B: Plasma glucose and insulin levels during the clamps. C: Steady-state glucose infusion rates during clamps. D: Whole body glucose turnover. E, F: Insulin-stimulated glucose uptake in skeletal muscle (quadriceps and gastrocnemius). Values are means ± SE for 6 mice in each group. *$P<0.05$. 

---

53
triglyceride levels tended to be elevated in chow-fed $\text{M}^{\text{IL10}}$ mice as compared to the levels in chow-fed WT mice (Fig. 2.5 C). After the HFD, intramuscular triglyceride levels were comparable between $\text{M}^{\text{IL10}}$ and WT mice. The local IL-10 mRNA levels in skeletal muscle were approximately 2-fold higher in HFD-fed $\text{M}^{\text{IL10}}$ than in HFD-fed WT (Fig. 2.5 D).

**Obesity-mediated inflammation in skeletal muscle is attenuated in $\text{M}^{\text{IL10}}$ mice** We have previously shown that inflammation develops in skeletal muscle after 3 weeks of HFD, and that this local inflammation may be causally associated with insulin resistance [93]. To that end, we assessed inflammation profile in skeletal muscle by performing qRT-PCR using samples obtained from HFD-fed $\text{M}^{\text{IL10}}$ and WT mice. Skeletal muscle mRNA levels of F4/80 and CD68, as markers of macrophage infiltration, were decreased significantly in HFD-fed $\text{M}^{\text{IL10}}$ mice as compared to HFD-fed WT mice (Fig. 2.6 A&B). Muscle MCP-1 mRNA levels also decreased in HFD-fed $\text{M}^{\text{IL10}}$ mice (Fig. 2.6 C). Using Luminex analysis, we found that local levels of IFN-γ, IL-1α and IL-6 were increased by 2- to 4-fold in skeletal muscle samples from WT mice after 16 weeks of HFD (Fig. 2.6 D-F). In contrast, muscle samples from HFD-fed $\text{M}^{\text{IL10}}$ mice showed completely normal levels of IFN-γ, IL-1α and IL-6 indicating that muscle IL-10 overexpression protected against diet-induced inflammation in skeletal muscle (Fig. 2.6 D-F). We did not observe a difference in circulating IL-10 levels in plasma (Fig 2.4 B). Plasma levels of IL-6 were not significantly different between WT and $\text{M}^{\text{IL10}}$ mice on chow or HFD. Plasma IL-1α levels tended to increase by more than 2-fold in WT mice after 16 weeks of HFD, whereas they were not significantly affected by HFD in $\text{M}^{\text{IL10}}$ mice. (Fig. 2.4 C&D).
Fig. 2.4  

A: Insulin-stimulated Akt phosphorylation of chow-fed WT and M_{IL10} mice quadriceps muscles.  

B, C, D: Plasma IL-10, IL-6 and IL-1α levels of same WT and M_{IL10} mice measured after 16 weeks of HFD (n=5-6/group).  

E, F: Liver CD68 and F4/80 macrophage marker mRNA levels in chow and HFD-fed WT and M_{IL10} mice (n=5-6/group).
Figure 2.5 Insulin signaling, muscle morphology and triglyceride and IL-10 levels in skeletal muscle. A: Insulin-stimulated Akt phosphorylation in quadriceps muscle. B: H&E staining of gastrocnemius muscles. C: Intramuscular triglyceride content (quadriceps) (n= 3-5/group). D: IL-10 mRNA levels in gastrocnemius muscles measured by quantitative RT-PCR (n= 4-5/group). Values are means ± SE for each group. *P<0.05.
Figure 2.6 Local inflammation in skeletal muscles of M$_{IL10}^{IL10}$ and WT mice after 16 weeks of chow or HFD. A-C: F4/80, CD68, and chemokine MCP-1 mRNA levels in quadriceps muscles of HFD-fed M$_{IL10}^{IL10}$ and WT mice as measured by qRT-PCR (n=3-5/group). D-F: IFN-γ, IL-1α, and IL-6 (F) levels (n=6/group). Values are means ± SE for each group. *$P<0.05$. 
**Obesity-mediated inflammation and insulin resistance in adipose tissue and liver**

Insulin-stimulated glucose uptake in white and brown adipose tissues (BAT) did not differ between chow-fed WT and $M^{IL10}$ mice, and HFD caused insulin resistance in adipose tissues in both groups of mice (Fig. 2.7 A&B). mRNA levels of the macrophage markers F4/80 and CD68 also increased in WAT by chronic HFD feeding but were not different between HFD-fed WT and $M^{IL10}$ mice (Fig. 2.7 C&D).

Basal HGP level did not differ between WT and $M^{IL10}$ mice on chow or HFD (Fig. 2.8 A). During the clamp, insulin decreased HGP in both chow-fed WT and $M^{IL10}$ mice (Fig. 2.8 B). HFD-fed WT mice developed insulin resistance in liver as indicated by increased clamp HGP, but HFD-fed $M^{IL10}$ mice showed lower clamp HGP than HFD-fed WT mice (Fig. 2.8B). Western blot analysis showed that neither phospho-Akt nor total Akt protein levels were altered between WT and $M^{IL10}$ mice on chow or HFD (Fig. 2.8 C). However, liver mRNA levels of G6Pase and phosphoenolpyruvate carboxykinase (PEPCK) tended to be lower in HFD-fed $M^{IL10}$ mice than HFD-fed WT mice (Fig. 2.8 D&E). mRNA levels of macrophage markers CD68 and F4/80 mRNA levels in liver were not significantly altered in HFD-fed $M^{IL10}$ mice, indicating that local inflammation was selectively suppressed in skeletal muscle (Fig. 2.4 E&F).

**IL-10 expressing leptin-deficient mice are protected from insulin resistance** Recent studies have shown deleterious effects of HFD on the intestinal epithelium and the role of gut microbes on obesity-mediated inflammation [378]. Thus, we have generated muscle-selective IL-10 expressing spontaneously obese mice by cross-breeding $M^{IL10}$ mice with heterozygous leptin-deficient $ob/+ $ mice. The offspring from this cross was further mated
Figure 2.7 Insulin-stimulated glucose uptake and inflammation in adipose tissues. A, B: Insulin-stimulated glucose uptake in white (epididymal) and brown (intrascapular) adipose tissues in WT and M\textsuperscript{IL10} mice fed chow or HFD. (n=4-7/group). C, D: F4/80 and CD68 mRNA levels in WAT of WT and M\textsuperscript{IL10} mice fed chow or HFD (n=5-6/group). Values are means ± SE for each group. *P<0.05.
Figure 2.8 Hepatic glucose metabolism and insulin signaling in WT and M<sup>IL10</sup> mice fed chow or HFD for 16 weeks. A: Basal hepatic glucose production. B: Clamp hepatic glucose production (n=5-6/group). C: Insulin-stimulated Akt phosphorylation in liver. D, E: Liver G6Pase and PEPCK mRNA levels (n=4-6/group). Values are means ± SE for each group. *P<0.05.
to obtain MCK-IL10\textsuperscript{ob/ob} mice. As expected, \textit{ob/ob} mice became obese spontaneously on chow diet, and by 16 weeks of age, whole-body fat mass accounted for more than 50% of body weight in \textit{ob/ob} mice (Fig. 2.9 \textit{A&B}). MCK-IL10\textsuperscript{ob/ob} mice also became spontaneously obese on chow diet and reached a comparable degree of obesity to \textit{ob/ob} mice at 16 weeks of age (Fig. 2.9 \textit{A&B}). Basal plasma glucose and fatty acids levels were similar in both groups of mice (Fig. 2.9 \textit{C & Fig. 2.10 \textit{A}}). A 3-day metabolic cage analysis showed that daily food intake and physical activity did not differ between groups (Fig. 2.9 \textit{D&E}). In contrast, MCK-IL10\textsuperscript{ob/ob} mice showed a modest but significant increase in energy expenditure that was largely due to elevated VO\textsubscript{2} consumption rates selectively at night cycle as compared to \textit{ob/ob} mice (Fig. 2.9 \textit{F&G}).

Hyperinsulinemic-euglycemic clamp study showed a significantly increased whole-body glucose turnover in MCK-IL10\textsuperscript{ob/ob} mice as compared to that in \textit{ob/ob} mice, suggesting increased insulin sensitivity in these mice (Fig. 2.11 \textit{A}). Consistent with this notion, insulin-stimulated glucose uptake in skeletal muscle tended to increase in MCK-IL10\textsuperscript{ob/ob} mice compared to that in \textit{ob/ob} mice although this difference did not reach a statistical significance (Fig. 2.11 \textit{B}). Glucose uptake in WAT and hepatic insulin action did not differ between groups (Fig. 2.11 \textit{C&D}).

**Mice lacking muscle IL-10 signaling are insulin resistant after HFD** To determine the cell type responsible for the downstream effects of IL-10, we have generated mice with muscle-specific ablation of IL-10 receptor (M-IL10R\textsuperscript{\textminus}) (Fig. 2.10 \textit{B&C}). Male M-IL10R\textsuperscript{\textminus} mice and MCK-Cre mice (as controls; WT) were fed HFD and chow diet \textit{ad libitum} starting at 7 weeks of age. Metabolic studies were performed after 6 weeks of HFD.
Figure 2.9 Metabolic effects of muscle-specific IL-10 overexpression in leptin-deficient ob/ob mice (MCK-IL10<sup>ob/ob</sup>). A-C: Body weight, whole-body fat mass using 1H-MRS, and fasting glucose levels were measured from 7 MCK-IL10<sup>ob/ob</sup> mice and 11 ob/ob mice. D-G: Daily food intake, physical activity, energy expenditure, and average of hourly VO<sub>2</sub> consumption were measured during a 3-day analysis of metabolic cages in 3 MCK-IL10<sup>ob/ob</sup> mice and 4 ob/ob mice. Values are means ± SE for each group. *P<0.05.
Fig 2.10 A: Plasma non-esterified FFA levels of ob/ob and MCK-IL10\(^{ob/ob}\) mice (n=6/group). Genotyping of M-IL10\(^{-/-}\) mice. B: PCR for Cre gene (450bp) C: IL10R LoxP PCR (WT band 220 bp floxed band 280 bp). D: Insulin-stimulated gastrocnemius and quadriceps glucose uptake levels in chow and HFD-fed WT and M-IL10R\(^{-/-}\) mice (n=5-8/group) and E: Skeletal muscle IL-10 levels in chow and HFD-fed WT and M-IL10R\(^{-/-}\) mice (n=4-6/group).
**Figure 2.11** A 2-hour hyperinsulinemic-euglycemic clamp in awake MCK-IL10\textsubscript{ob/ob} mice and ob/ob mice. A: Whole body glucose turnover in MCK-IL10\textsubscript{ob/ob} (n=7) and ob/ob (n=11) mice. B: Insulin-stimulated glucose uptake in skeletal muscle in gastrocnemius and quadriceps muscles. C: WAT (epidydimal) glucose uptake in MCK-IL10\textsubscript{ob/ob} (n=7) and ob/ob (n=11) mice. D: Hepatic insulin action reflected as insulin-mediated percent suppression of HGP in MCK-IL10\textsubscript{ob/ob} (n=7) and ob/ob (n=11) mice. Values are means ± SE for each group. *P<0.05.
and showed that both M-IL10R⁻/⁻ and WT mice gained similar fat masses after 6 weeks of HFD (Fig. 2.12A). Basal glucose levels were higher after HFD in M-IL10R⁻/⁻ mice than in WT mice (Fig. 2.12B). During hyperinsulinemic-euglycemic clamp, plasma glucose levels were kept at euglycemia (~7 mM) in both groups of mice. Strikingly, insulin-stimulated whole-body glucose turnover rates were significantly decreased and glucose infusion rates were lower in HFD-fed M-IL10R⁻/⁻ than HFD-fed WT mice (Fig. 2.12C&D). Hepatic insulin action during clamp did not differ between WT and M-IL10R⁻/⁻ groups under chow and HFD-fed conditions (Fig. 2.12E). Quadriceps and gastrocnemius muscle glucose uptake levels tend to be lower in M-IL10R⁻/⁻ mice than in WT mice after HFD (Fig. 2.10D). Also, local IL-10 levels in skeletal muscle tended to decrease in M-IL10R⁻/⁻ mice as a possible feedback loop of IL-10 signaling. (Fig. 2.10E). Skeletal muscle mRNA levels of F4/80 (macrophage marker) and inflammatory cytokine tumor necrosis factor (TNF)-α were significantly increased in WT mice after HFD (Fig. 2.12F&G). Importantly, HFD-fed M-IL10R⁻/⁻ mice showed significant further increases in F4/80, TNFα, IL-6 and IL-1β mRNA levels in skeletal muscle as compared to HFD-fed WT mice, indicating that muscle deletion of IL-10 receptor exacerbated HFD-induced local inflammation in skeletal muscle (Fig. 2.12F-I).
Figure 2.12 Metabolic profile, insulin action, and skeletal muscle inflammation were assessed in M-IL10R−/− and WT mice after 6 weeks of HFD or chow diet feeding. A: Whole body fat mass measured using 1H-MRS. B: Basal glucose levels. C: Glucose infusion rates during hyperinsulinemic-euglycemic clamp in awake mice (n=6-11). D: Whole body glucose turnover. E: Hepatic insulin action expressed as insulin-mediated percent suppression of HGP. F-I: F4/80, TNFα, IL-1β and IL-6 mRNA levels in skeletal muscles of M-IL10R−/− and WT mice fed chow or HFD (n=4-7/group). Values are means ± SE for each group. *P<0.05.
2.5 Discussion

Although adipose tissue macrophage accumulation and inflammation are characterized in obesity-mediated insulin resistance, recent studies refute the cause-and-effect relationship between adipose tissue inflammation and insulin resistance [30, 78, 133, 369, 371]. Lee et al. have shown that insulin resistance develops in the absence of adipose tissue inflammation after 3 days of high-fat feeding [371]. Our recent study also found that a short-term weight loss induced by low-calorie diet or exercise improves insulin sensitivity without altering adipose tissue inflammation [133]. These findings indicate that adipose tissue inflammation develops in obesity, but it may not be causally associated with insulin resistance in skeletal muscle, a major organ responsible for glucose disposal.

