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Innate Immunity in Type 2 Diabetes Pathogenesis: Role of the Lipopolysaccharide Signaling Cascade: A Dissertation

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Innate Immunity in Type 2 Diabetes Pathogenesis:

Role of the Lipopolysaccharide Signaling Cascade

A Dissertation Presented

By

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APPROVAL PAGE

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July 1, 2008

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DEDICATION

I dedicate this thesis to my family whose support, guidance, and unconditional love have been the foundation of my education

To Anna Lee for her unwavering support of my ambitions

And in loving memory of my Grandfather, Uncle, and Lita

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ABSTRACT

Once seen as a disease of wealthy nations, type 2 diabetes mellitus is now showing unprecedented growth throughout the world, fueling increases in microvascular and macrovascular complications. A compelling and growing body of evidence suggests that glucose intolerance and insulin resistance, hallmarks of the diabetic patient, may be driven by chronic inflammation. In particular, a predominance of visceral fat has been associated with enhanced inflammatory cytokine secretion that may contribute to enhanced risk of diabetes and comorbid cardiovascular disease in these individuals. As a function of its potency and wide environmental and biological distribution, we hypothesized that bacterial lipopolysaccharide (LPS, also known as endotoxin) may promote adipose inflammation and concomitant metabolic dysfunction.

Indeed, expression of the LPS receptor CD14 is enhanced on visceral adipocytes of *ob/ob* mice, paralleling enhanced IL-6 secretion *ex vivo*. Furthermore, rosiglitazone-fed *ob/ob* mice demonstrated a reduction in CD14 that coordinated with diminished IL-6 secretion, suggesting a basis for the touted anti-inflammatory effects of this commonly employed type 2 diabetes medication. Mice deficient in components of the LPS signaling cascade, namely CD14, TLR4, and MyD88, yielded adipocytes with markedly attenuated IL-6 secretion, corroborating the central importance of LPS in adipocyte inflammation and supporting the role of this signaling pathway in depot-specific inflammation.

Despite the prominent role of LPS signaling in adipocyte inflammation, CD14-, TLR4-, and MyD88-deficient mice failed to show resistance to diet induced obesity. Surprisingly, *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice had marked glucose intolerance without alteration in

total weight or adipose accumulation. In contrast, *myd88*$^{-/-}$ mice revealed minor glucose intolerance only with high fat diet challenge at an advanced age despite being overtly obese. In *cd14*$^{-/-}$ and *tlr4*$^{-/-}$, but not *myd88*$^{-/-}$, mice, an exaggerated rebound to hypoglycemia was associated with enhanced norepinephrine secretion, which could be abrogated by the adrenergic β-blocker propranolol. The overlay of these mouse models reveals a divergence of phenotypes that demonstrate LPS signaling disruption may lead to glucose intolerance and insulin resistance in part due to enhanced sympathoadrenal tone, uncovering an essential role of innate immunity in physiological stress and its impact upon glucose homeostasis.

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Diabetes Mellitus and Inflammation as a Unifying Concept in Insulin Resistance

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DIABETES MELLITUS AND THE METABOLIC SYNDROME

Diabetes mellitus, often referred to as diabetes, encompasses a constellation of disorders that can be generally characterized by a disturbance in metabolism that leads to high circulating glucose (hyperglycemia). Normally, accumulation of glucose in the circulation is counteracted by the secretion of pancreatic insulin that enhances glucose uptake by peripheral tissues, such as muscle, liver, and adipose, while suppresses formation of endogenous glucose production from metabolic substrates (gluconeogenesis) and from breakdown of glycogen (glycogenolysis). However, a loss of insulin supply or insulin action on these peripheral tissues can lead to an imbalance that favors glucose excess in the vascular tree.

Type 1 diabetes, also known as insulin dependent diabetes mellitus (IDDM), is believed to be caused by autoimmune destruction of the insulin-producing β-cells of the pancreas, leading to an absolute insulin deficiency that typically manifests in the first decade of life. Without an endogenous source of insulin, type 1 diabetes patients rely on exogenous administration of insulin in order to maintain glucose homeostasis (**Table 1.1**).

Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM), is associated with *insulin resistance*, referring to the inability of peripheral tissues to respond to insulin. With progressive insulin resistance, it is believed that pancreatic β-cells can secrete more insulin in order to compensate for this diminished insulin action and maintain glucose homeostasis. Therefore, despite insulin resistance, these individuals may not be overtly diabetic, which is defined clinically by a fasting

plasma glucose > 126mg/dL (7.0 mmol/L). However, it is widely believed that this compensated state, though not technically considered diabetes, heralds the beginning of a disease process. In fact, insulin resistance can be found in patients decades preceding disease and is a consistent finding amongst diabetic individuals.[1-7] Furthermore, prospective studies suggest that insulin resistance is the best predictor of diabetes.[1,4] However, recent data suggests that the positive predictive value of insulin resistance may be limited to those with a family history or genetic predisposition, such as offspring of diabetic patients and Pima Indians.[1,4,8]

Table 1.1: Diabetes Mellitus: Type 1 vs Type 2

Description	Type I (IDDM)	Type II (NIDDM)
Etiology	Viral or Immune Destruction of β cells of islet	Increased Resistance to Insulin Action
Serum Insulin	Low	Variable
Insulin Treatment	Always	Sometimes
Incidence	5-10%	>85%
Age	<30	>40
Obesity Association	No	Yes

This early insulin resistant yet normoglycemic state may progress to diabetes if either the β-cells can not keep up with the insulin demand, insulin resistance worsens, or likely a combination of the two. In the diabetic patient, insulin levels may still be above normal, but we can consider the system at a relative insulin deficiency that fails to maintain normoglycemia. At late stages in disease, the β-cells may even become exhausted due to high insulin demands and undergo apoptosis, leading to β-cell loss and reduction of insulin levels below normal. Hence, the type 2 diabetes patients may have a

range of circulating insulin levels contingent on the stage of disease. In accord, pharmacologic treatment in type 2 diabetes can vary from insulin sensitizing compounds, including biguanides (glucophage), sulfonylureas (glyburide), and the thiazolidinediones (rosiglitazone, pioglitazone), to exogenous insulin administration. (**Table 1.1**)

BURDEN OF DISEASE

Diabetes mellitus is a disease of antiquity, derived from the greek verb derivative *diabetes* that means "a compass, siphon" to represent the characteristic excess in urination in individuals with diabetes and later modified with the latin word *mellitus* that means "honey" to describe the sweet taste of urine in these patients. This theme of sweet and copious urination is echoed through many cultures, including ancient Indians that diagnosed patients for "sweet urine disease" (Madhumeha) based on the attraction of ants to the sugar in the urine. In fact, modern Korean, Chinese, and Japanese words for diabetes are derived from common ancient asian ideographs that translate to "sugar urine disease."[9] Despite the long existence of diabetes, it is widely believed that we are in the midst of a surge in diabetes supported by both anecdotal clinical evidence and formal disease surveillance studies. Moreover, with advances in average lifespan, the chronic complications of diabetes has become evident, representing a significant blight on human health and burden to modern day healthcare systems.

UNITED STATES ESTIMATES

In the United States, the American Diabetes Association (ADA) estimates that 1 million additional people have been diagnosed with diabetes every year for the last 5 years, culminating in an estimated diabetes population of 17.5 million in 2007[10]. In the United States as well as other industrialized countries, the majority of diabetes (90-95% in the US) is classified as type 2 diabetes, which is believed to be the major source of global growth. For the purpose of this thesis, we will primarily address type 2 diabetes and will commonly refer to it as simply "diabetes." As diabetes prevalence increases

with age, this growth can be partially attributed to an aging baby boomer generation. However, estimates also predict an increase in diabetes in the young, suggesting that one out of every three children born in 2000 will develop diabetes [11].

Though type 2 diabetes has a strong heritable component, the recent burst in diabetes in westernized countries within the span of a generation suggests that there is a strong environmental component as well. It is widely believed that obesity due to dietary excess coupled with a decrease in physical activity is the primary culprits in the recent diabetes pandemic. Obesity is defined clinically by the Body Mass Index (BMI) which is a ratio of total weight (in kilograms) divided by the square of body height (in meters). Based on some population studies, it appears that obesity is proportional to certain metrics of diabetes, including fasting glucose and 30-minute insulin concentration following oral glucose tolerance test.[12]

In the United States, it is estimated that 32.2% of adults are obese, defined as a BMI $\geq$30kg/m^2, while 2.8% of men and 6.9% of women are considered extremely obese with a BMI $\geq$40kg/m^2 (i.e., greater than 280-lbs with a height of 5 foot 10 inches). [13-20] The recent rise in obesity in the United States was recorded by The Center for Disease Control's Behavioral Risk Factor Surveillance System (BRFSS) which collected data on obesity from 1985 until 2006 through monthly telephone interviews conducted by individual state health departments. In summary, out of the 44 states participating in the BRFSS in 1990 there were no states with an equal or greater than 15% obesity prevalence. In contrast, within two decades, only 4 states had an obesity prevalence less than 20%, whereas 22 states had an obesity prevalence equal or greater than 25%,

including 2 states with an obesity prevalence greater than 30% (**Figure 1.1**).[21-27] In support of the linkage between obesity and diabetes, states with the highest prevalence of diabetes (>8%) all have an obesity prevalence greater than 25% (**Figure 1.2**).