Obesity is a state of systemic inflammation, and local inflammation develops in multiple organs including skeletal muscle and liver [89, 93]. Importantly, recent studies have suggested a direct and causal role of skeletal muscle inflammation and muscle-derived cytokines in the development of insulin resistance [94, 147, 153, 168, 379, 380]. Our previous study has found that after 3 weeks of high-fat feeding, skeletal muscle was characterized by inflammation, insulin resistance and reduced glucose metabolism in skeletal muscle [93]. Thus, these findings are consistent with a notion that HFD-mediated local inflammation in skeletal muscle is causally associated with insulin resistance. However, the metabolic and inflammatory processes ongoing in metabolic tissues through short-term high-fat feeding may differ from the events following chronic obesity. Additionally, chronic obesity models that are better reflecting type 2 diabetes conditions potentially involve an increased insult from immune cells and inflammatory signaling in
skeletal muscle, adipose tissue and kidney as well as more compromised lipid metabolism and inflammation in liver [371, 381, 382]. Therefore, our current study addressed the effects of anti-inflammatory cytokine IL-10 under chronically obese conditions induced by a 16-week feeding of HFD. Moreover, leptin is known to regulate inflammation and immunity with its effects on T cell and macrophage-secreted cytokines [383, 384]. Thus, leptin-deficient model sed in current study rules out the effect of leptin on inflammation. Our newly generated MCK-IL10$^{ob/ob}$ model also circumvent direct effects of excess dietary fatty acid uptake and fatty acid induced inflammation/insulin resistance because these mice develop spontaneous obesity on a chow diet.

To study a more suitable chronic obesity and type 2 diabetes model in our current study, we examined the effects of long-term HFD on glucose metabolism and local inflammation in M$^{{IL10}}$ mice. After 16 weeks of HFD, WT mice became markedly obese with a 4-fold increase in whole-body fat mass. Intramuscular levels of IL-6, IFN-$\gamma$, and IL-1$\alpha$ were low in chow-fed mice, as expected, but after 16 weeks of HFD, local cytokine levels in skeletal muscle were elevated by 3 to 4-fold in WT mice. Some of these inflammatory cytokines may be released by locally infiltrating macrophages and/or surrounding adipocytes in skeletal muscle fiber. IL-6 was previously shown to induce insulin resistance by activating STAT3 and increasing intracellular levels of SOCS3, which may target insulin signaling proteins for ubiquitin-mediated degradation, causing insulin resistance [79, 385]. In contrast, IL-6 has been shown to be released by exercising muscle and to promote glucose uptake, this cellular event involves a much higher level of IL-6 than what is observed in obesity [386]. Furthermore, IL-1$\alpha$ has been shown to inhibit
insulin signaling by inducing serine phosphorylation of IRS-1 in adipocytes [387]. Consistent with this notion, insulin-stimulated Akt phosphorylation was reduced in the skeletal muscle of HFD-fed WT mice, supporting the cause-and-effect relationship between HFD-mediated increase in local macrophage and inflammatory cytokines and skeletal muscle insulin resistance.

Despite marked obesity after 16 weeks of HFD, M<sup>IL10</sup> mice were significantly more insulin sensitive than WT mice, which was largely due to increased insulin signaling and glucose metabolism in skeletal muscle. Intramuscular lipid content was also similar between WT and M<sup>IL10</sup> mice after HFD indicating that suppressing obesity-mediated inflammation in skeletal muscle can improve insulin sensitivity without altering intramuscular lipid content in obese mice. IL-10 is an anti-inflammatory cytokine previously known as “cytokine synthesis inhibiting factor (CSIF)” and produced by many immune cell types including CD4- T helper cells and macrophages [255, 307]. Similar to other cytokines, such as IL-6 and TNFα, myocytes have also been shown to express IL-10 and IL-10 receptors [93, 285]. IL-10 suppresses local inflammation by inhibiting synthesis and action of pro-inflammatory cytokines including TNFα, IL-1β and IL-6, as well as inhibiting macrophage activation [259, 388]. To that end, intramuscular injection of IL-10 DNA was shown to be effective in suppressing inflammation and preventing autoimmune diabetes in mice [299]. Our findings that obesity-mediated increase in IL-6, IFN-γ, and IL-1α in skeletal muscle was normalized in M<sup>IL10</sup> mice support the anti-inflammatory action of IL-10 in these mice. Also, HFD-fed M<sup>IL10</sup> mice had decreased levels of macrophage marker F4/80 and CD68 as well as reduced MCP-1 mRNA levels.
Importantly, these data indicate that improved glucose metabolism in skeletal muscle may be due to IL-10 effects to rescue local inflammation following chronic high-fat feeding in these mice. Also, inflammation was suppressed only in skeletal muscles in HFD-fed M^{IL10} mice; there was no difference in liver and adipose tissue inflammation. Plasma IL-10 levels were also not different in HFD-fed M^{IL10} mice. Furthermore, in contrast to skeletal muscle, white and BAT remained insulin resistant after HFD in M^{IL10} mice, supporting the muscle-specific expression of IL-10 and its effects on muscle glucose metabolism. However, we found that HGP during clamp state was significantly reduced in M^{IL10} mice as compared to WT mice after 16 weeks of HFD, suggesting improved insulin action in liver of HFD-fed M^{IL10} mice. Since IL-10 has been shown to be released by myocytes and its level has been shown to modestly increase in circulation [93], IL-10 may also be responsible for suppressing gluconeogenesis in these mice. We did not see a difference in liver Akt phosphorylation between HFD-fed groups; however, gluconeogenesis gene expression levels tended to decrease in M^{IL10} mice consistent with reduced HGP in these mice.

Recent studies have shown that HFD with selective composition of fatty acids may be directly responsible for systemic inflammation possibly by altering intestinal permeability or gut microbe population [378]. We along with others have also suggested that obesity-mediated inflammation is due to excess nutrient availability, imbalanced nutrient flux and metabolism, and activation of intracellular endoplasmic reticulum and oxidative stress [30, 79, 373]. In order to delineate the important question pertaining to the source of obesity-mediated inflammation, we determined the effects of IL-10 in spontaneously obese mice by cross-breeding M^{IL10} mice with leptin-deficient ob/ob mice.
As expected, IL-10 expressing *ob/ob* mice (MCK-IL10\textsuperscript{ob/ob}) became profoundly obese while on a chow diet with whole-body fat mass accounting for more than 50% of their body weight. Despite being markedly obese and having same level of plasma FFA, MCK-IL10\textsuperscript{ob/ob} mice were more insulin sensitive than *ob/ob* mice, further demonstrating IL-10’s potent insulin–sensitizing effects associated with its anti-inflammatory effect in skeletal muscle. Thus, these data indicate that obesity-mediated inflammation and insulin resistance in skeletal muscle are not dependent on dietary composition. Our findings suggest that IL-10’s insulin sensitizing effects are independent of leptin.

The beneficial effects of IL-10 to protect against obesity-mediated insulin resistance may be due to IL-10’s ability to suppress local inflammation and the deleterious action of pro-inflammatory cytokines in skeletal muscle. Gao and colleagues have recently shown that hydrodynamic delivery of mouse IL-10 protects against the HFD-mediated glucose intolerance that was associated with reduced inflammation in adipose tissue. However, since IL-10 receptors are expressed in multiple cell types including immune cells and myocytes [93, 226], IL-10’s direct action on myocytes cannot be ruled out. To further determine the mechanism of IL-10’s insulin sensitizing effects, we generated mice with muscle-specific ablation of IL-10 receptor. Since deletion was specific to IL-10 receptor type 1 we excluded only IL-10 signaling not all IL-10 cytokine subfamily members. After 6 weeks of HFD, M-IL10R\textsuperscript{−/−} mice became more insulin resistant than WT mice, indicating that absence of IL-10 signaling in skeletal muscle exacerbated diet-induced insulin resistance. Also, mRNA levels of macrophage markers and inflammatory cytokines are elevated profoundly in HFD-fed KO mice. These data suggest that IL-10 effects may be
mediated by intracellular IL-10 signaling in skeletal muscle. To that end, we have previously found that obesity-induced signal to activate local inflammation may involve oxidative stress and altered Ca\(^{2+}\) homeostasis in skeletal muscle [389]. Muscle IL-10 levels also tended to be lower in M-IL10R\(^{-/-}\) mice that might be result of feedback mechanism. Thus, IL-10 signaling in myocytes may relieve oxidative stress and block local inflammation, resulting in improved glucose metabolism in skeletal muscle. Further studies are needed to understand how IL-10 may affect oxidative stress in skeletal muscle.

In conclusion, our findings demonstrate that obesity due to chronic high-fat feeding or leptin deficiency causes local inflammation with marked increases in IL-6, IL-1\(\alpha\), and IFN-\(\gamma\) levels in skeletal muscle. Obesity-mediated muscle inflammation is completely attenuated by IL-10, and this anti-inflammatory action of IL-10 protects against muscle insulin resistance. Additionally, IL-10 signaling in myocytes may relieve obesity-induced oxidative stress and inflammation in skeletal muscle. Taken together, these results support our previous notion that IL-10 may be a potential therapeutic target to treat insulin resistance in type 2 diabetes. Though it has short half-life in vivo, local delivery of IL-10 is safe and may have positive effects in skeletal muscle insulin sensitivity [307]. While the eventual clinical application and method of delivery need further investigation, the role of IL-10 as an anti-inflammatory and insulin-sensitizing agent may open new doors in type 2 diabetes research.
Chapter III: Interleukin-10 Prevents Aging-Associated Inflammation and Insulin Resistance in Skeletal Muscle


73
3.1 Abstract

Altered energy balance and insulin resistance are important characteristics of aging. Skeletal muscle is a major site of disposal, and the role of aging-associated inflammation in skeletal muscle insulin resistance remains unclear. To investigate, we examined glucose metabolism in 18-month-old transgenic mice with muscle-specific overexpression of anti-inflammatory cytokine interleukin-10 (M^{IL10}) and wild-type mice during hyperinsulinemic-euglycemic clamps. Despite similar fat mass and energy balance, aging M^{IL10} mice were protected from aging-associated insulin resistance with significant increases in glucose infusion rates, whole-body glucose turnover and skeletal muscle glucose uptake (~60%, \(p<0.05\)) as compared to age-matched wild-type mice. This protective effect was associated with decreased muscle inflammation, but no changes in adipose tissue inflammation in aging M^{IL10} mice. These results demonstrate the importance of skeletal muscle inflammation in aging-mediated insulin resistance, and our findings further implicate a potential therapeutic role of anti-inflammatory cytokine in the treatment of aging-mediated insulin resistance.
3.2 Introduction

Aging is characterized by alterations in metabolism that resemble a physiological state of obesity and insulin resistance [44]. Molecular events underlying insulin resistance have been extensively studied but the mechanism of aging-associated insulin resistance remains poorly understood.

Adipose tissue inflammation develops in obesity, and macrophages and inflammatory cytokines are causally associated with insulin resistance [370]. Aging is also associated with a low-grade chronic inflammation state referred to as “inflamm-aging” that involves a multitude of inflammation-related events contributing to morbidity and mortality [212, 390, 391]. In that regard, circulating levels of inflammatory cytokines are shown to be elevated in elderly human subjects, and these aging-associated events are observed independent of obesity [392]. Our recent study has also found that aging promotes moderate obesity, adipose tissue inflammation, and insulin resistance in mice [391], consistent with findings from human subjects.

Aging-associated inflammation and increased cytokine response may be caused by a declining level of IL-10 during aging [226, 290]. IL-10 is an essential cytokine, mainly produced by macrophages, and is responsible for suppressing pro-inflammatory response in various tissues, including skeletal muscle [307]. IL-10 prevents inflammation by suppressing macrophages and blocking the antigen presentation, as well as the release and activity of inflammatory cytokines, such as IL-6, TNFα, and IL-1β [226, 393]. Consistent with this, IL-10 KO mice showed elevated levels of inflammatory cytokines following LPS
stimulation, and aging further exacerbated the inflammatory response in these mice [290]. Furthermore, IL-10 has a major beneficial impact on common inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, chronic inflammatory bowel diseases and multiple sclerosis [307]. Therefore, IL-10 can be a suitable immunomodulatory candidate for anti-inflammatory therapy against inflammation-mediated conditions [307]. Interestingly, Hacham et al., have shown that IL-10 expression in skeletal muscle is reduced in aging mice [330]. Therefore, we asked the question ‘Can suppression of local inflammation in muscle by IL-10 prevent muscle insulin resistance?’.

In that regard, we have previously shown that mice with muscle-selective overexpression of IL-10 (M\textsuperscript{IL10}) are protected from diet-induced insulin resistance in skeletal muscle [93, 172]. So, we hypothesized that anti-inflammatory cytokine IL-10 can prevent inflammation and insulin resistance locally in skeletal muscle. Thus, in the current study, we aimed to examine the effects of aging on energy balance, inflammation and insulin resistance in M\textsuperscript{IL10} mice.
3.3 Materials and Methods

**Animals** Male muscle creatine kinase M<sup>IL10</sup> mice (13) and WT littermates (C57BL/6J background) were studied from young (4 months of age) to aging (18 months of age) (n=8/group). All mice were housed in controlled temperature and light/dark cycle with free access to a standard chow diet (Prolab Isopro RMH 3000 5P75; St. Louis, MO, USA) and water. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

**Body composition and in vivo assessment of energy balance** Whole body fat and lean mass were non-invasively measured using <sup>1</sup>H-MRS (Echo-MRI; Echo Medical Systems, Houston, TX, USA). Food intake, physical activity, VO2, VCO2, energy expenditure rates, and respiratory exchange ratios were measured via indirect calorimetry in specialized single-housed metabolic cages (TSE-Systems Inc., Bad Homburg, Germany). For these metabolic cage measurements, acclimatized WT and M<sup>IL10</sup> mice at 12, 14, 16 and 18 months of age were fed a standard chow diet *ad libitum* at ambient temperature (20-23 °C) under a 12-hour light/dark cycle for 72 hours. Data were collected via equipment-associated software.

**Hyperinsulinemic-euglycemic clamp to assess insulin sensitivity** At 18 months of age, we conducted a 2-hr hyperinsulinemic-euglycemic clamp to measure insulin action and glucose metabolism in awake mice [394]. Briefly, the clamp began with a primed and continuous infusion of human insulin (150 mU/kg bolus followed by 2.5 mU/kg/min), and 20% glucose was infused at variable rates to maintain euglycemia. During the clamp, [3-
$^3$H]glucose was infused to assess whole-body glucose turnover, and 2-$[^{14}\text{C}]$DG was bolus injected at 75 min of clamp to measure glucose uptake in individual organs [394].

**Biochemical and molecular analysis** Plasma glucose levels during clamp were measured with a glucose analyzer (Analox, North Yorkshire, UK). Plasma concentrations of $[^3\text{H}]$glucose, 2-$[^{14}\text{C}]$DG, and $^3\text{H}_2\text{O}$ were measured as described previously [394]. Tissue and serum triglyceride concentrations were measured using triglyceride assay kit (Sigma, St. Louis, MO, USA) using plasma and tissue homogenates. Plasma insulin levels were determined by ultra-sensitive ELISA kit (ALPCO Diagnostics, Salem, NH, USA).

For insulin signaling analysis, skeletal muscle samples (gastrocnemius) were collected at the end of clamp, and protein lysates were prepared for Western blots using rabbit monoclonal p-Akt-Ser473 and Akt-Ser473 antibodies (Cell signaling, Danvers, MA, USA) with β-actin as a loading control. Gastrocnemius lysates were also used to measure local muscle levels of IL-1β, MCP-1, IL-10 and IFNγ using multiplexed-ELISA assay with Luminex 200 Multiplex Bio-Plex 200 System (Millipore, Darmstadt, Germany) using MILLIPLEX® MAP kits (Millipore, Billerca, MA). Plasma leptin, resistin, and adiponectin levels were also measured with Luminex. Muscle human growth hormone (hGH) levels are measured by ELISA in muscle homogenates (Alpco, Salem, NH, USA).

For qRT-PCR, RNA isolation was performed with homogenized skeletal muscle samples (gastrocnemius) and visceral WAT using TRIzol (Life Sciences, Carlsbad, CA, USA) following manufacturer’s protocols. cDNA was synthesized from 2 μg of total RNA by use of Omniscript cDNA synthesis kit (Qiagen, Venlo, Netherlands). cDNA and SYBERgreen supermix (Bio-rad, Hercules,CA) was run in Biorad-CFX96 (Hercules, CA)
real time system with the listed primers (Table 3.1). Relative gene expression was calculated using Bio-rad CFX manager software normalized to the RPL32 housekeeping gene.

**Histological analysis** Gastrocnemius muscles were extracted freshly and fixed in 10% formalin for overnight, and embedded in paraffin blocks. 5 µm sections were cut and stained with H&E to be observed under X20 magnification.

**3.3.6 Exercise study** Male M\(^{IL10}\) and WT mice at 18 months of age were used for exercise study using treadmills (n=4~5/group). After a 30-minute acclimatization period, treadmill speed gradually increased every 3 min between 7.5-15.9 m/min until the mouse was exhausted and stopped running. The stopping time and total distance were recorded for each mouse.

**Statistical analysis** Data are expressed as means ± S.E., and differences between groups were examined for statistical significance using analysis of co-variant analysis (ANCOVA) and 2 or 1-tailed Student’s t test. A probability value of p<0.05 was used as the criterion for statistical significance.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68-Forward</td>
<td>TGTCTGATCTTGCTAGGACCG</td>
</tr>
<tr>
<td>CD68-Reverse</td>
<td>GAGAGTAAACGGCCTTTTTGTG</td>
</tr>
<tr>
<td>Citrate synthase-Forward</td>
<td>GGGAAGGGCTAAGAACCCCTTG</td>
</tr>
<tr>
<td>Citrate synthase-Reverse</td>
<td>TTCATCTCCGTCATGCCATA</td>
</tr>
<tr>
<td>F4/80 (Emr1)-Forward</td>
<td>TGACTCACCTTTGGTGCTCTAA</td>
</tr>
<tr>
<td>F4/80 (Emr1)-Reverse</td>
<td>CTTCCCAAGATCCAGTCTTTCC</td>
</tr>
<tr>
<td>IL-6-Forward</td>
<td>AGTTGCCCTTCTGGGACTGA</td>
</tr>
<tr>
<td>IL-6-Reverse</td>
<td>TCCACGATTCCCAGAGAAC</td>
</tr>
<tr>
<td>IL-10-Forward</td>
<td>TGAATTCCCTGGGTGAGAAG</td>
</tr>
<tr>
<td>IL-10-Reverse</td>
<td>GCTCCACTGCCTTGCTCTTA</td>
</tr>
<tr>
<td>SDHA-Forward</td>
<td>GCTGTGTGGATGTCACTAAG</td>
</tr>
<tr>
<td>SDHA-Reverse</td>
<td>CCCACCCATGTTGTAATGCA</td>
</tr>
</tbody>
</table>

Table 3.1 List of primers
3.4 Results

**Body composition profile in aging mice** Longitudinal changes in body composition were recorded in male M\textsuperscript{IL10} and WT mice fed a standard chow diet from 4 to 18 months of age. Body weights of M\textsuperscript{IL10} and WT mice increased gradually over the first 12 months of age and plateaued with further aging (Fig. 3.1 A). Whole body lean mass also increased gradually with aging in both groups of mice, but between 13 to 16 months of age, M\textsuperscript{IL10} mice showed significantly higher lean mass than WT mice (Fig. 3.1 B). Whole body fat mass increased more rapidly in M\textsuperscript{IL10} mice than in WT mice during the first 8 months of age, but they were not significantly different with further aging (Fig. 3.1 C).

**Altered energy balance in aging mice** Aging-associated changes in energy balance were non-invasively assessed with metabolic cages in male M\textsuperscript{IL10} and WT mice at 12, 14, 16, and 18 months of age. Energy expenditure and VO\textsubscript{2} rates were significantly elevated in M\textsuperscript{IL10} mice as compared to WT mice at 16 months of age (Fig. 3.2 A and Fig. 3.3 A). VCO\textsubscript{2} rate also tended to be increased in M\textsuperscript{IL10} mice as compared to WT mice at 16 months of age (p=0.07; Fig. 3.3 B). Respiratory exchange ratio did not differ between aging M\textsuperscript{IL10} and WT mice and reflected consumption of chow diet (Fig. 3.2 B). Daily caloric intake was not significantly different between aging M\textsuperscript{IL10} and WT mice (Fig. 3.2 C). We measured circulating levels of leptin, a major hormone regulating feeding behavior, and found that serum leptin levels were significantly reduced in aging M\textsuperscript{IL10} mice as compared to aging WT mice (Table 3.2).

We performed qRT-PCR analysis in muscle samples obtained from young (4 months of age) and aging (18 months of age) mice to assess mitochondrial metabolic genes.
Figure 3.1 Body weight and body composition of M\textsuperscript{IL10}, and wild-type (WT) mice between 4-18 months of age (n=10-12 per group). A) Body weight. B&C) Whole body lean and fat masses measured using \textsuperscript{1}H-MRS. *P < 0.05 vs. WT mice.
Figure 3.2 Longitudinal assessment of energy balance using metabolic cages in aging $M^{IL10}$ and wild-type (WT) mice (n=6 per group). Data are averaged from a 3-day continuous measurement in mice at 12, 14, 16, and 18 months of age. A) Energy expenditure rate. B) Respiratory exchange ratio. C) Daily food intake (caloric intake normalized to body weight) D) Hourly physical activity. *P < 0.05 vs. WT mice. #P < 0.05 for vs. physical activity in WT mice at 12 months of age.
Figure 3.3 VO2 rate normalized to lean mass (A), VCO2 rate normalized to lean mass (B), and VO2 rate normalized to fat mass (C) in male M\textsuperscript{IL10} and WT mice at 12, 14, 16, and 18 months of age. Values are means ± SE for 6 mice per group. *P<0.05 vs. WT mice.
Skeletal muscle mRNA levels of uncoupling protein (UCP)3 and succinate dehydrogenase were significantly decreased in both aging groups of WT and $M^{IL10}$ mice when compared with young cohorts (Figure 3.4). Expression of citrate synthase in muscle also tended to decline in both groups of aging mice vs. young mice ($p=0.06$; Fig. 3.4 C). There were no significant differences in any of the mitochondrial respiration gene expressions between aging WT and aging $M^{IL10}$ mice.

Physical activity declined with aging and was significantly reduced in WT mice at 18 months of age compared with the same group of WT mice at 12 months of age (Fig 3.2 D). In contrast, this aging-associated decline in physical activity was not observed in $M^{IL10}$ mice (Fig. 3.2 D). As a result, $M^{IL10}$ mice showed a tendency for higher activity than WT mice at 18 months of age ($p=0.06$). To determine whether this affects the exercise capacity, we performed a treadmill exercise test and found no significant difference between aging $M^{IL10}$ and WT mice in total distance running on treadmill until exhaustion (Fig 3.5 A). Although the hGH sequences are not supposed to be translated, we measured muscle levels of growth hormone and found no difference between $M^{IL-10}$ and WT mice (Fig 3.5 B).

**$M^{IL10}$ mice are protected from aging-mediated insulin resistance** Basal glucose and insulin levels in overnight-fasted state did not differ between $M^{IL10}$ and WT mice at 18 months of age (Table 3.2). A 2-hour hyperinsulinemic-euglycemic clamp was performed to measure insulin action and glucose metabolism in awake age-matched $M^{IL10}$ and WT mice at 18 months of age. Steady-state rates of glucose infusion to maintain euglycemia during clamp were significantly increased by 60% in aging $M^{IL10}$ mice vs. aging WT mice.
Figure 3.4 Skeletal muscle mRNA levels of uncoupling protein (UCP) 3, succinate dehydrogenase, and citrate synthase in young (4 months of age) and aging (18 months of age) M<sup>IL10</sup> and WT mice. Values are means ± SE for 3-4 mice per group. *P<0.05 vs. young mice.
Metabolic profile in WT vs. aging M\textsuperscript{IL10} mice

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mg/dl)</th>
<th>Insulin (ng/ml)</th>
<th>Leptin (µg/ml)</th>
<th>Resistin (µg/ml)</th>
<th>Adiponectin (µg/ml)</th>
<th>Triglyceride (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>151±4</td>
<td>0.61±0.16</td>
<td>14±2</td>
<td>12±1</td>
<td>13±1</td>
<td>66±3</td>
</tr>
<tr>
<td>M\textsuperscript{IL10}</td>
<td>135±10</td>
<td>0.56±0.15</td>
<td>5±1*</td>
<td>7±1*</td>
<td>10±1</td>
<td>52±3*</td>
</tr>
</tbody>
</table>

Table 3.2 Metabolic profile in aging WT vs. M\textsuperscript{IL10} mice. Plasma levels of glucose, insulin, leptin, resistin, adiponectin, and triglyceride in overnight-fasted WT and M\textsuperscript{IL10} mice at 18 months of age (n=3-5/group). Values are means ± SE. *P<0.05 vs. WT mice.
Tracer analysis showed that insulin-stimulated whole-body glucose turnover rate was also increased by ~60%, and whole-body glycogen synthesis was increased 2-fold in aging M<sup>IL10</sup> mice (Fig. 3.6 B&C). Whole body glycolysis was not significantly different between the groups (Fig. 3.6 D). These data indicate that aging M<sup>IL10</sup> mice are more insulin sensitive than aging WT mice.