GLOBAL ESTIMATES

This trend of excess is readily exported to developing countries. In 2005, 1.6 billion people were estimated to be overweight (BMI $\geq$25kg/m^2) with 400 million of those obese. Predictions to 2015 elevate these numbers to 2.3 billion overweight individuals with 700 million of those obese (**Figure 1.3**).[28] Indeed, the type 2 diabetes population shows similar expansion with currently 180 million people afflicted globally with an anticipated doubling by the year 2030.[28] The mortality attributed to diabetes is difficult to quantify because death certificates typically record conditions which are predisposed by diabetes, such as cardiovascular disease and renal failure; however the World Health Organization (WHO) estimates global diabetes-attributable death at ~2.9 million on an annual basis.[28] With the majority of diabetes deaths currently occurring in low- to middle- income countries as well as an anticipated 80% increase in mortality expected in upper-middle income countries, diabetes is truly a global pandemic.[28]

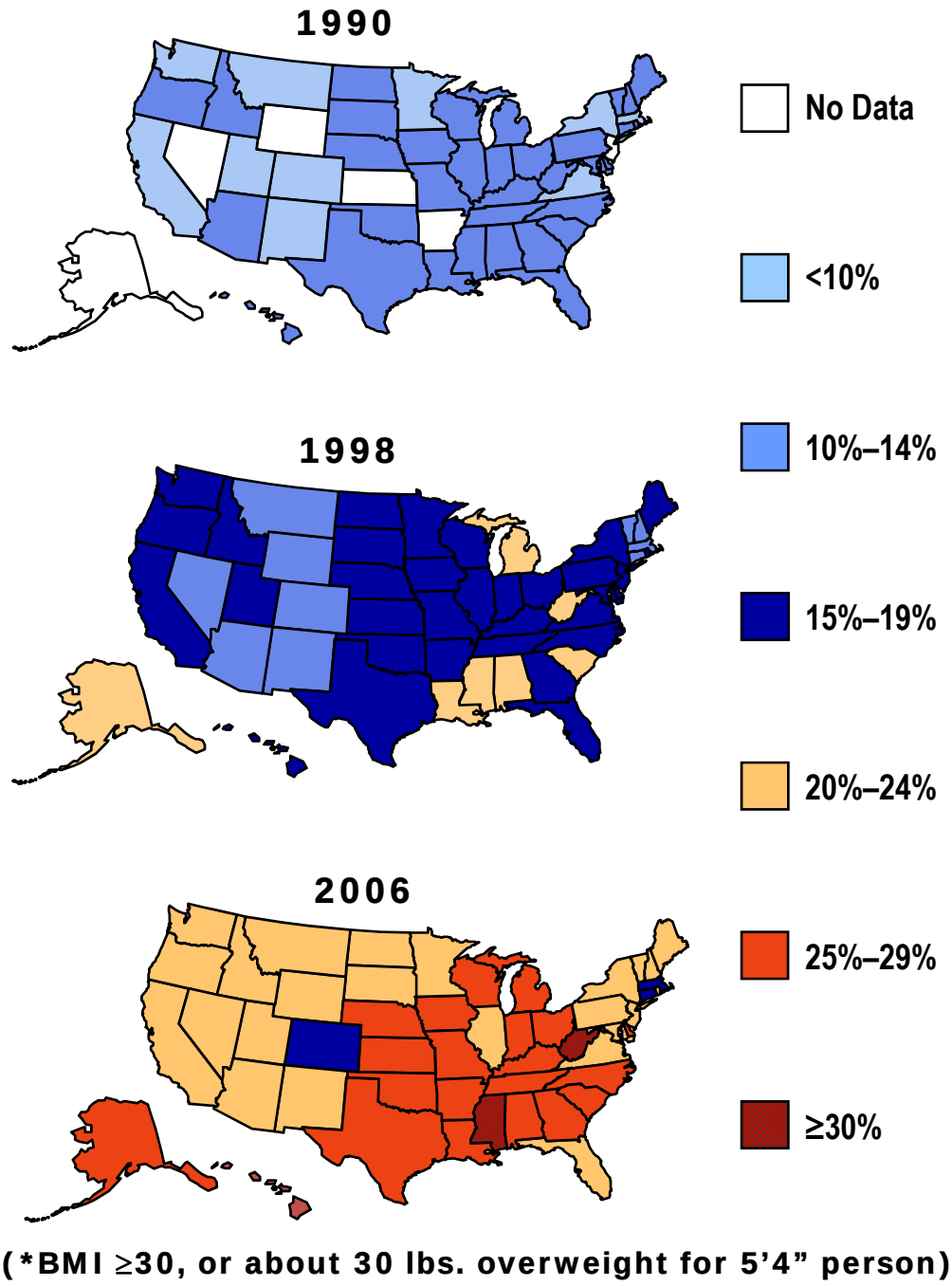


Figure 1.1: Obesity* Trends Among US Adults – Center for Disease Control and Prevention, Behavioral Risk Factor Surveillance System (BRFSS)

BRFSS is the world's largest ongoing telephone health survey conducted by individual states on a monthly basis. This data suggests a significant rise in obesity in the United States over the last two decades.

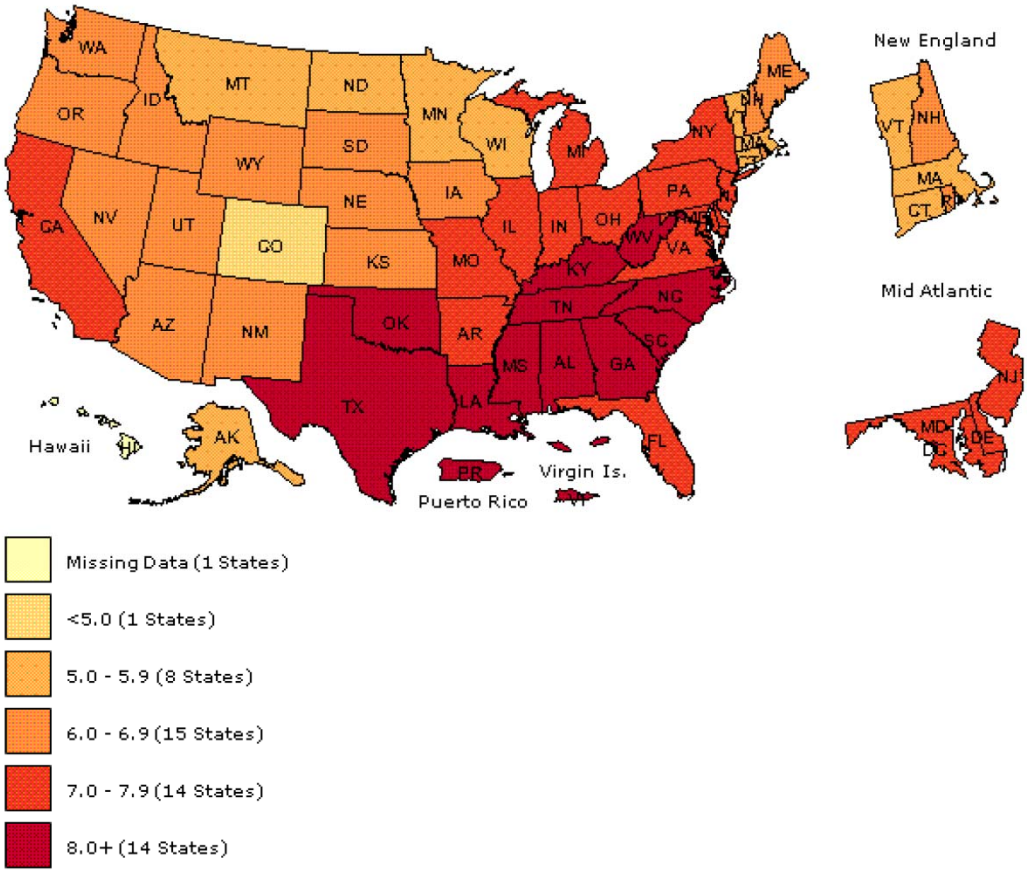


Figure 1.2: Age-Adjusted Percentage of Adults with Diagnosed Diabetes by State (2005)

States with the highest percentage of diagnosed diabetes tend to correlate with states with a higher percentage of obese citizens.

Source:http://apps.nccd.cdc.gov/DDTSTRS/Index.aspx?stateId=25&state=Massachusetts&cat=prevalence&Data=map&view=TO&trend=prevalence&id=1

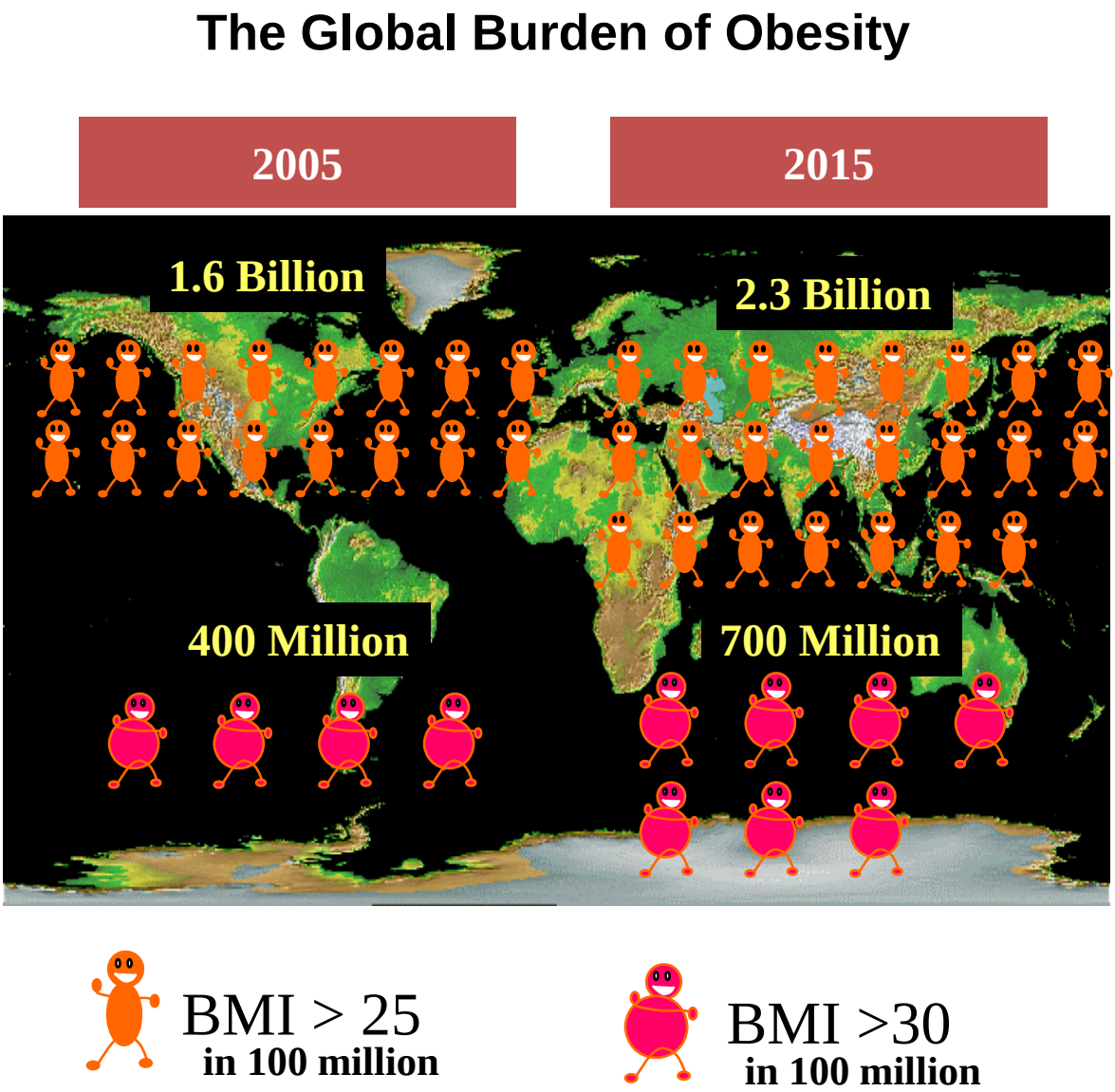


Figure 1.3: Global Prevalence of Overweight and Obese Individuals – Projections to 2015