Increased insulin action in aging M<sup>IL10</sup> mice was largely caused by a 70% increase in insulin-stimulated glucose uptake in skeletal muscle (gastrocnemius) (Fig. 3.7 A). Because intramuscular lipid content is a major determinant of insulin action, we measured muscle triglyceride levels. They did not differ between aging M<sup>IL10</sup> and WT mice (Fig. 3.7 B). Muscle insulin signaling was determined with Western blots using antibodies against Akt and phospho-Akt. Total Akt protein levels in skeletal muscle did not differ between groups, but phospho-Akt levels were increased by more than 2-fold in aging M<sup>IL10</sup> mice vs. aging WT mice (p=0.05; Fig. 3.7 C). H&E staining showed that there were no obvious changes in skeletal muscle morphology between the groups (Fig. 3.7 D). We measured circulating hormones and metabolites known to affect insulin action using multiplexed Luminex and Cobas analyzer (Roche Diagnostics, Indianapolis, IN, USA), and found significant decreases in plasma leptin, resistin and triglyceride levels in aging M<sup>IL10</sup> mice vs. aging WT mice (Table 3.2). Plasma adiponectin levels did not significantly differ between groups.
Figure 3.5 (A) Total distance run on treadmill till exhaustion in $M^{IL10}$ and WT mice at 24 months of age. Values are means ± SE for 4-5 mice per group. (B) Muscle hGH levels in $M^{IL10}$ and WT mice at 18 months of age. Values are means ± SE for 7 mice per group.
Figure 3.6 A 2-hour hyperinsulinemic-euglycemic clamp to assess insulin action and glucose metabolism in awake M\textsuperscript{IL-10} and wild-type (WT) mice at 18 months of age (n=9-10 per group). A) Steady-state glucose infusion rates during clamps. B) Whole body glucose turnover. C) Whole body glycogen synthesis. D) Whole body glycolysis. *P < 0.05 vs. WT mice.
Figure 3.7 Insulin signaling and glucose/lipid metabolism in skeletal muscle of M<sup>IL10</sup> and wild-type (WT) mice at 18 months of age (n=7-10 per group). A) Insulin-stimulated glucose uptake in skeletal muscle (gastrocnemius). B) Intramuscular (quadriceps) triglyceride levels. C) Total Akt and insulin-stimulated Akt phosphorylation at Ser<sup>473</sup> in skeletal muscle (gastrocnemius). D) H&E staining of gastrocnemius muscle. *P < 0.05 vs. WT mice.
Aging-associated inflammation in skeletal muscle is reduced in M^{IL10} mice We have previously shown that local inflammation develops in skeletal muscle in obesity is causally associated with insulin resistance [93]. The qRT-PCR analysis of skeletal muscle samples showed that IL-10 mRNA expression was significantly reduced (>70%) with aging in WT mice (Fig. 3.8 A), and this is consistent with previous observations in aging humans [395]. Muscle IL-10 mRNA level in aging M^{IL10} mice was significantly increased (2.5-fold) compared with that in aging WT mice (Fig. 3.8 B). This finding was associated with significant decreases in CD68 (macrophage marker) and IL-6 mRNA levels in aging M^{IL10} mice vs. aging WT mice. Expression of TNFα mRNA in skeletal muscle also tended to be lower in aging M^{IL10} mice. These important changes in inflammatory gene expressions were further confirmed at the protein levels. Skeletal muscle IL-10 levels were significantly higher in aging M^{IL10} mice than in aging WT mice (Fig. 3.8 C). Local levels of inflammatory cytokines (IFN-γ and IL-1β) and chemokine (MCP-1) in skeletal muscles were all significantly reduced in aging M^{IL10} mice vs. aging WT mice (Fig. 3.8 C).

In contrast to the profound alterations in glucose metabolism and inflammation in skeletal muscle, insulin-stimulated glucose uptake in WAT and expression of F4/80 and TNFα in adipose tissue did not differ between aging M^{IL10} and WT mice (Fig. 3.9 A-C). Interestingly, glucose metabolism in BAT was significantly increased in aging M^{IL10} mice vs. aging WT mice (Fig. 3.9 D). Insulin-stimulated glucose uptake in the heart was not altered in aging M^{IL10} mice (Fig. 3.9 D). Furthermore, basal and clamp hepatic glucose production, hepatic insulin action and intrahepatic triglyceride levels were not significantly
Figure 3.8 Skeletal muscle mRNA and protein levels of inflammatory cytokines, macrophages, and chemokines in M\textsuperscript{IL10} and wild-type (WT) mice (n=3-8 per group). A) Muscle IL-10 mRNA levels in WT mice at 4 months (Young) and 18 months of age (Aging). B) Muscle mRNA levels of IL-10, CD68, IL-6, and TNFα in aging M\textsuperscript{IL10} and WT mice at 18 months of age. C) Muscle protein levels of IL-10, IFN-γ, IL-1β, and MCP-1 in aging M\textsuperscript{IL10} and WT mice at 18 months of age. *P < 0.05 vs. Young WT mice or WT mice.
Figure 3.9 Glucose metabolism and inflammation in adipose tissue and heart of aging M<sup>IL10</sup> and wild-type (WT) mice at 18 months of age. A) Insulin-stimulated glucose uptake in WAT (epidydimal) (n=9-10 per group). B) WAT (epidydimal) mRNA levels of F4/80 and TNFα (n=4-9 per group). C) Insulin-stimulated glucose uptake in BAT (intrascapular) (n=9-10/group). D) Insulin-stimulated glucose uptake in heart (n=4-5 per group). *P < 0.05 vs. WT mice.
Figure 3.10 Hepatic glucose production (HGP) and hepatic insulin action during hyperinsulinemic-euglycemic clamp in awake M<sup>IL10<sup>−/−</sup> and wild-type mice at 18 months of age (n=9-10 per group). A) Basal HGP. B) Insulin-stimulated HGP during clamp. C) Hepatic insulin action expressed as insulin-mediated percent suppression of basal HGP. D) Intrahepatic triglyceride levels. *P < 0.05 vs. wild-type mice.
altered in $M^{IL10}$ mice (Fig. 3.10). These data indicate that aging-associated inflammation and insulin resistance were selectively attenuated in skeletal muscle of $M^{IL10}$ mice.

### 3.5 Discussion

Metabolic changes during aging are closely related to several prevalent medical conditions, such as obesity, insulin resistance, type 2 diabetes, cardiovascular diseases and neurodegenerative disorders. We have previously shown that aging promotes modest obesity, adipose tissue inflammation and peripheral insulin resistance, and that these aging-associated disorders are ameliorated in mice with global deletion of protein tyrosine phosphatase 1B, a negative regulator of insulin signaling [391]. Because obesity is an aging-related phenomenon that is causally associated with insulin resistance, it is difficult to delineate the contribution of aging vs. obesity effects on insulin resistance. In that regard, our current results demonstrate that aging mice with muscle-selective overexpression of IL-10 are more insulin sensitive than aging WT mice with matched adiposity and demonstrate that aging-associated insulin resistance can be rescued independent of obesity. Our findings also indicate that beneficial effects of IL-10 were caused by reduced local inflammation and improved insulin signaling and glucose metabolism in skeletal muscle.

A preponderance of recent evidence has highlighted the role of adipose tissue in obesity-mediated inflammation and insulin resistance and contributed to an “adipocentric” view of type 2 diabetes [30, 78, 370]. However, aging-associated inflammation and insulin resistance may develop independent of obesity, suggesting that adipose tissue is not the primary organ responsible for dysregulated glucose metabolism in aging [392]. Likewise,
in our current study, the aging WT mice gained body weight and fat mass minimally from 4 to 18 months of age and did not reach a pronounced obese state despite developing insulin resistance in multiple organs. Energy expenditure tended to decline slightly, whereas physical activity was significantly reduced with aging in WT mice, consistent with previous findings [362, 396, 397]. However, both energy expenditure and physical activity were markedly elevated in aging M<sup>IL10</sup> mice as compared to aging WT mice, suggesting that M<sup>IL10</sup> mice were more metabolically active. Although we did not find significant differences in total distance running on treadmill until exhaustion, other exercise capacities may be altered in aging M<sup>IL10</sup> mice.

We have shown that IL-10 overexpression in skeletal muscle prevents HFD-induced insulin resistance in mice [93]. Our current study demonstrates that muscle-specific expression of IL-10 also protects against aging-associated insulin resistance, given that aging M<sup>IL10</sup> mice showed marked increases in whole-body glucose turnover and muscle glucose metabolism vs. aging WT mice. This effect was in part caused by increased muscle Akt phosphorylation in aging M<sup>IL10</sup> mice, supporting the previous notion that defects in muscle insulin signaling may underlie aging-associated insulin resistance [391]. Although ectopic lipid accumulation is causally related to obesity-mediated defects in insulin signaling [398], our findings of comparable intramuscular triglyceride levels in M<sup>IL10</sup> and WT mice suggest that other aging-associated factors are responsible for affecting muscle insulin signaling in aging mice.

Adipocyte-derived hormones, such as resistin, adiponectin, and leptin, are known to modulate insulin action, and their serum levels have been shown to increase with aging.
Resistin is a negative regulator of insulin sensitivity and has been shown to increase HGP and induce hyperglycemia [399, 400]. Aging M$^{IL10}$ mice showed significantly lower plasma resistin levels vs. aging WT mice. However, neither basal HGP nor hepatic insulin action was affected in aging M$^{IL10}$ mice, suggesting minimal effects of resistin in this model. In addition, aging M$^{IL10}$ mice showed significantly lower leptin levels than aging WT mice, although both groups had similar fat mass. In that regard, leptin levels usually correlate with adiposity, but this relationship has been shown to be affected by aging [403]. Last, serum triglyceride levels also increase with aging in both humans and animals [404, 405], and aging M$^{IL10}$ mice had a lower plasma triglyceride level than aging WT mice. Thus, aging M$^{IL10}$ mice had an overall improved metabolic profile vs. aging WT mice.

Previous studies have reported that IL-10 levels and signaling in skeletal muscle decrease with aging, and those findings are consistent with our current data showing lower IL-10 levels in aging WT mice [330, 406-408]. In contrast, M$^{IL10}$ mice were protected from this aging-associated effect as muscle IL-10 levels remained consistently higher and within a physiological range. This increase in IL-10 expression was able to suppress local inflammation in skeletal muscle during aging, because macrophage marker (CD68), chemokine (MCP-1), and inflammatory cytokines (IL-6, IFN-γ, IL-1β and TNFα) were all reduced in aging M$^{IL10}$ mice. The physiologic role of IL-10 during aging is largely unknown. IL-10 polymorphisms were found to be associated with longevity in men [409], and global IL-10 KO mice were shown to develop frailty and altered muscle energy metabolism [410].
It is important to point out that improved insulin sensitivity in aging $\text{M}^{\text{IL10}}$ mice is a result of selective increase in muscle glucose metabolism without affecting inflammation and metabolism in WAT, which is generally a major focus of the “obesity-inflammation-diabetes” paradigm. However, there was an increase in BAT glucose uptake in aging $\text{M}^{\text{IL10}}$ mice. This increase may be caused by similarities in the metabolic machinery and developmental origin between skeletal muscle and brown fat [411] and secondary effects of improved muscle insulin action. To that end, myocardial glucose uptake was not affected in aging $\text{M}^{\text{IL10}}$ mice. Furthermore, liver is an important organ in maintaining glucose homeostasis [412], and there were no significant effects of HGP or intrahepatic lipid levels in aging $\text{M}^{\text{IL10}}$ mice. Although the main source of IL-10 is macrophages [226], several studies have shown that IL-10 is expressed in mouse skeletal muscle and myoblasts [285, 330]. Thus, the profound effects of IL-10 in skeletal muscle may imply a physiologic function of IL-10 signaling on muscle inflammation and glucose metabolism.

As aging-related diseases often involve mitochondrial dysfunction [362, 413-415], we also examined muscle mitochondrial oxidative enzyme expressions in aging mice. Both WT and $\text{M}^{\text{IL10}}$ mice manifested significant decreases in expressions of mitochondrial oxidative enzymes in skeletal muscle with aging, and this effect was comparable between WT and $\text{M}^{\text{IL10}}$ mice. Thus, aging-associated alterations in mitochondrial metabolic genes did not presumably play a major role in improved metabolic activity in $\text{M}^{\text{IL10}}$ mice. In addition, suppression of muscle inflammation was shown to correlate with increased muscle function [242], but the aging $\text{M}^{\text{IL10}}$ mice did not exhibit obvious improvement in
muscle function based on the treadmill exhaustion test. However, future studies examining different aspects of muscle function and exercise capacity are clearly warranted.

In the transgenic construct of M^{IL10} mouse model the IL-10 sequence to be expressed were flanked downstream by a 2.2-kb segment of human growth hormone (hGH) sequence. The purpose of the addition of these sequences was to provide polyadenylation/termination signals and to increase transgene expression efficiency; these sequences were not to be translated. Even so, we measured the hGH levels in M^{IL10} and WT mice, and found no difference. This was important, because recently some researchers suggested that the inserted hGH sequences in some constructs might be translated [416]. If growth hormone was to be expressed in skeletal muscle it could effect muscle mass and muscle glucose metabolism, because growth hormone regulates the cellular growth and excess growth hormone was shown to decrease insulin sensitivity [417].

In summary, our results demonstrate that IL-10, as an anti-inflammatory immunomodulator, attenuates aging-associated inflammation and improves insulin signaling and glucose metabolism in skeletal muscle. Also, our findings imply an important role of skeletal muscle inflammation in aging-associated insulin resistance and further suggest a therapeutic potential of anti-inflammatory modulators in treating insulin resistance and metabolic abnormalities during aging. To that end, application of recombinant IL-10 in human has turned out to be safe in most of the clinical trials for the treatment of autoimmune diseases, neurodegenerative disorders, and several other conditions [307, 418, 419]. Thus, IL-10 may be a promising therapeutic agent for the prevention of aging-associated decline in muscle glucose metabolism.
Chapter IV: The Role of IL-10 in Myogenesis and Maintaining Lean Mass
4.1 Abstract

Obesity exerts pathophysiological effects on multiple organs including the musculoskeletal system. In the previous chapters, we have shown that mice with muscle-specific overexpression of IL-10 (MIL10) are protected from obesity-mediated inflammation and insulin resistance in the muscle. Serendipitously, while we were studying the IL-10-insulin resistance relationship, we found that MIL10 mice showed an increased lean mass phenotype compared to the WT mice during chronic high-fat diet (HFD) feeding (P<0.05). X-ray computerized tomography scans confirmed that the higher lean mass phenotype was due to the higher skeletal muscle mass. IL-10-expressing leptin-deficient ob/ob mice (MCK-IL10\textsuperscript{ob/ob}) also developed higher lean mass than ob/ob mice after 8 weeks of age. Moreover, aging MIL10 mice similarly showed higher lean mass than WT mice, whereas M-IL10R\textsuperscript{-/-} mice had less lean mass than the controls. Therefore, according to our observations, we hypothesized that IL-10 may be inducing skeletal muscle mass increase, especially during insulin resistant states. To investigate the effects of IL-10 on muscle growth, we incubated C2C12 myoblasts with IL-6, IL-10 and IL-10+IL-6 combination. IL-10 promoted myoblast proliferation but not differentiation. To determine the mechanism, the skeletal muscles of 16-week standard chow or HFD-fed WT and MIL10 mice were examined. qRT-PCR was performed to measure the expression of growth-associated proliferation and differentiation genes in the skeletal muscle. Expressions of proliferative myoblast determination protein (MyoD) and myogenic factor 5 (Myf5) genes in skeletal muscle were increased by 2 to 5-fold in MIL10 mice, whereas no difference was observed between groups in differentiation genes. Lean mass was found to be correlated with IL-10
and MyoD expression levels in the skeletal muscles of the $M^{IL10}$ mice. Overall, our findings suggest that physiological growth of skeletal muscle to support increased body weight may be affected by the obesity-induced inflammation and insulin resistance and IL-10-mediated suppression of inflammation and insulin resistance restores healthy muscle growth during obesity.
4.2 Introduction

Increased fat mass during obesity stimulates muscle growth due to the weight-bearing effect. In early obesity, muscle and fat masses are correlated with each other [420]. Later on, chronic obesity-induced insulin resistance may impair the ability of the skeletal muscle to undergo load-induced muscle growth [421]. Moreover, increased insulin levels during early obesity due to insulin resistance is another factor that may promote muscle growth at the initial stage, since it is known that resting muscle needs insulin to increase in size [422]. Total muscle mass in extremities were shown to be significantly associated with increased insulin levels in overweight and obese subjects [423]. We hypothesize that after morbid obesity during diabetes the muscle cannot compensate the growth anymore, and insulin signaling is impaired, so compensatory muscle growth ceases. At the extremely obese and insulin resistant state, skeletal muscle also secretes myostatin which stops skeletal muscle growth [424, 425]. Since skeletal muscle is the primary glucose uptake site in the body [426], increased muscle mass also improves insulin sensitivity as a feedback loop [427].

One phenomenon occurring in this extremely obese-diabetic phase is the chronic low-grade inflammation of the skeletal muscle [93, 147]. Elevated inflammation in the skeletal muscle results in decreased insulin signaling and increased insulin resistance [93]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine that suppresses macrophage activation and pro-inflammatory cytokine synthesis and action [226]. We have previously shown that anti-inflammatory cytokine IL-10 improved insulin sensitivity in skeletal muscles of obese mice [93, 172]. Serendipitously, while studying insulin resistance, we
discovered that transgenic M^{IL10} mice that are overexpressing IL-10 in skeletal muscles had higher lean mass than WT mice after long-term HFD. This finding led us to study the relation between IL-10 and skeletal muscle growth during insulin resistant states. Thus, we asked the question ‘Does IL-10 induce muscle mass increase during insulin resistant states?’ To answer this question, we analyzed the muscles of the transgenic animals during insulin resistant conditions.

Myogenesis is regulated by transcription factors called as ‘Myogenic regulatory factors’ (MRFs). The four MRFs: -MyoD, Myf5, Myogenin and Mrf4- are the major regulators of myoblast growth and differentiation [428]. MyoD and Myf5 are the early myoblast proliferation genes and in the absences of MyoD and Myf5 muscle does not develop [429]. MyoD is shown to convert fibroblasts into myoblasts and is required for adult stem cell function [430, 431]. It is shown to be activated 3 hours after the satellite cell activation [432]. Myogenin, on the other hand, is expressed following MyoD, and is the major determinant for the myoblast differentiation [430]. Myocyte enhancer factor 2C (MEF2C) is another transcription factor that takes place during muscle differentiation [433-435]. Major muscle catabolism mediator MAFbx ubiquitin ligase degrades MyoD to inhibit muscle growth [431]. Skeletal muscle alpha-actin and myosin heavy chain I (MHCI) are also fundamental for the control of muscle mass and function since they comprise most of the structural and contractile proteins in the skeletal muscle [436, 437]. We hypothesized that IL-10 might be affecting the expression of these regulatory genes to increase muscle growth.
Myoblasts are somite-derived muscle cell progenitors that can differentiate to form skeletal muscle myofibers [438]. C2C12 cells are commonly used murine myoblasts to study myogenesis [439]. Therefore, in this study, we measured the expression levels of myogenic markers after long-term obesity in IL-10-expressing mouse muscles and IL-10 induced C2C12 myoblasts to investigate the role of IL-10 on proliferation of myocytes and muscle tissue growth. Overall, here we show that lean mass correlates with IL-10 and MyoD mRNA expressions in M\(^{IL10}\) transgenic mice, suggesting a constructive role for IL-10 in maintaining muscle mass during extremely obese and insulin resistant states.

4.3 Materials and Methods

**Animals** For the HFD study, 12 week-old M\(^{IL10}\) male transgenic mice with muscle-specific overexpression of IL-10 and WT littermates were fed with HFD (55% fat by calories; Harlan Teklad TD93075, Madison, WI, USA) or standard chow diet (Labdiet Prolab Isopro RMH 3000 5P75, St. Louis, MO, USA) *ad libitum* for 16 weeks (n=9/group). For spontaneous obesity study, leptin-deficient transgenic mice with muscle-specific overexpression of IL-10 (MCK-IL10\(^{ob/ob}\)) and ob/ob controls were fed with a standard chow diet *ad libitum* for 11 weeks (n=6/group). For the aging group, M\(^{IL10}\) male transgenic mice and WT littermates were fed with a standard chow diet for 12 months. For the last study, male muscle-specific knock down of IL-10 receptor 1 mice and MCK-Cre controls are fed with HFD for 5 weeks. All mice were housed under controlled temperature (23°C) and light/dark cycle with free access to food and water. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.
Lean mass measurements in mice  Whole body fat and lean masses were non-invasively measured using Echo-MRI (Echo Medical Systems, Houston, TX, USA) weekly or monthly. X-ray computerized tomography of mice are performed by The UMMS Small Animal Imaging Core Facility. The muscle:fat ratios were calculated according to the average densities calculated for skeletal muscle and adipose tissue by Vivoquant 1.22 program (Invicro, Boston, MA, USA). Muscle wet weights of freshly collected mouse muscles were measured on a sensitive balance.

Biochemical and molecular analysis  For qRT-PCR, RNA is isolated from gastrocnemius muscle samples using TRIzol (Life Sciences, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA was synthesized from 2 µg of total RNA by use of Omniscript cDNA synthesis kit (Qiagen, Venlo, Netherlands). cDNA and SYBRgreen supermix (Bio-rad, Hercules, CA, USA) was run in Biorad-CFX96 real time system with the listed primers (Table 4.1). Relative mRNA expressions were calculated compared to the housekeeping gene.

Flow cytometric analysis  Left quadriceps muscles from anesthetized animals were isolated and minced in Dulbecco’s modified Eagle’s Medium (DMEM) (Gibco, Waltham, MA, USA) + 5% fetal bovine serum and 0.2% collagenase (Sigma-Aldrich, St Louis, MO, USA). They were shaken at 37°C for one hour, passed through a 70-µm filter, and centrifuged with a 70/40 Percoll (Sigma-Aldrich, St Louis, MO, USA) gradient to isolate mononuclear cells. Cells were counted and resuspended in the same medium. Single-cell suspensions were incubated in 2.4G2 hybridoma supernatant to block FcγR binding for 15 minutes and combined with CD11b, CD11c, F4/80, CD86 and CD206 conjugated
antibodies (Ebioscience, San Jose, CA, USA). Cells were fixed in 4% paraformaldehyde and then resuspended in fluorescence-activated cell sorting buffer (1× PBS/1.5% FCS/0.05% NaN3). Suspensions were analyzed on a Becton Dickinson (Franklin Lakes, NJ, USA) LSRII flow cytometer. At least 20,000 events were collected for each sample, and data were analyzed using FlowJo software (Treestar, CA, USA).

**Cell culture experiments** For proliferation experiment, 1x10⁴ C2C12 mouse myoblast cells in DMEM+10% FBS+Pen/Strep medium were seeded as 3 wells per group in 12-well plates. 25 ng IL-10, IL-6 or IL-10+IL-6 (Cell signaling, Danver, MA, USA) were added to each well daily. No cytokines were added to the negative control. Cells were cultured for 48 and 72 hours in the growth medium. At the end of 48 and 72 hours, the cells were trypsinized and counted twice per well using a hematocytometer. For differentiation experiment, 2x10⁶ C2C12 myoblast cells were seeded as 2 well/group into 12-well plates in DMEM+10% FBS growth medium and were allowed to reach confluency for 2 days. After 90% confluence was reached, medium was changed to differentiation medium (High glucose DMEM+2% Horse serum+ Pen/Strep) and 10nM insulin and 25 ng IL-10, IL-6, IL-10+IL-6 or no cytokine were added to each well daily for 3 days except the control wells. At day 4, RNAs were isolated with TRIzol (Life Sciences, Carslbad, CA, USA) for cDNA synthesis. qRT-PCR was performed as described above.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-actin-Forward</td>
<td>CCCAAAGCTAACGGGGAGAAG</td>
</tr>
<tr>
<td>Alpha-actin-Reverse</td>
<td>CCAGATCACAACAGATGGCC</td>
</tr>
<tr>
<td>IL-10-Forward</td>
<td>TGAATCCCTGGGTGAGAAG</td>
</tr>
<tr>
<td>IL-10-Reverse</td>
<td>GCTCCACTGCCCTGCTCTTA</td>
</tr>
<tr>
<td>MAFbx-Forward</td>
<td>GGCGATCGAGAATGCAGTC</td>
</tr>
<tr>
<td>MAFbx-Reverse</td>
<td>CAGCTTCGTGCAGACCT</td>
</tr>
<tr>
<td>MEF2C-Forward</td>
<td>ATCCGATGCGACGATTCAG</td>
</tr>
<tr>
<td>MEF2C-Reverse</td>
<td>AACAGCACAATCTTTGCCT</td>
</tr>
<tr>
<td>MHCII-Forward</td>
<td>TGGAGGCCAGGTTCTTGAA</td>
</tr>
<tr>
<td>MHCII-Reverse</td>
<td>ATTTTCTCCGCTTTCTCAG</td>
</tr>
<tr>
<td>Myf5-Forward</td>
<td>TATGAAGGCTCTGTATCCC</td>
</tr>
<tr>
<td>Myf5-Reverse</td>
<td>ACGTGCTCTCATGCTCTG</td>
</tr>
<tr>
<td>MyoD-Forward</td>
<td>CACTCCGGGACATAGACTTG</td>
</tr>
<tr>
<td>MyoD-Reverse</td>
<td>AAAACGCAGGTCTGGTGA</td>
</tr>
<tr>
<td>Myogenin-Forward</td>
<td>CTACAGGGCCTGCTCAGTC</td>
</tr>
<tr>
<td>Myogenin-Reverse</td>
<td>AGATTGAGGGGTGTACCTG</td>
</tr>
</tbody>
</table>