Source: http://www.who.int/ncd_surveillance/infobase/web/InfoBasePolicyMaker/reports/Reporter.aspx?id=1

CLINICAL COMPLICATIONS

Diabetes is a chronic disease that leads to accumulation of damage to the vasculature tree, the nervous system, and the kidney. Major complications can be divided into damage to the microvasculature and microvasculature (**Table 1.2**).

Table 1.2: Chronic Complications of Hyperglycemia

Microvascular	Macrovascular
Eye Disease	Coronary artery disease
Neuropathy	Peripheral vascular disease
Nephropathy	Cerebrovascular disease

Microvascular Complications

Diabetes predisposes individuals to common eye disorders, increasing the risk of glaucoma by 40% and cataracts by 60%.[9] However, diabetes can also lead to a group of eye maladies, termed diabetic retinopathy, that lead to 12,000-24,000 new cases of blindness each year, making diabetes a leading cause of blindness in adults between 20-74 years old in the United States.[9] In a global arena, the WHO estimates that after 15 years of diabetes 2% of people become blind and 10% will develop visual impairment.[28]

Damage to the small vessels supplying nerves (*vasa nervorum*) leads to diabetic neuropathies that can range from changes in sensory sensations to loss of biological functions (e.g., erectile dysfunction, impaired gastric motility, and vascular compromise). Roughly half of diabetic patients will develop some form of neuropathy.[9]

Due to hyperglycemia, 40% of type 1 diabetic patients and 5-15% of type 2 diabetic patients will develop diabetic nephropathy – the most common cause of end stage renal disease in the United States.[29,30] Though the cause of diabetic nephropathy is

not clear, it is believed that intraglomerular hypertension is an early event contributing to glomerular sclerosis.[30]

Macrovascular Complications

The macrovasculature refers to the large blood vessels, including the aorta, the coronary arteries, and larger vessels of the brain and limbs. Diabetes is considered an anginal equivalent (>20% chance of major coronary events per 10 years), giving diabetic patients the same risk profile as an individual with pre-existing heart disease. This roughly translates into a 2-4 times increased risk for heart disease and stroke in comparison to individuals without diabetes. As the majority of our risk assessment studies are conducted in American or European populations, the INTERHEART study expanded analysis to include 52 countries with varying ethnic and racial backgrounds, validating that diabetes is amongst the greatest risks for heart disease (**Figure 1.4**). Indeed, it is estimated that 64% of diabetic patients will succumb to heart disease and stroke.

INTERHEART STUDY (2004)

	OR	PAR
ApoB/ApoA1 ratio (Abnormal Lipid)	**3.25**	**49.2%**
Smoking	**2.87**	**35.7%**
Hypertension	**1.91**	**17.9%**
Diabetes	**2.37**	**9.9%**
Abdominal obesity	**1.12-1.62**	**20.1%**
Regular physical activity	**0.86**	**12.2%**

From Young JL, Libby P. Atherosclerosis. Chapter in: Lilly LS, editor. *Pathophysiology of Heart Disease*, 4th edition. Philadelphia: Lippincott Williams & Wilkins 2006: 118-140.

Figure 1.4: INTERHEART Study

The INTERHEART study identifies major modifiable cardiovascular risks from 29,000 survivors of myocardial infarction in 52 countries. These risks are presented as odds ratios (OR), representing the odds of a patient suffering an adverse event in comparison to the control, and population attributable risk (PAR), representing the proportion of deaths preventable if the risk was completely eliminated.

ETIOLOGY OF DISEASE – GLUCOSE PERSPECTIVE

Despite a predominance of obesity in the type 2 diabetic patient population, it is estimated that the majority of obese individuals are not overtly diabetic, suggesting that nutrient excess alone is a strong but not singular risk factor for glucometabolic dysfunction.[31,32] First, we will discuss the predominant "glucocentric" theory in the field that focuses on damage caused by high glucose. Second, we will discuss the "lipocentric" view that reveals how lipids can initiate and propagate dysfunction. Third, we will discuss the growing appreciation of the role of inflammation in diabetes pathogenesis, potentially serving as a unifying concept in "glucocentric" and "lipocentric" viewpoints.

Excessive circulating plasma glucose is by definition diabetes, but why is this extra sugar detrimental? In fact, glucose is a reactive compound that must be regulated from improper reaction and modification of existing proteins, e.g., aberrant glycosylation. In addition to traditional biochemical studies, hints from the cells involved in microvascular complications suggest that hyperglycemia introduces a cellular stress that leads to dysfunction. Michael Brownlee summarizes the glucocentric perspective into four key pathways: 1) Polyol pathway, 2) Advanced Glycation Endproducts, 3) PKC activation, and 4) Hexosamine pathway.[33]

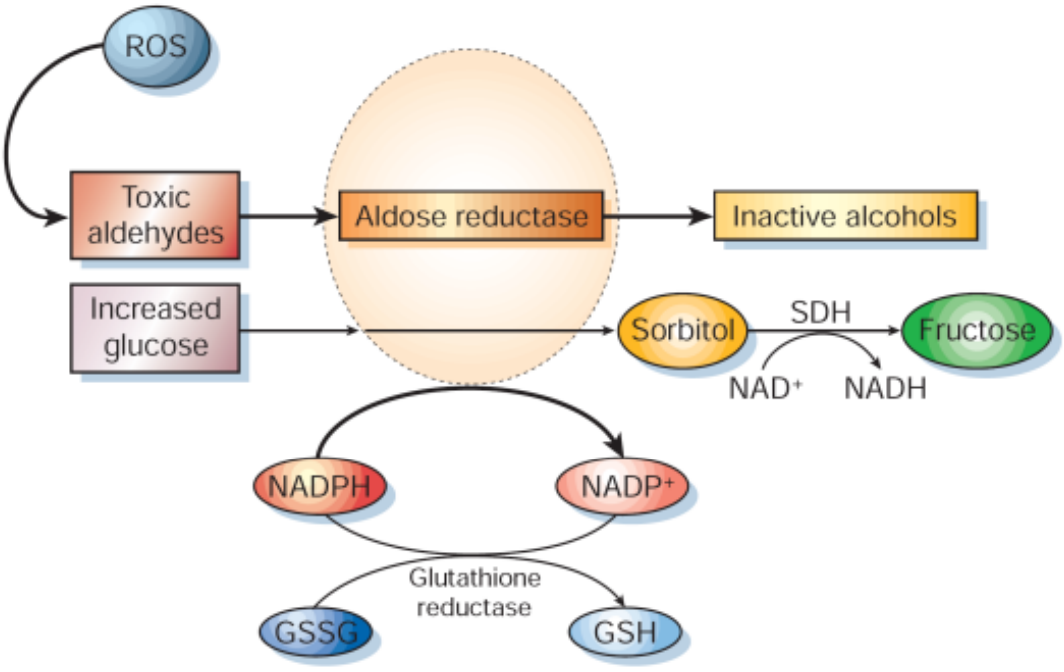
THE POLYOL PATHWAY

The polyol pathway is an important pathway to deal with excess glucose, converting intracellular glucose through aldose reductase into sorbitol, which can be later

oxidized by sorbitol dehydrogenase to fructose to be used for glycolysis and glyconeogenesis. Aldose reductase serves a similar salutary function to detoxify reactive aldehydes created by reactive oxygen species (ROS) into non-reactive alcohols. However, in these redox reactions, NADPH is required as a reducing agent; therefore, excess activity in these pathways diminishes the available supply of NADPH necessary for generating the cellular antioxidant glutathione (GSH) (**Figure 1.5**). The combination of excess sorbitol, which causes oncotic stress, with diminished GSH, which limits cell antioxidant capabilities, is believed to play a role in damage of susceptible cells of the microvasculature. To exemplify the clinical importance of the polyol pathway in neuropathies, *Engerman et al.* found that the aldose reductase inhibitor Sorbinil could prevent the loss of nerve conduction velocity in diabetic dogs.[34]

ADVANCED GLYCATION ENDPRODUCTS

Advanced glycation endproduct (AGE) formation is believed to be fostered by stoichiometric pressures in the high intracellular glucose environments of diabetic patients through both auto-oxidation and enzymatic processes, resulting in a reactive dicarbonyl that forms a covalent linkage with the amino group of proteins. This aberrant reaction is believed to cause cellular damage via three pathways: 1) alteration of intracellular proteins and associated function, 2) alteration of extracellular proteins that leads to dysfunction of appropriate cellular reactions, and 3) circulating AGE proteins can modify plasma proteins and signal through the receptor of AGE (RAGE) to initiate reactive oxygen species and activate nuclear factor kappa B (NFκB) – a prominent pro-



From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001

Figure 1.5: The Polyol Pathway

inflammatory transcription factor leading to release of inflammatory cytokines (IL-1, TNFα, IL-6), growth factors (TGFβ, PDGF, M-CSF, GM-CSF), and pro-coagulant molecules (thrombomodulin, PAI-1) (**Figure 1.6**). The role of AGE inhibitors is actively under investigation and early results suggest that they may be effective in reducing diabetic microvascular complications.[35-41]

PROTEIN KINASE C ACTIVATION

Increased intracellular glucose may serve as a substrate for *de novo* formation of diacylglycerol (DAG) – the activating cofactor for several members of the protein kinase C (PKC) family. The PKC family mediates many processes that may be involved in microvascular complications, including alterations in vascular reactivity, permeability, angiogenesis, and occlusion that may contribute to retinal and glomerular damage (**Figure 1.7**). Furthermore, generation of ROS and activation of NFκB enhances secretion of pro-inflammatory factors, discussed earlier, that have the potential of changing the local microenvironment as well as affect distant tissues. Despite general PKC activation with wide and disparate features, isoform-specific PKC inhibitors have shown improvement in retinal and glomerular dysfunction.[42,43] Of interest, the first 2 pathways discussed may also contribute to PKC activation via net increases in reactive oxygen species either by shifts in the polyol pathway towards glucose reduction or receptor-mediated production with AGE ligation.