Table 4.1 List of primers
4.4 Results

**IL-10 promotes muscle growth in mice** M$^{IL10}$ mice that are overexpressing IL-10 in the skeletal muscle and WT littermates were fed with a 55% fat by calorie-diet for 16 weeks. During the first weeks of HFD feeding, lean mass of both groups increased with time. But after 10 weeks of HFD, WT group lean mass levels began to reach a plateau. Lean mass of M$^{IL10}$ group measured by $^3$H-MRI were lower than WT group at the beginning. However, lean mass of M$^{IL10}$ mice continued to increase linearly, and after 12 weeks of chronic HFD feeding became significantly higher than WT mice (Fig. 4.1 A). Lean mass of chow-fed MCK-IL10$^{ob/ob}$ mice also became significantly higher than ob/ob mice by 7 weeks of age (Fig. 4.1 B). When chow-fed M$^{IL10}$ mice gained weight, and started to become insulin resistant after 8 months of age, they also showed higher lean mass than WT mice (Fig. 4.1 C). Last, we fed M-IL10R$^{-/-}$ mice that are lacking IL-10 receptor in skeletal muscles along with controls with HFD for 6 weeks to confirm our findings. Confirming our hypothesis, M-IL10R$^{-/-}$ mice started to demonstrate lower lean mass phenotype by the first week of HFD (Fig. 4.1 D). To verify that higher lean mass measurement by $^3$H-NMR is reflecting the higher skeletal muscle mass, we calculated muscle:fat ratio using X-ray computerized tomography. By using the density values of skeletal muscle and fat, we calculated muscle:fat ratios in the whole-body, upper, and lower extremities in WT and M$^{IL10}$ mice fed with 16 weeks of HFD (Fig. 4.2). There wasn’t any difference between chow-fed M$^{IL10}$ and WT mice. WT-HFD group muscle:fat ratio was decreased compared to the WT-chow group as expected. Muscle:fat ratio of the M$^{IL10}$-HFD mice were almost two-fold higher.
Figure 4.1 Lean mass phenotypes of IL-10 transgenic and M-IL10R⁻/⁻ mice. Whole body lean masses of A: M$_{IL10}^+$ and WT mice on 16 weeks of HFD (n=9) B: MCK-IL10$^{ob/ob}$ and ob/ob mice on chow diet (n=6) C: aging M$_{IL10}^+$ and WT mice on chow diet (n=20) and D: M-IL10R⁻/⁻ mice on chow diet (n=6-11) as calculated by H$^1$-MRI. Values are means ± SE in each group. *P<0.05.
Figure 4.2 Muscle: fat ratios calculated by X-ray computed tomography of WT and $\mathbf{M}^{IL_{10}}$ mice. Whole body, upper extremities and lower extremities muscle: fat ratio of WT and $\mathbf{M}^{IL_{10}}$ mice on 16 weeks of HFD or chow diet calculated according to densities of the scanned tissues.
Figure 4.3 Muscle weights of WT and M\textsuperscript{IL10} mice fed with HFD for 16 weeks. Measured wet weights of A: Quadriceps (QD), B: Tibialis anterior (TA) and C: Soleus (SOL) muscles of WT and M\textsuperscript{IL10} mice fed with HFD for 16 weeks (n=3-4). Values are means ± SE in each group. *P<0.05.
than WT-HFD mice, agreeing with the higher lean mass phenotype calculated by the MRI. We also isolated fresh skeletal muscles to measure their wet weights. Fast-twitch quadriiceps and tibialis anterior muscles were measured to be slightly higher in HFD-fed M\textsuperscript{IL10} group compared to WT group (Fig. 4.3 \textit{A&B}). Slow-twitch soleus muscles interestingly measured to be slightly less in HFD-fed M\textsuperscript{IL10} group than WT group (Fig. 4.3 \textit{C}). None of the differences reached statistical significance.

**IL-10 increases proliferation but not differentiation of C2C12 myoblasts** To investigate the direct effect of IL-10 on myoblast proliferation we seeded C2C12 cells in culture plates and added IL-10, IL-6, IL-10+IL-6 or no cytokine into the wells daily and counted cells after day 2 and 3. When only IL-10 was added, the cell numbers increased by 50% during the first 2 days (Fig. 4.4). After day 2, the cell numbers declined. IL-6 didn’t cause a significant increase by day 2. The IL-6+IL-10 combination might also have created a cytotoxic effect and decreased cell numbers. To inspect the effect of IL-10 on myoblast differentiation, we seeded C2C12 myoblasts into plates and differentiated them. After 4 days of IL-10, IL-6 or IL-10+IL-6 treatment myogenin gene expressions were detected in collected cells. All insulin-added, differentiated groups had higher myogenin mRNA expression levels compared to the negative control, but there was no significant difference between IL-10, IL-6 and IL-10+IL-6 groups (Fig. 4.5).

**IL-10 increases myogenic gene expression in HFD-fed mice** To determine the effects of IL-10 on myogenic gene expressions in obese mouse muscles; we isolated RNA from 16-week chow or HFD-fed M\textsuperscript{IL10} and WT mice. There wasn’t a significant difference between chow-fed groups in major myogenic genes MyoD and Myf5 expressions (Fig. 4.6 \textit{A&B}).
Figure 4.4 IL-10 increased proliferation in myoblasts. C2C12 mouse myoblasts are seeded and added with IL-10, IL-6 or both cytokines daily and cells are counted after 48 and 72 hours for each group. Data are shown as fold change in respect to control wells as an average of 3 different plates. Values are means ± SE in each group. *P<0.05.
Figure 4.5 IL-10 does not affect myoblast differentiation. C2C12 mouse myoblasts are differentiated and added with IL-10, IL-6 or both cytokines daily for 3 days and myogenin mRNA levels are measured in duplicate wells. Values are means ± SE in each group.
Interestingly, there were prominent increases in MyoD and Myf5 mRNA levels in HFD-fed M^{IL10} group compared to WT group (Fig. 4.6 A&B). We also measured MAFbx ubiquitin ligase expression in the muscles of HFD mice since it degrades MyoD. MAFbx mRNA levels tended to be lower in M^{IL10} group, but it didn’t reach statistical significance (Fig. 4.6 C). To confirm the increased MyoD expression in transgenic IL-10 mouse models, we also checked MyoD mRNA levels in MCK-IL10^{ob/ob} mice. MCK-IL10^{ob/ob} mice also showed higher MyoD expression compared to ob/ob controls in the muscle (Fig. 4.6 D).

We also measured skeletal muscle alpha-actin and MHCI mRNA expression levels to determine muscle structural protein amounts for muscle function. Even though the levels were similar, HFD-fed M^{IL10} groups had slightly higher mRNA levels than WT-HFD mice (Fig. 4.6 E&F). We also checked the differentiation markers in the skeletal muscles. There was no significant difference in myogenin and MEF2C differentiation marker mRNA levels between the groups (Fig. 4.6 G&H). But, there was a trend of increase in WT-HFD mice mRNA levels compared to chow-fed WT mice. This increase was not observed in HFD-fed M^{IL10} mice.

**M2 macrophages are more abundant in HFD-fed M^{IL10} group than WT group** To identify immune cell frequencies in the skeletal muscles of the HFD-fed mice we performed flow cytometry. Activated M1 macrophages that are known to be recruited during obesity [156], and pro-inflammatory CD11b^{+} F4/80^{+} M1 macrophages had higher frequencies in WT-HFD group muscles than M^{IL10}-HFD group in the preliminary study (Fig. 4.7). Interestingly, anti-inflammatory M2 macrophage frequencies were higher in the
Figure 4.6 mRNA levels of muscle growth genes in WT and $M^{IL10}$ mice gastrocnemius muscles. A: MyoD, B: Myf5 C: MAFbx mRNA levels in WT and $M^{IL10}$ mice (n=7). D: MyoD mRNA levels in $ob/ob$ and MCK-IL10$^{ob/ob}$ mice (n=3). E: MHCI, F: Alpha-actin G: Myogenin and H: MEF2C mRNA levels in WT and $M^{IL10}$ mice (n=3-5). Values are means ± SE in each group. *P<0.05.
Figure 4.7 Flow cytometry analysis of skeletal muscle immune cell population in quadriceps muscles of WT and $M^{IL10}$ mice fed with HFD for 16 weeks. M1 and M2 macrophage frequencies detected by flow cytometry in skeletal muscles of each group (n=3). Values are means in each group.
skeletal muscles of $M^{IL10}$-HFD group than WT-HFD group suggesting that M2 polarization may be occurring in the IL-10 expressing skeletal muscles of $M^{IL10}$ mice (Fig. 4.7).

**Lean mass is correlated with IL-10 and MyoD expression levels in HFD-fed $M^{IL10}$ mice** To investigate the association between increased lean mass, MyoD and IL-10 mRNA expression levels in the mouse muscles, we performed correlation analysis. MyoD mRNA expression levels in the combination of all groups did not have a correlation with lean mass (Fig. 4.8 A). When we analyzed only HFD-fed $M^{IL10}$ group, we observed a strong correlation between $M^{IL10}$ mice lean mass and MyoD and Myf5 mRNA expression levels suggesting that lean mass increase was associated with the increased myogenic gene expression levels in obese $M^{IL10}$ mice (Fig. 4.8 B&C). To examine if IL-10 levels have a positive effect on lean mass we made correlation graphs of IL-10 mRNA levels and lean mass in HFD-fed mice. When we separated WT-HFD and $M^{IL10}$-HFD groups, we observed that there’s no correlation in WT-HFD mice where endogenous IL-10 levels are low. But IL-10 levels and lean mass were strongly correlated in $M^{IL10}$-HFD mice suggesting a dose effect of IL-10 in muscle growth (Fig. 4.9).
Figure 4.8 Muscle MyoD and Myf5 mRNA expressions are correlated with lean mass in HFD fed $\text{M}^{\text{IL10}}$ mice. Muscle MyoD mRNA levels versus lean mass in A: Both WT and $\text{M}^{\text{IL10}}$ mice fed with HFD or chow diet (n=30), B: in $\text{M}^{\text{IL10}}$ mice fed HFD (n=8), C: Muscle Myf5 mRNA levels versus lean mass in $\text{M}^{\text{IL10}}$ mice fed HFD (n=7).
Figure 4.9 Muscle IL-10 mRNA expressions are correlated with lean mass in HFD fed M$^{IL10}$ mice. Muscle IL-10 mRNA levels versus lean mass in A: in WT mice fed with HFD, B: in M$^{IL10}$ mice fed with HFD.
4.5 Discussion

IL-10 is a major cytokine responsible for the termination of anti-inflammatory responses in tissues [307]. We have previously found that IL-10 has a preventive effect on skeletal muscle insulin resistance [93, 172]. While we were studying with skeletal muscle IL-10 overexpressing mice, we discovered the constructive effects of IL-10 in maintaining muscle mass in obese mice. This study confirms the beneficial effects of IL-10 in muscle growth during insulin resistant-obese conditions.

During natural process of muscle growth, skeletal muscle mass increases with elevating body weight to compensate for the additional load [197]. During the state of extreme obesity and insulin resistance, linear growth of muscle is ceased, and muscle mass reaches a plateau. We hypothesize that this phenomenon might be caused by the increasing inflammation in the muscle with obesity [147], increasing insensitivity to insulin which is a major growth stimulator, or decreasing Akt signaling for muscle growth.

A low-state chronic inflammation in the muscles takes place during chronic obesity, manifesting itself by increased pro-inflammatory cytokine production and macrophage infiltration [93, 133, 147]. The adverse effect of inflammatory cytokines in the skeletal muscle growth was previously shown [440-444]. Major pro-inflammatory cytokines TNFα and IL-1β activate NF-κB pathway and downregulate major myogenesis proteins including MyoD and myogenin [443, 445]. TNFα also blocks IGF-I pathway through JNK signaling [338] and decreases protein content [342], and synthesis [446] in myocytes. C2C12 cell line that we used in this study is responsive to inflammatory cytokines TNFα and IL-1β in myoblast stage, and therefore, is a good model to study myogenesis [447]. The effect of
IL-10 to suppress synthesis, release and action of these inflammatory cytokines may be the factor restoring the skeletal muscle growth during obesity.

During insulin-resistant, extremely obese states insulin sensitivity and Akt signaling are impaired in skeletal muscle. Insulin is at the same time a major growth hormone acting in skeletal muscle growth and preventing muscle protein breakdown [422, 448, 449]. Akt is also a signaling mediator downstream of the insulin pathway. It is important for Akt/mTOR pathway, a key pathway for skeletal muscle hypertrophy in muscle [450]. Increased Akt signaling is shown to result in muscle hypertrophy itself through the mTOR pathway [117-119]. Researchers also demonstrated that inducible Akt-dependent muscle hypertrophy in adult mice led to proliferation of the interstitial cells, but not satellite cell activation [119]. Akt also phosphorylates forkhead box O (FOXO) proteins and decreases the transcription of ubiquitin ligases like MAFbx and MURF-1[120]. We have shown that IL-10 expression in the skeletal muscle improved insulin sensitivity and Akt signaling during chronic obesity. This may be another factor restoring muscle growth during chronic obesity states in mice. This preventive effect of IL-10 in muscle mass loss did not only appear in chronic HFD-fed conditions but also in other chronic obesity conditions like leptin-deficiency in ob/ob mice. Aging is also associated with changes in body composition that lead to a shift toward increased fat mass and decreased muscle mass in normal adults [451]. The same effect was observed in the aging mice skeletal muscles. Another important point for validation of our observations was showing the opposite effect when IL-10 signaling was blocked in the skeletal muscle. HFD-fed mice that are lacking insulin receptor in skeletal muscle displayed the opposite phenotype with lower lean mass
compared to controls, which supported our hypothesis. Other studies have also found that ablation of IL-10 expression in dystrophic mice increased muscle damage in vivo and reduced mouse strength, in concordance with our results [282].

When exposed to saturated fatty acids muscle cells co-cultured with macrophages tend to polarize macrophages towards M1 type which may promote insulin resistance [452]. M1 macrophages are known to play an important role in the obesity-mediated inflammation [156]. Lower M1 and higher M2 macrophage frequencies in the muscles of M$^{IL10}$ mice were also interesting in the context that it suggests M2 polarization of macrophages by IL-10 may also be contributing to the improved muscle growth in mice. In another study, researchers suggested that IL-10 does not have a direct effect on proliferation or myogenin expression. However, when muscle cells are co-cultured with macrophages, IL-10 treatment polarized macrophages to M2 phenotype and M2 macrophages indorse the initial, proliferative stage of myogenesis which supports our hypothesis [355].