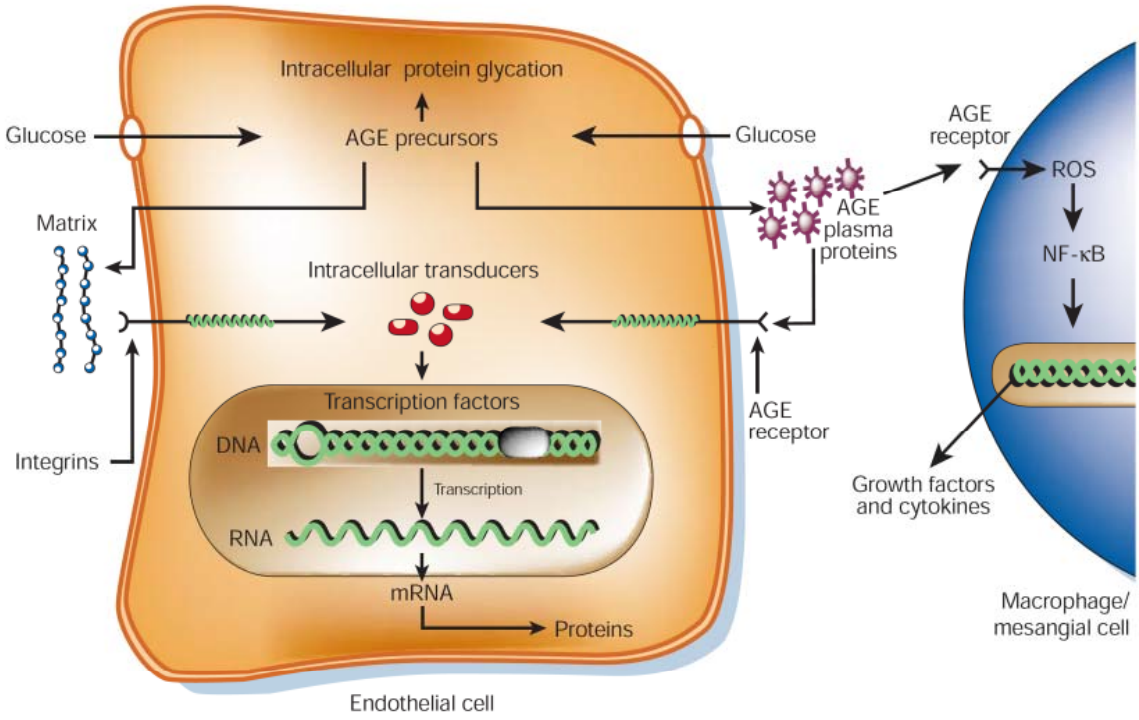


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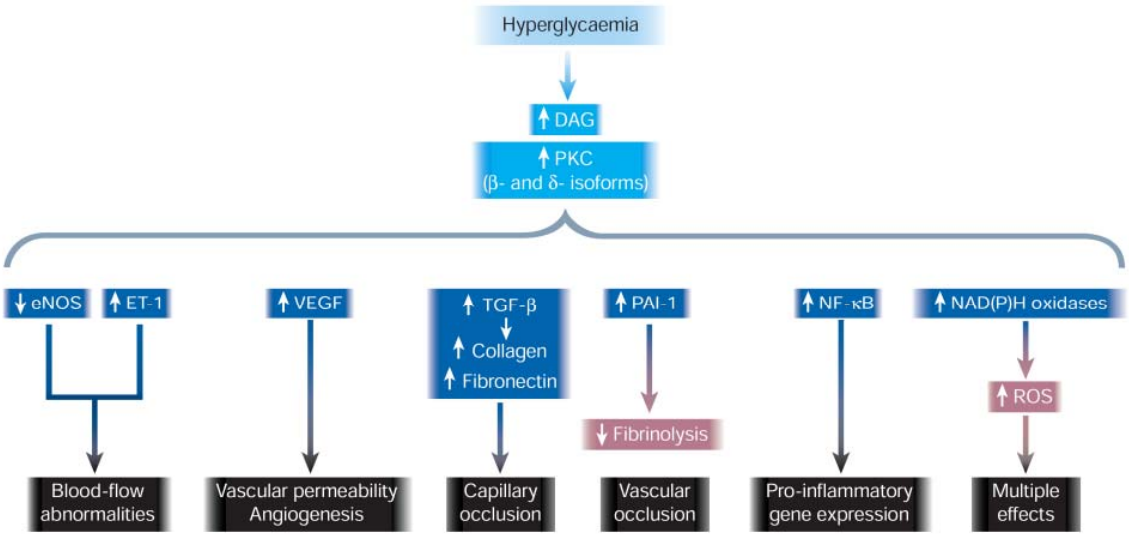
Figure 1.6: Advanced Glycation Endproducts



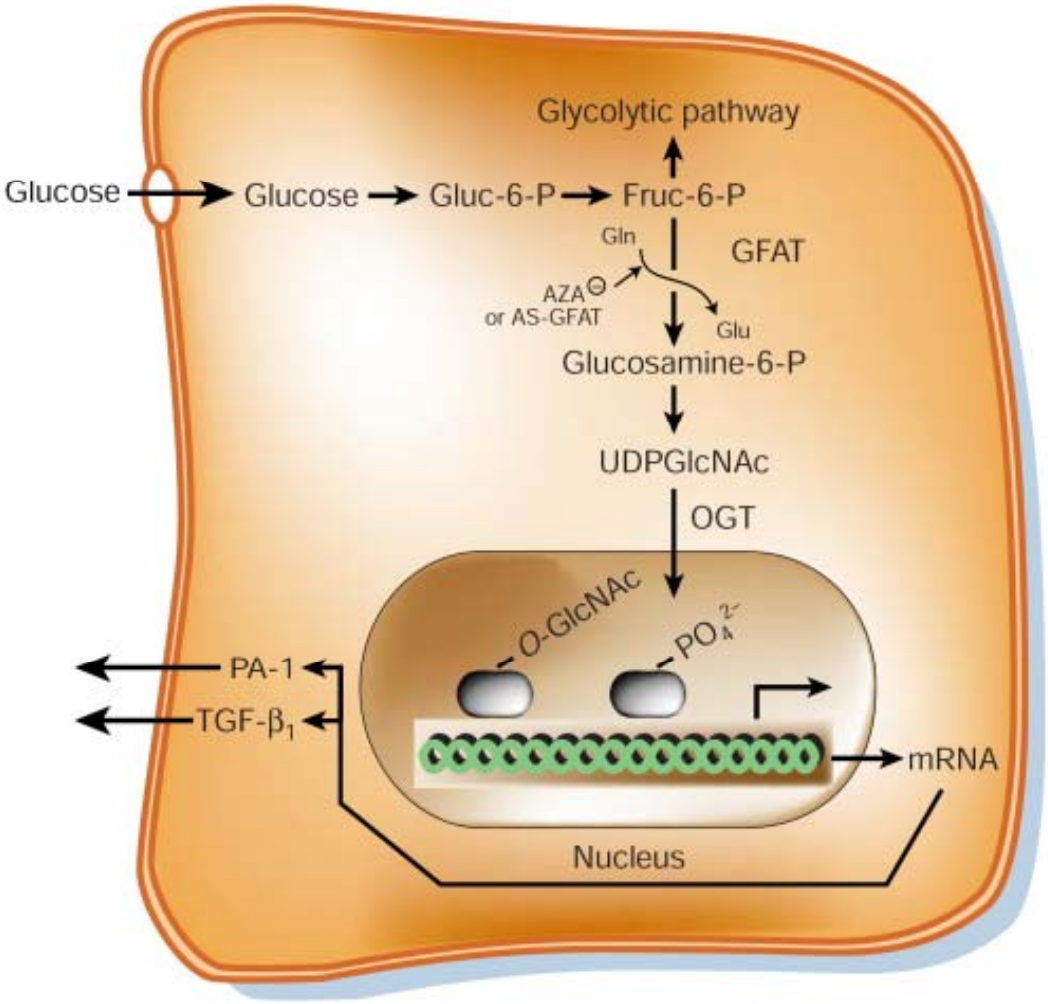
From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001