MyoD and Myf5 are activated in the early stage of muscle formation and they help remodeling of the chromatin for further differentiation factors [453]. Myogenin and MEF2C, on the other hand, are expressed later and work together for successful differentiation of myoblasts into myotubes [454]. To understand which step of muscle formation (early myogenesis or myofibril differentiation) IL-10 is promoting, we worked with C2C12 myoblast cultures. IL-10 directly increased myoblast proliferation for the first 2 days but didn’t have an effect on myogenic gene expression in the differentiated myotubes. The decrease in the cell numbers after day-2 might be due to cytotoxicity of
cytokines or confluency. According to these results, IL-10 seems to be directly affecting early stage myogenesis genes. We also observed that some $M^{IL10}$ mice had a more prominent increase in lean mass than their littermates. The correlation graphs that we plotted demonstrated that the increase in the lean mass was associated with the increase in MyoD expression which also correlated with the amount of IL-10 expressed in the skeletal muscles of $M^{IL10}$ mice that explains this phenomenon. These results suggested that IL-10 increases muscle mass in a dose-dependent manner.

Overall, our working model is: during early obesity, skeletal muscle growth is induced to compensate for the increased load due to weight-bearing effect. However, in extreme obesity and insulin resistance, the healthy environment the growth of muscle is disturbed. Muscle cannot respond to growth signals and muscle growth is stopped, even though body weight is still increasing. At this level, suppression of inflammation and increase in insulin and Akt signaling are restored by the IL-10 expression, which results in the continuation of the linear increase of the muscle mass along with the weight gain. These findings define the anti-inflammatory IL-10 as a therapeutic target for the healthy homeostasis of the muscle mass during insulin resistant states.
Figure 4.10 IL-10 overexpression in skeletal muscles of HFD fed mice is protecting mice against muscle growth cessation due to insulin resistance in late-stage obesity.
Chapter V: Concluding Remarks and Future Directions

5.1 Concluding Remarks

Defining the relationship between chronic inflammation and diabetes is essential to understand the reasons and mechanisms lying behind the pathology of this disease. As the largest insulin-sensitive tissue, glucose metabolism of the skeletal muscle is fundamental for the whole-body glucose homeostasis. Our lab, as well as others, pointed out the importance of chronic inflammation in the skeletal muscles of insulin resistant subjects. Here in this thesis, I investigated the effects of the anti-inflammatory cytokine IL-10 on suppressing the chronic obesity- and aging-associated skeletal muscle inflammation and insulin resistance.

In chapters II and III, I reported the positive effects of IL-10 in obesity and aging-associated inflammation and insulin resistance. Our lab had already shown the preventive effect of IL-10 against insulin resistance during short-term HFD feeding. However, the consequences of short-term lipid exposition are different than chronic obesity. Researchers have shown that different immune cell types and responses are associated with short-term and long-term HFD feeding [371, 381]. In my thesis, I investigated the effects of IL-10 in a chronically obese and hyperinsulinemic model that mimics the pathology of type 2 diabetes more realistically. Also, to eliminate the effects of lipid toxicity in the chronic obesity model, I generated MCK-IL10^{ob/ob} mouse model. In both studies, local expression of IL-10 in the skeletal muscle improved the insulin sensitivity. These results suggested that IL-10’s preventive effects were not only against the fatty acid-induced toxicity, but also against the chronic obesity-mediated inflammation.
Another significant finding of this thesis is that IL-10 can be effective against aging-associated insulin resistance. This is the first study to show IL-10’s protective role on aging-driven insulin resistance in skeletal muscle. In our model, adiposity of aging M^{IL10} and WT mice were comparable. This fact allowed us to differentiate the effects of obesity from aging in our experimental setup. Similar to the result of the obesity studies, IL-10 preserved the muscle insulin sensitivity in aging mice. In both obesity- and aging-associated insulin-resistant models, IL-10 improved glucose metabolism by suppressing the local inflammation as evident by decreased macrophage recruitment and lowered pro-inflammatory cytokine levels in the skeletal muscle. These data supported the importance of skeletal muscle inflammation in insulin resistance.

Furthermore, using an HFD-fed muscle IL-10 receptor knock out mouse model, I have shown that the direct effect of IL-10 on skeletal muscle fibers is important for muscle insulin sensitivity. This was the first in vivo study to show that the direct effect of IL-10 through IL-10 receptor is required to suppress the local inflammation and improve glucose metabolism in skeletal muscle. Although we have shown the importance of direct IL-10-skeletal muscle signaling, the exact metabolic pathway that IL-10 is triggering in skeletal muscle fibers is not determined by our studies yet. Therefore, revealing the detailed pathway that IL-10 is triggering in skeletal muscle fibers to improve insulin sensitivity is a crucial next step for this study.

The hypothetical schematic representation of the effects of IL-10 that is derived from our results is summarized in Figure 5.1. Obesity and aging can trigger M1 macrophage recruitment and inflammatory cytokine release from these macrophages.
These inflammatory cytokines can interfere with insulin signaling in the skeletal muscle through inhibition of tyrosine phosphorylation of IRS-I, and activation of stress kinase pathways. This phenomenon can cause insulin resistance and hyperinsulinemia in skeletal muscles due to the loss of sensitivity to the insulin. IL-10 can decrease these negative effects on insulin signaling by suppressing the macrophage recruitment and pro-inflammatory cytokine release from the macrophages. Also, IL-10 signaling directly on the skeletal muscle fibers can activate STAT3/SOCS3 pathway to inhibit inflammatory cytokine synthesis from muscles, which would interfere with the insulin signaling otherwise. Overall, the local expression of IL-10 in the skeletal muscle can improve muscle insulin sensitivity.

We showed that IL-10 levels decrease with obesity and aging. Contradictory results in literature on increased/decreased serum IL-10 levels during obesity and aging [191, 320, 322, 323, 455-457] may be due to the fact that the regulation of the IL-10 production and the different responses from different immune cell type sources are much more complex than we think, and that they show different characteristics according to the exact conditions triggering them.
**Figure 5.1** Schematic representation of potential IL-10’s effects on skeletal muscle glucose metabolism
IL-10 can be synthesized directly from the myocytes, as evident from the IL-10 secretion in myoblast-only cultures and increased levels of IL-10 release after exercise [284, 285]. But we believe the primary sources in our models are M2 macrophages. IL-10 was suggested to cause M2 polarization in dystrophic and atrophic mouse models previously [282, 355]. Also in our pilot study, we saw that IL-10 over-expression in skeletal muscle decreased M1 macrophage and increased M2 macrophage frequencies after HFD-feeding. Since the main source of IL-10 is M2 macrophages, IL-10 levels may increase as a positive feedback loop in this model.

In chapter IV, I defined a novel role for IL-10 to promote and maintain muscle growth in insulin resistant conditions in vivo for the first time. Although there were cell culture studies and dystrophic mouse models suggesting an active role for IL-10 in muscle growth, our study was the first to demonstrate the in vivo effect of IL-10 on muscle mass during obesity- and aging-associated insulin resistance to our knowledge. It was an intriguing finding that the positive effect of IL-10 on lean mass was just observed in the insulin resistant conditions and not during the healthy state. Some possible mechanisms are: - increased inflammation may accompany increased insulin resistance and suppressing inflammation rescues inflammation-mediated muscle growth cessation and; - increased insulin/IGF signaling in the skeletal muscle due to IL-10’s effects on skeletal muscle may prevent muscle growth cessation in skeletal muscle. Type 2 macrophage polarization is critical for myogenesis after an injury [458]. Although other researchers did not observe the proliferative effect of IL-10 directly on myocyte cultures like we observed; they support
our finding which IL-10 causes a type 2 macrophage polarization that increases myoblast proliferation [355].

Although we haven’t conducted experiments on them, we can speculate about the mechanism of IL-10-mediated muscle growth. For example IL-10 mediated M2 macrophage polarization may induce myogenin expression [458]. Or IL-10 itself may induce Cyclin D1/c-Myc through Jak/STAT3 pathway to trigger muscle cell proliferation [459]. Also, IL-10-activated Jak/STAT3 pathway may interact with MyoD to induce satellite cell differentiation [460]. Another possibility is the suppression of TNFα by IL-10 may prevent TNFα-mediated MyoD destabilization [461]. The IL-10-mediated increase in Akt signaling may inhibit FOXO pathway too, which induces muscle ubiquitin ligases MAFbx and MURF1 to breakdown muscle [462]. Likewise, the IL-10-mediated increase in Akt signaling may inhibit GSK3 which inhibits eukaryotic initiation factor (eif)2b that induces muscle protein synthesis [463]. Similarly, the IL-10-mediated increase in PI3K/Akt signaling may initiate mTOR pathway that increases muscle protein synthesis and growth [464].

Muscle loss was already associated with chronic inflammation during aging in several studies [465, 466]. Therefore, suppressing the inflammation specifically in skeletal muscle to rescue age-related muscle mass decline in our studies was on the same line with the general view. IL-10 global knock out mice was previously defined as a model of decreased muscle strength and muscle wasting [328]. So, our study shows that IL-10’s effects are local in the skeletal muscle, and endorses IL-10 as a therapeutic agent to prevent these muscular deformities. Although several studies tried to deliver IL-10 DNA by
hydrodynamic gene delivery, vector viruses and degradable carriers, no successful direct targeting of IL-10 into skeletal muscle was observed up to date [302, 334, 467]. In those delivery systems, even when it is injected directly into the skeletal muscle, DNA is delivered to the other metabolic organs and IL-10 levels increase in circulation. IL-10’s half-life is very short, so it needs to be directly delivered to the target organ efficiently. IL-10 is meant to suppress all inflammatory processes including the natural vital ones, so high levels of IL-10 can form a threat to the general physiological homeostasis. A safe, on-target delivery strategy is yet to be developed for the therapeutic use of IL-10.

To sum up, obesity and insulin resistance induce activation of M1 macrophages and pro-inflammatory cytokine release. This in return, blocks the insulin sensitivity and the linear growth of skeletal muscles. IL-10 protects against the insulin resistance and growth cessation in the skeletal muscles by blocking the recruitment and activation of M1 macrophages, and release and action of pro-inflammatory cytokines (Fig. 5.2). Overall, the role of IL-10 in skeletal muscle as an anti-inflammatory agent requires deeper understanding and may hold therapeutic potential in future.
**Figure 5.2** The schematic of effects of IL-10 on skeletal muscle
5.2 Experimental Caveats and Alternative Explanations:

We observed the positive effects of IL-10 on both muscle insulin sensitivity and muscle growth. One thing in common in both scenarios is the insulin signaling. If IL-10 has an effect of improving insulin signaling, this might explain how it can increase both insulin sensitivity and insulin-induced growth. However, detailed studies on the IL-10-insulin pathway interaction should be conducted to give an answer to this question.

Our current study design did not explore the origin of the increased muscle mass. There is another hypothesis suggested by researchers for the IL-10-mediated muscle regeneration. Mesangioblasts are stem cells associated with microvasculature walls in the skeletal muscle [468]. Researchers suggested that IL-10 by driving mesangioblast differentiation to myogenic lineage can promote muscle growth and regeneration [469]. The effect of insulin resistance on mesangioblast differentiation and effect of IL-10 can be investigated in the future.

In this project, we focused on the IL-10’s anti-inflammatory effect to suppress insulin resistance. Another mechanism that IL-10 can improve glucose metabolism without the anti-inflammatory properties of IL-10, is the insulin-independent activation of Akt. IL-10 through IL-10 receptor is shown to induce PI3K and Akt pathway itself in fibroblasts [470], macrophages [471, 472], cardiomyocytes [473, 474] and adipocytes [206]. Even though there is no direct evidence that IL-10 can induce PI3K in skeletal muscle fibers yet, it is possible that IL-10 might be inducing Akt pathway independent of insulin to improve glucose metabolism in muscle.
Another caveat in this study is some of the measurements in skeletal muscles were performed on post-clamp samples. Given that during clamp there is a long stimulation of insulin, the effect of insulin in inflammation should not be avoided [475]. The experiments can be repeated with non-clamped samples for confirmation.

Since insulin goes through trans-endothelial transport before reaching the skeletal muscle fiber surface [106, 476], it is possible that IL-10 has an effect on this transport process. For example, IL-10 induces the PI3K/Akt pathway which is important for endothelial insulin signaling [477]. The current study is unable to differentiate between effects of IL-10 directly on muscle fibers or muscle interstitium including endothelial cells.

In our experiments, we observed that aging M\textsuperscript{IL10} mice tended to get exhausted faster compared to wildtypes. The current study did not examine contraction force or grip strength values and sample number was low. Although, our interpretation was due to a single parameter, this tendency to get exhausted earlier may be a sign of higher type II fibers in the skeletal muscle. Type I fibers in skeletal muscle have an oxidative profile and contract slowly [478], whereas type II fibers are more glycolytic and contract faster [479]. A high ratio of fast-twitch type II fibers to slow-twitch fibers is good for short-term intense exercise but decreases endurance [480, 481]. Type 2 diabetes promotes an increase in type II fibers and a decrease in type I fibers [482]. For example, researchers showed that PPAR\textdelta expression in skeletal muscle caused a fiber type switch to type I fibers, improving exhaustion limit and improving the obesity-induced metabolic phenotype in mice [483]. Another group showed that PGC-1\alpha knock out animals exhibited a type I to type II fiber switch in muscle and reduced endurance capacity [484]. In our primary results, we saw
also a slight increase in fast twitch muscles mass (quadriceps and tibialis anterior) and a slight decrease in slow twitch muscle mass (soleus) in $M^{IL10}$ group which agrees with their more rapid exhaustion phenotype. These data suggest that IL-10 expression in skeletal muscle may not have prevented slow to fast fiber switch, but on the contrary, may have enhanced it. Reduced fast twitch muscle fiber size (tibialis anterior) observed in IL-10 KO animals also agree with these results [485]. Effects of IL-10 on skeletal muscle fiber type switching has not been investigated, suggesting future avenues of study.