Figure 1.7: Protein Kinase C Pathway

THE HEXOSAMINE PATHWAY

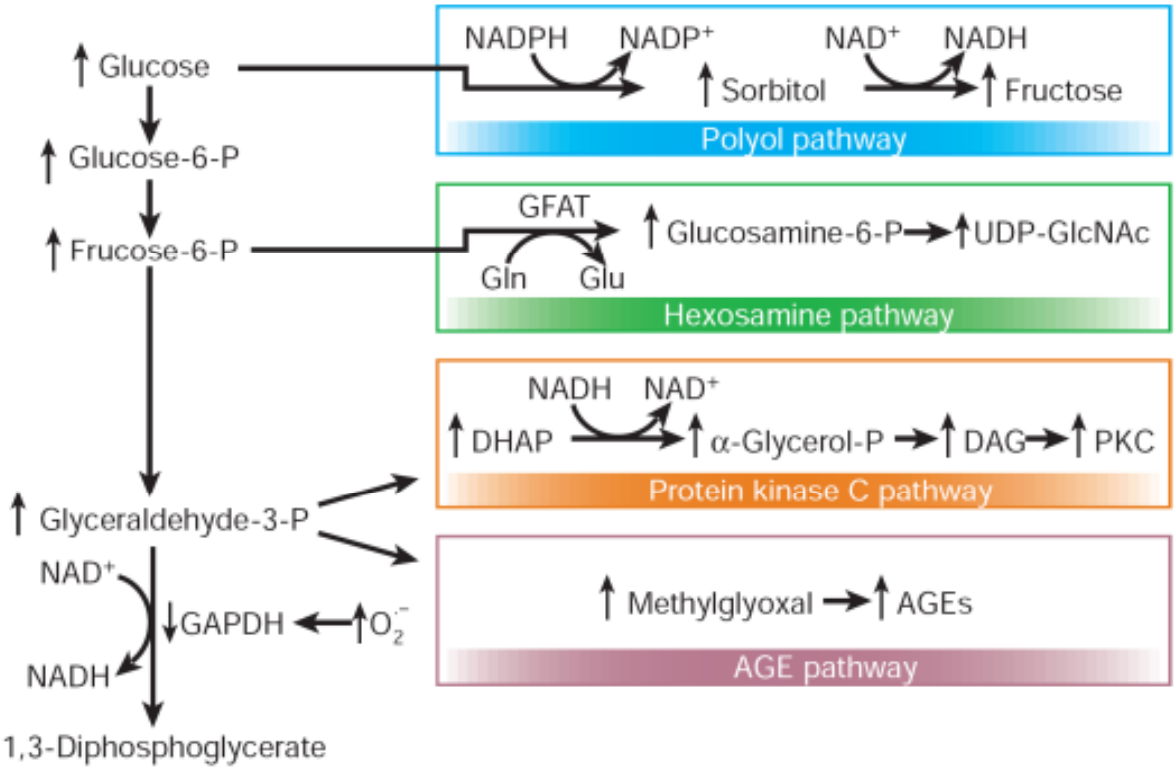
The majority of excess intracellular glucose is destined to form ATP; however, an increase in glycolytic intermediates may lead to diversion into other metabolic pathways. Exemplified in aortic endothelial cells, hyperglycemia led to the shuttling into the hexosamine pathway with the conversion of the fructose-6-phosphate intermediate to glucosamine-6-phosphate and then uridine diphosphate *N*-acetyl glucosamine (UDP-GlcNAc) (**Figure 1.8**). UDP-GlcNAc modification of serine and threonine residues of transcription factors can lead to alteration of transcriptional activity. As an example, modification of Sp1 enhances PAI-1 and TGFβ expression, contributing to both glomerular and vascular complications (**Figure 1.8**).

Although each of these pathways contributes to microvascular complications and potential exacerbation of macrovascular complications via pro-inflammatory and pro-coagulant molecules, it is believed that these four pathways can be unified by the observation of enhanced intracellular oxidative stress from superoxide production and ROS. In concert with the ability of ROS to enhance stress- and inflammatory-associated pathways, ROS inhibition of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) can lead to a "back-up" of the glycolytic pathway, diverting intermediates into each of these four hyperglycemia-induced pathways (**Figure 1.9**).



From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001

Figure 1.8: Hexosamine Pathway



From Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001

Figure 1.9: The Central Role of Reactive Oxygen Species in the Glucocentric Perspective

ETIOLOGY OF DISEASE – LIPID PERSPECTIVE

The common clinical finding of hepatic steatosis and hypertriglyceridemia in type 2 diabetic patients coupled with epidemiological associations of diabetes with obesity suggests that alteration in lipids may contribute to diabetes pathogenesis. In human studies employing MRI-based techniques to quantify intracellular lipid accumulation in skeletal muscle, it was shown that high intramyocellular lipids correlated better with insulin resistance than traditional metrics, including BMI and total body fat.[44,45] Insulin resistance with short-term lipid infusions corroborate that excess circulating free fatty acids can play a causative role; however, it is not clear what the mechanism of action is and whether this approximates obesity in human populations. One theory proposed by Randle suggests that increased β-oxidation of intracellular fatty acids favors a higher NADH:NAD+ ratio; thus, preventing forward progress of the glycolytic pathway that require NAD+ substrate. According to Randle's hypothesis there would be an increase in intracellular glucose-6-phosphate levels; however, this theory is challenged by studies demonstrating decreased glucose-6-phosphate in lipid-infusion studies of healthy individuals as well as in normoglycemic offspring of diabetic parents.[46-48]

To account for this difference, it is believed that free fatty acids may impair glucose uptake rather than serve as a competitor for intracellular substrate oxidation. Indeed, molecular mechanisms have been proposed demonstrating that free fatty acids can lead to inhibition of IRS-1 activity – a key component of the insulin signaling pathway necessary for glucose uptake. However, the direct mechanism of action is elusive, but could involve other inhibitors of IRS-1 activity – namely inflammatory

cytokines. In the circulation, free fatty acids have wide access to vascular cells and circulating monocytes. In cell culture systems, free fatty acids, in particular those saturated, have been shown to induce inflammatory molecules such as IL-6, IL-8, and intercellular adhesion molecule 1 (ICAM-1), which may contribute to tissue insulin resistance in many ways, including direct inhibition of the insulin signaling pathway as well as recruitment of professional inflammatory cells that can alter the inflammatory and metabolic tone.[49-58] Indeed, obese individuals are associated with a chronic inflammatory state which will be discussed in this emerging viewpoint on diabetes pathogenesis.

ETIOLOGY OF DISEASE – INFLAMMATORY PERSPECTIVE

As an ancient disease, empirical treatments for "sugar urine disease" have existed anecdotally for generations; however, we are just beginning to understand their molecular implications today. As an example, the bitter melon is a traditional Chinese treatment for diabetes that has recently been discovered to harbor potent AMPK activators, which by themselves have just recently been appreciated in diabetes pathogenesis over the last decade.[59] Similarly, the French lilac was used in medieval times to treat diabetes due to its active biguanide components. Although, biguanide-based medications has been is use pharmacologically for the last 50 years, its mode of action remains unclear, but may involve both AMPK activation as well as novel anti-inflammatory actions in the cells of the vascular wall.[60,61] Stemming from the innovation of analgesics in 1800s Germany, Ebstein observed that the anti-inflammatory salicylates could ameliorate glycosuria, representing one of earliest examples implicating inflammation in diabetes pathogenesis as well as suggesting that modulation of inflammatory pathways may be a potential therapeutic strategy.[62] However, further investigations into the role of inflammation would remain quiet in the diabetes field for the next century, perhaps muffled by the excitement surrounding the discovery of insulin by Frederick Banting and Charles Best. We will first discuss the reemergence of the inflammatory hypothesis with epidemiological studies linking obesity/diabetes with activation of the innate immune response, followed by a review of how inflammatory agents can directly cause insulin resistance, and finally a discussion on modulators of inflammation.

INFLAMMATORY MARKERS ASSOCIATED WITH DIABETES - THE ACUTE PHASE RESPONSE

Epidemiological studies of the 1950s and 1960s would mark the next major association of inflammation and diabetes with the finding that the acute phase reactant fibrinogen is elevated in obese and diabetic individuals[63-65]. Systemic inflammation initiates the acute phase response, signaling the liver to produce numerous immune-modulating proteins, including coagulation factors, complement, C-reactive protein (CRP), mannose-binding protein, and serum amyloid A. Of note, these secreted proteins are also known as *positive acute-phase proteins* in contrast to *negative acute phase proteins* that are decreased, such as albumin and transferrin.[66] With the emergence of antibody-based diagnostics, the ability to study additional acute phase reactants and inflammatory cytokines became possible in large population studies (**Table 1.3**). CRP, fibrinogen, and PAI-1 have emerged as leading candidates to predict development of insulin resistance and diabetes independent of classical risk factors, such as BMI, truncal obesity, elevated fasting triglycerides, and even pre-existing insulin resistance in some studies. Although the association of inflammatory mediators, independent of anthropometric parameters, with diabetes risk was well characterized, it still remained unclear whether inflammation initiated insulin resistance or merely served as a marker.

Table 1.3: Increased Acute Phase Reactants and Inflammatory Markers with Obesity or Diabetes

Molecule	Cohort
Fibrinogen	Predict development of diabetes over 5 years independent of BMI, fasting triglyceride, insulin sensitivity, HDL, or blood pressure [67-70] ↑ in Insulin resistant individuals with and without diabetes [71] ↑ Microalbuminuric patients[72] ↑ Caucasian participants, but not African Americans [73] ↑ BMI, truncal obesity, and bioelectric impedance [74] ↑ in patients from Atherosclerosis Risk in Communities study that develop diabetes[75] ↔ Eldery patients with new onset diabetes [76]
CRP	Predict development of diabetes over 5 years independent of BMI, fasting triglyceride, insulin sensitivity, HDL, or blood pressure [67,68,70] ↑ baseline values in non-diabetics that become diabetes[69] ↑ Insulin resistance in non-diabetic individuals [77] ↑ Impaired oral glucose tolerance in non-diabetic individuals [78] ↑ Non-diabetic, hyperinsulemic patients that become diabetic [79] ↑ Microalbuminuric patients[72] ↑ BMI, truncal obesity, and bioelectric impedance [74] ↑ Eldery patients with new onset diabetes [76] Predict development of diabetes in Women's Health Study independent of hormone replacement therapy [80,81]
IL-6	↑ Caucasian participants, but not African Americans [73] Predict development of diabetes in Women's Health Study independent of hormone replacement therapy [80,81] ↑ baseline levels in concert with IL-1β prospectively predict diabetes[82]
PAI-1	Predict development of diabetes over 5 years independent of BMI, fasting triglyceride, insulin sensitivity, HDL, or blood pressure [67,68,70,83] ↑ in insulin resistant individuals with and without diabetes [71] ↑ in Non-diabetic, hyperinsulemic patients that become diabetic [79] ↑ baseline values in non-diabetics that become diabetic independent of BMI or insulin sensitivity [69]
Orosomucoid/ Sialic Acid	↑ in patients from Atherosclerosis Risk in Communities study that develop diabetes[75]
WBC	Predict development of impaired fasting glucose and diabetes in non-smokers[84] ↑ in Caucasian participants, but not African Americans, predict diabetes development [73] ↔ Eldery patients with new onset diabetes [76] ↔ NHANES cohort[85] ↑ in patients from Atherosclerosis Risk in Communities study that develop diabetes[75] ↑ in baseline levels predict increase in insulin resistance nda diabetes development in Pima Indians[86]
Platelets	↔ Eldery patients with new onset diabetes [76]
Factor VIIIc	↔ Eldery patients with new onset diabetes [76]
Albumin	↓ in patients from Atherosclerosis Risk in Communities study that develop diabetes[75] ↔ Eldery patients with new onset diabetes [76]

INSULIN SENSITIVE TISSUES: INFLAMMATORY TARGETS AND SOURCES

Insulin resistance is not uniquely limited to the development of diabetes – its clinical presence can be seen in diverse conditions, including lipodystrophy, liver disease, polycystic ovarian syndrome, infection, and sepsis. Inflammatory cues are common denominators in these maladies, suggesting their potential role in curbing insulin signaling cascades and generating tissue insulin resistance.

The role of inflammatory mediators in the molecular pathogenesis of type 2 diabetes has gained appreciation over the last decade and a half with a seminal paper by *Hotamisligil et al.* revealing that the inflammatory cytokine TNFα can inhibit the insulin signaling pathway by prevention of insulin receptor phosphorylation as well as its main cytosolic substrate insulin receptor substrate (IRS)-1 in adipose and muscle tissues.[87] Although inflammatory cytokines are soluble and often act distally, the discovery by *Hotamisligil et al.* that TNFα was expressed by adipose tissue itself opened up new possibilities where cytokines can act in a paracrine or autocrine fashion – effectively increasing physiological concentrations when isolated to a local environment.[88]

We will examine current literature supporting the ability of inflammatory cytokines and mediators to alter insulin signaling pathways and glucose regulation in peripheral tissues, namely the liver, skeletal muscle, and adipose.

Liver

The importance of the liver in glucose homeostasis is exemplified by both infectious and non-infectious etiologies of liver dysfunction, which commonly present

with hepatic insulin resistance and impaired whole body glucose regulation. In fact, nonalcoholic fatty liver disease (NAFLD) is believed to be present in 5% of the general population and about 25% of the obese and type 2 diabetes populations, highlighting the liver's important role in glucose homeostasis in the human population.[89-92]

Productive insulin signaling in the liver inhibits hepatic gluconeogenesis and glycogenolysis, which in sum can be termed hepatic glucose production (HGP). In the fed state, insulin binds to the insulin tyrosine kinase receptor which phosphorylates tyrosine moieties on insulin receptor substrates (IRS), activating multiple parallel cascades: 1) MAPK cascade to activate Erk leading to gene transcription, and 2) PI3K cascade to activate Akt leading to protein synthesis through mTOR, to lipolysis through PKA and HSL, to GLUT4 exocytosis through AS160, and to inhibition of glycogen synthesis and fatty acid synthesis through GSK-3. As a central metabolic hub, the liver accepts gluconeogenic substrates and free fatty acids from other tissues of the body - liberated during fasting states in order to provide additional glucose. Given the reciprocal state when fed, insulin-mediated suppression of substrate release in the periphery may have accounted for decreased HGP. However, this non-hepatic (indirect) insulin effect on HGP has been partially discounted by the liver-specific insulin receptor knockout (LIRKO) mouse, which demonstrated the inability of insulin to suppress HGP despite functional peripheral insulin action, favoring a model where hepatic (direct) insulin action is an essential component for HGP regulation.[93]

Early studies of inflammation in hepatic glucose output employed injection of Escherichia coli endotoxin – a potent stimulus of multiple inflammatory cytokines.

Indeed, injection in both normoglycemic and diabetic rats led to marked elevation in serum glucose associated with depletion in hepatic glycogen content.[94,95] To specify which inflammatory player may be involved, *in vitro* experiments in primary murine hepatocytes and human HepG2 liver cell lines demonstrated that pretreatment with IL-6 led to a time-dependent decrease in IRS-1 tyrosine phosphorylation and Akt phosphorylation – key events in the insulin signaling pathway.[96] Moreover, pre-infusion of IL-6 *in vivo* prior to hyperinsulemic-euglycemic clamping shows no changes in basal HGP, but following insulin administration, demonstrates a reduced ability to suppress HGP.[97] On a molecular level, the inability of insulin to suppress HGP in IL-6 pretreated mice was associated with a 50% decrease in insulin-stimulated IRS-2-associated PI3K activity.[97,98] However, co-infusion with the anti-inflammatory cytokine IL-10 was able to restore hepatic insulin sensitivity without changes in basal metabolism.[97]

TNFα neutralization utilizing TNF receptor (TNFR)- IgG fusion proteins in the Zucker (fa/fa) rat model of obesity failed to demonstrate *in vivo* improvement in hepatic insulin signaling.[87] However, studies by *Cheung et al.* using adenoviral strategies to express a chimeric fusion protein using human TNFR with mouse IgG demonstrated improvement in hepatic insulin resistance in obese animals, without changes in insulin receptor tyrosine phosphorylation.[99] On the other hand, enhancing TNFα via exogenous infusion in obese Zucker rats corroborated hepatic insulin resistance, suggesting TNFα could inhibit insulin action distal to insulin receptor phosphorylation.[100] However, *in vitro* studies in rat hepatoma Fao cells suggests that TNFα inhibits insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1.[101] Although the mechanism

of action remains unclear as well as complicated by the ability of TNFα to induce other cytokines, it appears that TNFα is sufficient to induce hepatic insulin resistance.

In response to inflammatory stimuli, negative feedback is promoted by a group of proteins names suppressors of cytokine signaling (SOCS). *In vitro*, IL-6-induced insulin resistance in HepG2 cells correlated temporally with increases in SOCS-3 expression.[102,103] To highlight the relevance of inflammation in obesity and their shared role in insulin resistance, *Ueki et al.* used obesity as well as LPS-induced endotoxemia models in parallel to demonstrate similar increases in hepatic and muscle SOCS associated with hepatic insulin resistance that could be recapitulated with adenoviral-mediated SOCS-3 overexpression alone.[104]

IL-6 may affect glucose homeostasis through its close interaction with STAT3. Loss of hepatic STAT3 abrogates insulin-mediated suppression of gluconeogenic enzymes, while reconstitution via adenovirus ameliorates this hepatic insulin resistance.[105] On the other hand, constituitive activation of hepatic STAT3 leads to unimpeded gluconeogenic suppression with diminished blood glucose and insulin.[105] In studies of diet and genetic obesity, STAT3 activity and hepatic insulin resistance were reduced by IL-6 neutralizing antibodies, exemplified by a 3-fold increase in insulin-induced suppression of gluconeogenesis and a 50% increase in hepatic Akt phosphorylation.[106]. The role of IL-6 and STAT3 in glucose homeostasis has central nervous system (CNS) origins as intracerebral ventricular (ICV) injection of insulin suppresses HGP, while loss of hepatic STAT3 or IL-6 impairs this normal response.[107]

The liver is an important organ mediating whole body glucose homeostasis with significant potential intersections with inflammation. In addition to the systemic inflammatory state in obesity, primary liver inflammation, e.g., chronic hepatitis C virus (HCV) infection, is associated temporally with insulin resistance, while HCV clearance with diminished inflammation parallels improvement in insulin sensitivity in these patients.[108-110] Moreover, transgenic models expressing HCV core protein alone was sufficient to produce insulin resistance that was associated with elevated TNFα.[111] Therefore, to appreciate the role of inflammation in insulin resistance and the importance of the liver in glucose homeostasis, we need only look to other clinical examples.

Skeletal Muscle

Skeletal muscle is the main site of insulin-stimulated glucose uptake, serving as an important glucose sink in the body with significant glycogen storage capability.[112] However, muscle-specific insulin receptor knockout mice (MIRKO) remain normoglycemic due to a redistribution of glucose uptake function to adipose tissue – exemplified by 3-fold increase in insulin-stimulated glucose uptake and enhanced adipose mass.[113] Therefore, although it is helpful to organize our thoughts on specific roles each tissue plays, there is remarkable plasticity in the system in order to maintain glucose homeostasis.

Multiple modalities of TNFα neutralization has been employed in models of obesity, including direct and adenoviral-mediated expression of TNFR-IgG fusion proteins and genetic knockouts, indicating a clear role of TNFα in skeletal muscle insulin

resistance with inhibition at the level of the insulin receptor and IRS.[87,88,99,100,114] Similar mechanisms of action were confirmed in human vastus lateralis muscles from healthy volunteers injected with TNFα, demonstrating direct suppression of insulin receptor signaling and glucose uptake in human skeletal muscle via inhibition of Akt-mediated phosphorylation of Akt substrate 160 (AS160).[115] Again, TNFα-induced insulin resistance in skeletal muscle may also be mediated by upregulation of SOC-3.[116] In sum, TNFα-mediated inhibition of skeletal muscle insulin signaling in rodent models shows a promising opportunity for anti-TNFα treatments in human diabetes, but their effectiveness remains unclear.[117]

Working skeletal muscle, e.g. during exercise, enhances expression of pro- and anti-inflammatory cytokines, but IL-6 secretion is the most prominent.[118,119] IL-6 secretion is proportional to the duration and intensity of exercise, and may be in part mediated by release of epinephrine during exercise which was demonstrated to enhance IL-6 secretion *in vitro*.[118,120]. Loss of IL-6 in genetic knockout models led to mature-onset obesity with glucose intolerance that could be reversed with exogenous IL-6 replacement, suggesting beneficial functions in contrast to those described earlier in the liver.[121] IL-6 administered centrally enhanced energy expenditure and fat loss, while low IL-6 in cerebrospinal fluid was associated with severe obesity in humans – suggestive of IL-6 action in the CNS. The role of IL-6 in whole body glucose homeostasis is likely tissue-specific, but remains an intriguing candidate as a muscle-derived cytokine enhanced by exercise and pluripotent in action.

Adipose Tissue

Expansion of adipose tissue is the most blatant feature associated with human obesity. Traditionally, adipose tissue has been viewed as an inert tissue that merely harbors excess energy in the form of calorie-dense lipids. However, recent studies have elucidated that adipose is indeed an endocrine organ, mediating diverse processes from energy metabolism and feeding behavior to vascular tone and hemostasis (**Figure 1.10**). In the last decade and a half, adipocytes have been shown to be professional secretors of: 1) traditional inflammatory cytokines, 2) adipose-specific cytokines also known as adipokines, and 3) chemokines that direct other cell types into adipose tissue.