The more rapid exhaustion profile in aging $M^{IL10}$ mice might also be caused by a fuel switch from FFA to glucose. Randle glucose-fatty acid cycle describes the fuel selection by muscle and liver [486]. During exhaustive exercise (e.g. running a marathon) lipid was found to be the preferred fuel, and utilization of lipid was dependent on plasma concentrations of FFA [487]. IL-10 knock out mice were shown to have 75% increased free fatty acid levels after fasting suggesting that IL-10 might have a role in decreasing plasma FFA levels [488]. We did not measure plasma FFA levels in aging $M^{IL10}$ mice. However, if plasma FFA levels are lower in $M^{IL10}$ mice, it might cause a more rapid exhaustion and a switch from fatty acid to glucose metabolism. Plasma FFA levels should also be evaluated in aging $M^{IL10}$ mice.

Our study mainly observed the effect of IL-10 on glucose metabolism in insulin-induced and glucose-infused conditions. However, the glucose uptake of skeletal muscle in other states such as during exercise is insulin-independent [99]. Therefore, as a limitation, our study has only explained the effects of IL-10 on muscle glucose metabolism in post-absorptive insulin-dependent states. It did not include effects of IL-10 in insulin-
independent glucose uptake. For example, in the endurance exercise study, exhaustion limits of aging M\(^{IL10}\) mice tended to be lower compared to wild types. Therefore, it is possible that while IL-10 is improving insulin-dependent muscle glucose uptake, it might not have a positive effect on contraction induced glucose uptake.

5.3 Future directions

In this thesis, I investigated the local effects of IL-10 in skeletal muscle during chronic obesity- and aging-induced insulin resistance and muscle growth cessation. Even though we have reached some conclusions on the positive effect of IL-10 on muscle glucose metabolism and growth, clearly more research is warranted to unravel the exact mechanism of IL-10’s actions.

In chapters II and III, I discussed that IL-10 signaling in skeletal muscle is preventive against chronic obesity and aging-mediated insulin resistance in skeletal muscle. Our M-IL10R\(^{-/-}\) mouse model was important to show that IL-10 directly acts on the skeletal muscle fibers through the IL-10 receptor. Now that we have revealed the direct effect of IL-10 on the muscle, it is essential to find the signaling pathways that IL-10 triggers in the muscle fiber. We know that IL-10 induces STAT3/SOCS3 pathway in the skeletal muscles of short-term HFD-fed M\(^{IL10}\) mice [93]. It is also important to see whether IL-10 signaling in the skeletal muscle is affecting cellular stress pathways like JNK, IKK\(\beta\), or ROS. HFD-fed M-IL10R\(^{-/-}\) mice could be a good model to compare the activation of these pathways in KO and WT mice by immunoblotting of phosphorylated proteins.

Inflammasomes are multi-protein complexes/sensors that control Caspase-1 activation and induce inflammation [489]. Researchers linked inflammasome activation in
the macrophages to diabetes and β cell death recently [490, 491]. Inflammasomes were detected in the skeletal muscles of IL-10 KO mouse model which had increased inflammation [485]. It might be interesting to test the existence of activated inflammasomes in the skeletal muscles of HFD-fed or aged M\textsuperscript{IL10} and WT mice by measuring Caspase-1 cleavage or activity, or NACHT-LRR-PYD domains-containing protein 3 (NLRP3) expression levels.

The direct effect of IL-10 on the skeletal muscle mass of insulin resistant \textit{in vivo} models is also a novel finding of this thesis. Next step is to find the downstream mechanisms of IL-10. Following IL-10 induction of skeletal muscle, Akt phosphorylation is increased, which we already confirmed in chapter II. Akt/mTOR pathway is one of the major growth pathways, and increased insulin signaling and Akt phosphorylation in skeletal muscle can directly trigger mTOR pathway to increase muscle mass. To test this hypothesis, we can measure p70S6K phosphorylation by immunoblotting, which is a direct downstream target of mTOR, in skeletal muscles of HFD-fed M\textsuperscript{IL10} and M-IL10R\textsuperscript{−/−} mice.

Another effect of IL-10 on the muscle growth can be its insulin-sensitizing action. To test if IL-10 is acting through the insulin/IGF-I pathway to promote muscle growth, we can place an osmotic S961 pump onto the mouse, which is a peptide that blocks the insulin receptors [492], and then test myogenic gene expressions in the muscles of HFD-fed M\textsuperscript{IL10}, M-IL10R\textsuperscript{−/−} and WT mice. Also, we can inject insulin-like growth factor I (IGF-I) to the skeletal muscles of HFD-fed M\textsuperscript{IL10} and M-IL10R\textsuperscript{−/−} mice acutely and immediately look at downstream Akt phosphorylation to see if IL-10 signaling has any effect on IGF-I-mediated growth mechanism.
Since adult muscle fiber precursors (satellite cells) are mostly quiescent after maturation, it is hard to study myogenesis in healthy adult mice. To observe the effect of IL-10 in muscle generation we need to trigger injury or atrophy in skeletal muscles. To investigate the role of IL-10 on muscle regeneration and maintenance, tail suspension of obese or aging $M^{IL10}$ mice (with controls) as a model of induced, reversible atrophy can be used in which we analyze myogenesis marker expression profiles. Tail suspension is an easy method to apply, and it mimics muscle wasting due to disuse atrophy that is observed during immobility and aging [493]. The differentially expressed myogenic genes after the mice are reloaded may give us a clue about the effects of IL-10 in promoting muscle mass increase.

Another study to understand the direct effects of IL-10 on the skeletal muscle fibers for promoting muscle growth may be crossing the dystrophic $mdx$ mouse model with $M^{IL10}$ and $M$-$IL10R^{-/-}$ mice. Previously Villalta and colleagues showed that crossing $mdx$ mice with the global IL-10 knock out mice exacerbated the muscle damage in dystrophic mice [282]. Also, they have shown that when $mdx$ mice are treated with IL-10, regeneration increased. Since all these effects are systemic, they don’t differentiate the local effects of IL-10 in the skeletal muscle. By generating $M^{IL10}mdx$ mice or $M$-$IL10R^{-/-}mdx$ mice, we can test whether IL-10 can influence the pathology of the dystrophy and muscle regeneration by its direct local effect on skeletal muscle. We can determine the numbers of satellite cells by immunolabeling or flow cytometry. Another quick method of muscle injury to assess the effects of IL-10 in muscle regeneration is the freeze-injury of the muscle, where a liquid nitrogen cooled rod is pressed onto exposed skeletal muscle several times to generate
freeze-thaw cycles of the muscle [494]. We can apply this method to the HFD-fed $M^{IL_{10}}$ and in M-IL10R$^{-/-}$ mouse models, then excise the skeletal muscles again to count satellite cell numbers and measure myogenic markers. We can also check the presence of the centrally located nuclei to detect newly formed fibers with conventional histological staining in the skeletal muscle.

We performed our proliferation studies in C2C12 cell line. Showing the effect of IL-10 on primary myocytes that are directly isolated from $M^{IL_{10}}$ and in M-IL10R$^{-/-}$ mice is also essential to determine whether the proliferation/differentiation phenotype of primary myocytes exposed to high or no IL-10 stimulation for a prolonged time has changed. We may isolate primary satellite cells by fluorescence-activated cell sorting according to their special surface marker profiles and separate them from non-myogenic and differentiated cells [495]. Using the original satellite cells isolated from our mouse models, we can perform clonal myogenesis assays to assess their colony-forming ability and determine the oxygen consumption and glycolysis using extracellular flux analyzer [496].

Another important thing to determine is the effects of IL-10 on fiber type composition/switching in the skeletal muscle. Researchers suggest that skeletal muscle fiber type composition is related to the obesity and diabetes and fiber type switching can occur due to insulin resistance and aging [497-500]. The frequency of slow-oxidative type I fibers and fast-glycolytic type II fibers can be analyzed by simple nicotinamide adenine dinucleotide (NAD)H staining in the skeletal muscle cryosections of aged and obese $M^{IL_{10}}$ and M-IL10R$^{-/-}$ mice. Also, we can check myosin heavy chain variants MHCI, MHCII with immunofluorescence microscopy. We may furthermore test if improved insulin signaling
and suppressed inflammation are specific to a particular fiber type. Researchers previously showed that slow-twitch muscle fiber proportion correlates with the insulin responsiveness in muscles, and high type I-content muscles like soleus are more insulin sensitive and can handle more glucose [501-503]. Therefore, it would be interesting to test if IL-10 regulates insulin signaling differently in different muscle fiber types. Inflammation in the muscle may also be altered due to the dominant fiber type. Inflammatory cytokine release from different fiber types showed different patterns in several studies [504, 505]. Chronic inflammation was suggested to increase type II muscle fibers in diaphragm muscle [506]. So it is possible that elevated inflammatory cytokines in our obese models with or without IL-10 expression can show different fiber type compositions in skeletal muscles. A possible way to test this is to compare a slow-twitch fiber dominant muscle (ex. soleus) with a fast-twitch fiber dominant muscle (ex. extensor digitorium longus, tibialis anterior) for inflammation and insulin sensitivity in our IL-10 mouse models. We can also look at the structure of the skeletal muscles by electron microscopy to see if there is any difference. The orientation of the contractile proteins and structure, and the number of mitochondria can give us idea about possible effects of IL-10 on muscle structure and oxidative capacity.

One possible mechanism that IL-10 is helping maintain muscle mass in insulin resistant conditions might be a positive effect of IL-10 on muscle activity. It would be interesting to test muscle function in $M^{IL-10}$ and M-IL10R−/− mice looking at different parameters such as grip-strength, isometric contraction force and exhaustion limits.

IL-10 KO mouse model showed decreased longevity compared to wild-type mice in studies [328]. Therefore it might be interesting to test the longevity in $M^{IL-10}$ mice, and
in M-IL10R⁻/⁻ mouse models. We actually started a pilot study to observe the longevity of M<sup>IL10</sup> on a chow diet, but the sample size was not enough to do a thorough statistical analysis. Assessing longevities of M<sup>IL10</sup> and in M-IL10R⁻/⁻ mouse models in chow and HFD-fed conditions and looking at the expressions of the longevity genes like sirtuins might be a potential future aim for this study.

In our pilot study, we found that M2 macrophage markers were higher and M1 macrophage markers were lower in skeletal muscles of HFD-fed M<sup>IL10</sup> mice than WT mice. It was suggesting that the polarization of skeletal muscle macrophages towards M2 phenotype in obesity due to IL-10 stimulation. To further investigate possible polarization effects of IL-10 on skeletal muscle resident macrophages we need to confirm M2 macrophage increase with higher sample numbers. In addition, we may perform qRT-PCR for M2 macrophage marker genes such as Arg1, CD206 and CD163 in skeletal muscles to strengthen our hypothesis.

Another thing we can analyze in M<sup>IL10</sup> mouse model is the levels of IL-10 receptor expression in muscle. We have seen that in M-IL10R⁻/⁻ mice IL-10 levels decreased due to the absence of positive feedback loop. We can test whether IL-10 receptor expression is increased in the M<sup>IL10</sup> mice to confirm the feedback mechanism.

Last, we defined IL-10 as a potential therapeutic agent against inflammation-induced insulin resistance and growth cessation. Under these circumstances, local delivery of IL-10 into skeletal muscle is gaining great importance. There are recent studies demonstrating delivery of IL-10 DNA by degradable polymer carriers, adeno-associated virus or hydrodynamic delivery [302, 334, 467], yet in these studies serum levels of IL-10
increase too. Since IL-10 is a powerful immuno-suppressant agent, it might be dangerous to increase systemic levels of IL-10. Instead, we should define a method for targeted delivery of IL-10 into the skeletal muscle in future.

Overall in the light of our findings, the next step is to define the mechanism IL-10 is acting directly on muscle fibers to increase insulin sensitivity and growth and design a site-specific delivery method for IL-10 for future applications.
References


[121] DeFronzo RA. From the Triumvirate to the Ominous Octet: A New Paradigm for the Treatment of Type 2 Diabetes Mellitus. Diabetes. 2009;58:773-95.


[125] Rothman DL, Magnusson I, Cline G, Gerard D, Kahn CR, Shulman RG, et al. Decreased muscle glucose transport/phosphorylation is an early defect in the pathogenesis of non-insulin-


159


Bluher M, Fasshauer M, Tonjes A, Kratzsch J, Schon MR, Paschke R. Association of interleukin-6, C-reactive protein, interleukin-10 and adiponectin plasma concentrations with measures of obesity, insulin sensitivity and glucose metabolism. Experimental and clinical


Levast B, Li Z, Madrenas J. The role of IL-10 in microbiome-associated immune modulation and disease tolerance. Cytokine. 2015;75:291-301.


Lyons JA, Haring JS, Biga PR. Myostatin expression, lymphocyte population, and potential cytokine production correlate with predisposition to high-fat diet induced obesity in mice. PloS one. 2010;5:e12928.


[441] Li YP, Reid MB. NF-kappaB mediates the protein loss induced by TNF-alpha in differentiated skeletal muscle myotubes. Am J Physiol Regul Integr Comp Physiol. 2000;279:R1165-70.

[442] Li YP, Lecker SH, Chen Y, Waddell ID, Goldberg AL, Reid MB. TNF-alpha increases ubiquitin-conjugating activity in skeletal muscle by up-regulating UbcH2/E220k. FASEB journal