TNFα was one of the first cytokines shown to be expressed in adipose and enhanced in rodent and human obesity, paralleling systemic and tissue insulin resistance closely.[88,122] Indeed, similar to experiments discussed in skeletal muscle, enhanced TNFα with obesity or direct infusion was able to elicit insulin resistance in adipose tissue and subsequently reversed with TNFα antagonists and genetic deletion.[87,114] Similar to skeletal muscle, TNFα action may be mediated by SOCS-3 expression.[116]

Several agonists common in the adipose microenvironment induce IL-6 secretion, including hormones (insulin, TSH, GH), inflammatory stimuli (TNFα, LPS, IL-1, IL-6, Prostaglandin D2), the fatty acid palmitate and the β-receptor agonist isoproterenol.[123-131] Sustained adipocyte exposure to IL-6 *in vitro* led to a feed forward mechanism that amplified IL-6 concentration from endogenous sources.[123,124] Though insulin resistance was a common end result of IL-6 treatment in both 3T3-L1 and 3T3-F442A adipocytes, the mechanism of action is conflicted with mixed results suggesting downregulation of

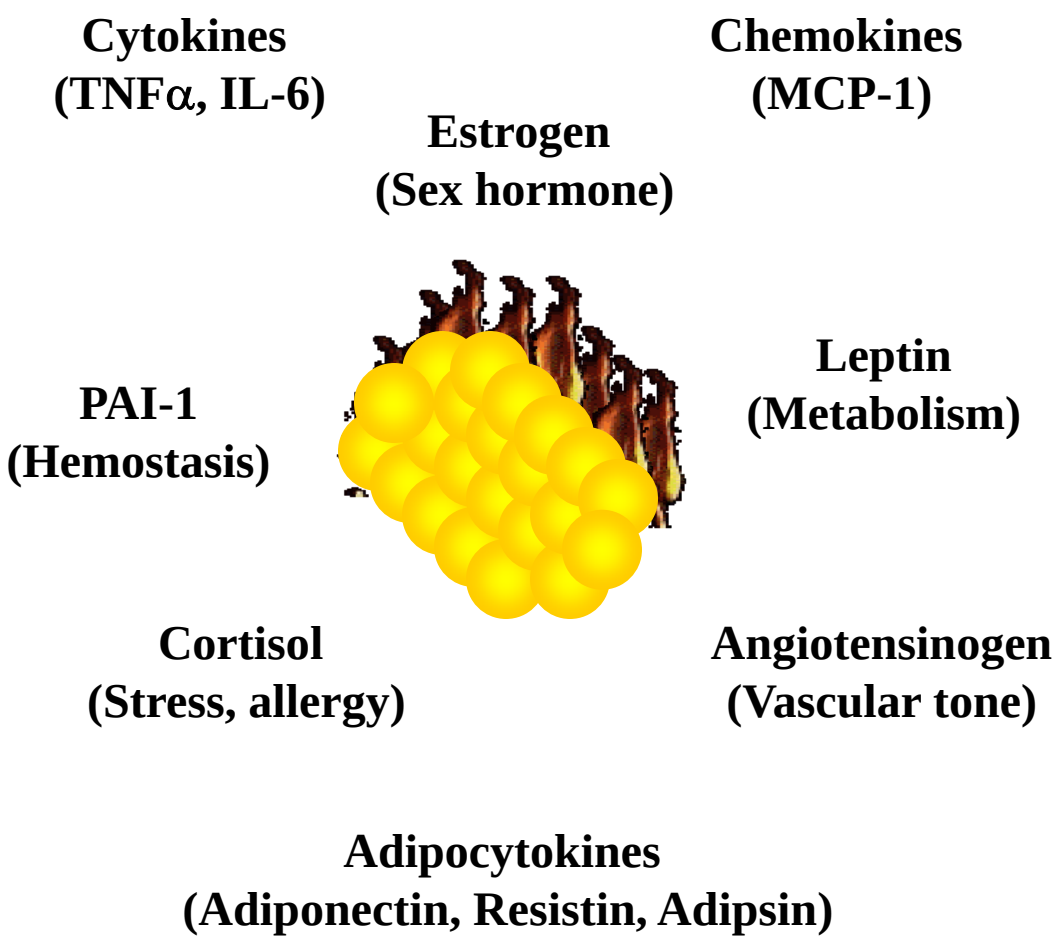


Figure 1.10: Adipose as an Endocrine Organ

Former views of adipose as an inert storehouse for lipids has been supplanted with recent discoveries that adipose is in fact a major source of secreted factors. The variety of factors secreted include inflammatory cytokines, chemokines, thrombogenic factors, metabolic hormones, sex hormones, mediators of vascular tone, and adipocytokines.

insulin receptor β, IRS-1, and GLUT-4 via translational or post-translational mechanisms.[123,124] IL-6 may also contribute to insulin resistance via the paracrine inhibition of the adipocyte-restricted insulin-mimetic visfatin expression in 3T3-L1 adipoctyes. [132]

Although IL-1β has a prominent role in other chronic inflammatory conditions, the *in vivo* contribution to tissue-specific glucose tolerance is difficult to disassociate from roles of IL-1 in food intake, lipid metabolism, and obesity.[133,134] However, continuous addition of IL-1β to 3T3-L1 adipocytes *in vitro* led to an Erk-dependent downregulation of IRS-1 mRNA which could be prevented with an Erk inhibitor.[135]

Inflammatory mediators clearly can modulate insulin signaling pathways with hepatocyte-, myocyte-, and adipocyte-derived cytokine production representing an effective local source of inflammatory stimuli; however, the role of adipose tissue in mediating systemic inflammatory tone remains controversial. Emerging data using a model of inducible fat-specific apoptosis (FAT Apoptosis Through Triggered Activation of Caspase-3; FAT-ATTAC) has led to insight into the role of adipose in directing systemic inflammation. Injection of the potent inflammatory stimulus LPS normally leads to rapid systemic inflammation and acute insulin resistance; however, fatless FAT-ATTAC show marked attenuation of LPS-induced inflammation, alluding to the importance of adipose in systemic inflammatory responses.[136]

Though the inflammatory hypothesis in diabetes is not a new theory, the ability to test the molecular intersections of these two areas has required both the development of the insulin signaling pathway as a framework for investigation as well as advancements

in recombinant protein and immunological technologies in order to create investigatory tools. Nonetheless, the role of inflammation in insulin-sensitive peripheral tissues is an active area of development and there is significant confidence that additional roles of inflammatory mediators in metabolic homeostasis will be uncovered.

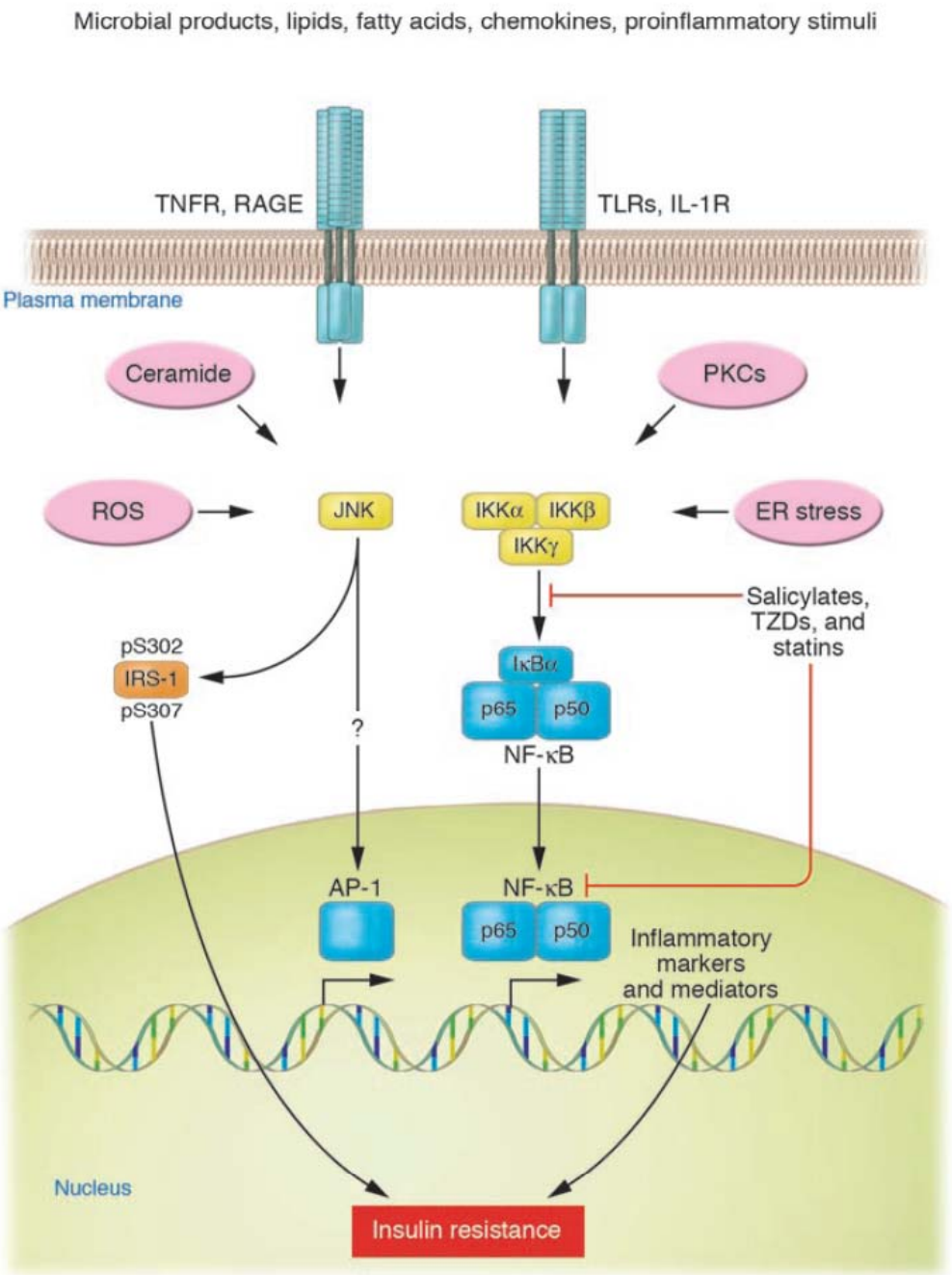
INFLAMMATION-DIRECTED INSULIN RESISTANCE – MOLECULAR BLUEPRINT

Though cytokine-induced insulin resistance is a concept in its infancy with the majority of studies conducted in the last decade, convergence of insulin signaling and inflammatory pathways have been revealed. In particular, the Jnk and NFκB inflammatory pathways intersect with components of the insulin signaling cascade.

C-Jun N-terminal kinase (Jnk) is a stress-responsive kinase, increasing in activity with cytokine exposure, intracellular oxidative stress, and free fatty acids. Activated Jnk phosphorylates IRS-1 at serine 307, inhibiting productive insulin-mediated tyrosine phosphorylation.[137] In contrast, substitution of alanine for serine at the 307 position on IRS abrogates Jnk-induced serine phosphorylation and prevents TNFα-mediated inhibition of insulin signaling.[137] In accord, Jnk-1 deficient mice are refractory to diet-induced obesity with enhanced insulin sensitivity (**Figure 1.11**).[138]

Nuclear Factor kappa B (NFκB) is the prototypical stress-responsive transcription factor that resides in the cytosol bound to inhibitor of kappa B (IκBα). Inflammatory cytokines, AGE, fatty acids, and bacterial lipopolysaccharides activate inhibitor of kappa B kinase (IKK), which phosphorylates IκBα to foster disassociation and allow NFκB nuclear translocation, driving transcription inflammatory cytokines and chemokines such

as IL-1, IL-6, IL-18, TNFα, and MCP-1. Of note, NFκB may also transcribe anti-inflammatory molecules as well, including IL-10 and manganese superoxide dismutase. Similar to Jnk, IKK phosphorylates IRS-1 at serine 307 in both cell models as well as using recombinant proteins.[139] Attenuating IKK activity via salicylate treatment or heterozygous gene disruption was sufficient to prevent diet-induced obesity and improve insulin sensitivity.[140,141] On the other hand, low-level constitutive expression of IKKβ in hepatocytes was sufficient to approximate the diet-driven insulin-resistant phenotype; however, hyperglycemia and systemic insulin resistance may have stemmed from severe hepatic insulin resistance.[142] Despite direct intracellular interaction of IKK and IRS-1 in this model, systemic IL-6 neutralization was still beneficial, suggesting that secreted factors as well as intracellular events both influence insulin resistance.[142](**Figure 1.11**)



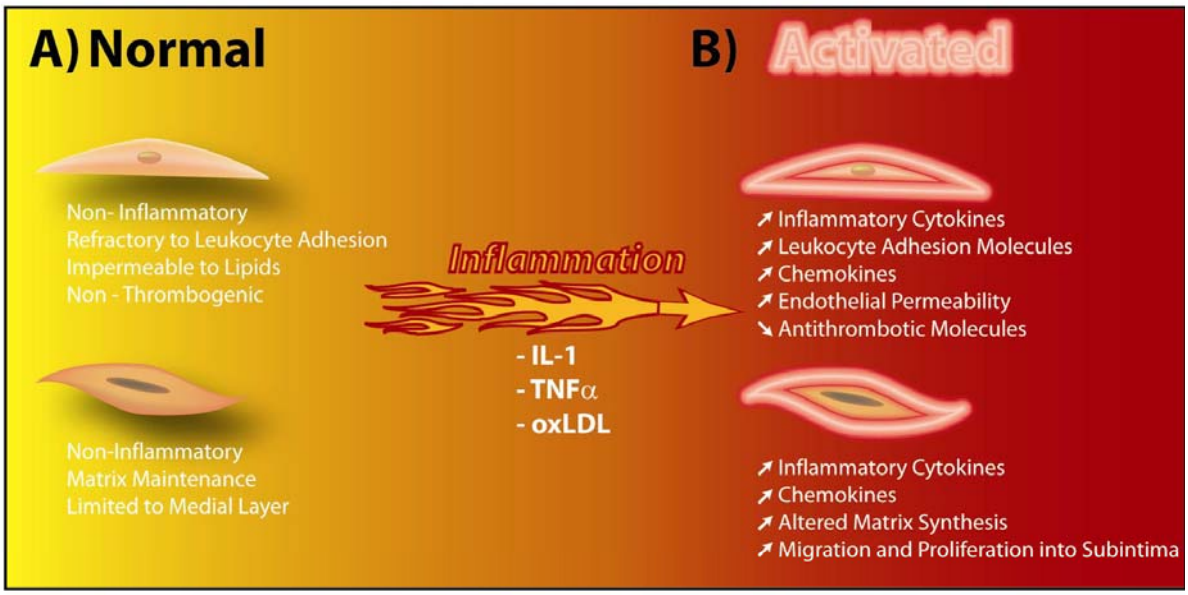
From Shoelson SE, Lee J, Goldfine AB: Inflammation and Insulin Resistance. *J Clin Invest* 116:1793-1801, 2006

Figure 1.11: Molecular Mechanisms of Inflammation-Induced Insulin Resistance

INFLAMMATION AS A UNIFYING CONCEPT IN INSULIN RESISTANCE: WHAT FANS THE FLAMES?

Numerous lines of evidence suggest that inflammation is present with insulin resistance in both animal models as well as human disease. The discovery that peripheral insulin-sensitive tissues themselves can produce many of these inflammatory mediators has put forward an intriguing hypothesis of inflammation-driven insulin resistance. Nonetheless, what environmental or biological cue drives this inflammatory insult to the point of insulin resistance?

Vascular inflammation has close clinical and molecular relationships with diabetes that maintains relevance for future inquiries; however, it may be outside the scope of this thesis. Therefore, we will merely summarize the main points that: 1) vascular smooth muscle cells (SMC) and endothelial cells (EC) normally maintain a non-inflammatory, non-thrombogenic vascular surface that limits leukocyte adhesion and lipid penetration (**Figure 1.12**), 2) activation of SMC and EC by inflammation (e.g., cytokines, LPS, ROS) stimulates feed forward cytokine expression, pro-thrombotic molecules, chemokines, and leukocyte adhesion molecules (**Figure 1.12**), 3) concomitant hyperglycemia in patients fosters accumulation of glycated LDL in the vessel wall, which serves as a potent inflammatory stimulus, and 4) the "activated" vessel wall recruits macrophages that serve as an important source of pro-inflammatory cytokines. Truly, the cast of players in any tissue goes beyond the parenchyma to include the cells of the vasculature that serve as the bouncers of the tissue – denying and directing entrance of



From Young JL, Libby P. Atherosclerosis. Chapter in: Lilly LS, editor. *Pathophysiology of Heart Disease*, 4th edition. Philadelphia: Lippincott Williams & Wilkins 2006: 118-140.

Figure 1.12: Endothelial and Smooth Muscle Cell Activation by Inflammation.

(A) Normal endothelial and smooth muscle cells maintain the strength and elasticity of the normal arterial wall, while limiting immune cell infiltration in the uninjured state. (B) Inflammatory "activation" of these vascular cells corrupts their normal function and favor pro-atherogenic mechanisms (e.g. immune cell infiltration) that drive plaque development. IL-1, interleukin-1; TNF-a, tumor necrosis factor-a; oxLDL, oxidized LDL; ROS, reactive oxygen species.

circulating cell populations. A more detailed description of these processes is provided in the context of atherosclerosis in the **Supplement**.

Glucocentric and lipocentric perspectives are not mutually exclusive of the inflammatory perspective. First, we will discuss how the cornerstones of diabetes, namely hyperglycemia, hyperlipidemia, insulin, and leptin, modulate inflammation. Second, we will introduce the LPS signaling pathway, which has been employed previously as an experimental method to drive inflammation-induced insulin resistance, but also represents an attractive etiologic agent that is both ubiquitous and potent. In conclusion, we will address whether inflammation is the driver or the bystander as well as present the specific aims for our scientific inquiry.

MODULATORS OF INFLAMMATION

Inflammation is a normal physiologic response to injurious stimuli with the intention to protect from further damage as well as initiate a healing response. Appropriately, the number of noxious stimuli that may activate inflammation are diverse and numerous. But when does an initially salutary response lead to pathophysiological consequences? We will address a subset of these pro- and anti- inflammatory signals that are relevant to diabetes from the glucocentric and lipocentric perspectives, demonstrating their overlap with inflammatory mechanisms.

Hyperglycemia

Excess glucose is the primary inciting factor in the glucocentric perspective. Beyond direct contribution of activated PKC and AGE formation in activating the pro-

inflammatory transcription factor NFκB, all four pathways outlined by Brownlee have been linked through a common denominator – reactive oxygen species (ROS). Increased intracellular glucose and processing through the electron transport chain leads to an excess of free electrons that must be donated to molecular oxygen to form oxygen radicals. The elimination of these radicals via increased degradation by manganese superoxide dismutase, inhibition of proximal steps of the electron transport chain, or uncoupling of the mitochondrial gradient prevents hyperglycemia-induced activation of major inflammatory pathways.[143] Indeed, *in vitro* culturing of 3T3-L1 in high glucose media leads to excess ROS production associated with IL-6 production.[144] However, we must take into account that this process of hyperglycemia-induced ROS generation with subsequent inflammatory activation may not be a cell-specific response, expanding potential sources of inflammation to many if not the majority of cells.

Hyperlipidemia

Elevated free fatty acids are common in the type 2 diabetes clinical picture with concordant elevation of triglycerides – a potential culprit linking the western diet that is high in saturated fats with the obesity and diabetes epidemic. As a metabolic substrate, excess fatty acid oxidation in peripheral tissues can also generate oxidative stress similar to hyperglycemia.[145] Additionally, fatty acid metabolites are potent activators of PKCθ, mediating acute fatty-acid induced insulin resistance with concordant serine phosphorylation of IRS-1 in skeletal muscle.[146] Moreover, increased accumulation of fatty acid metabolites with transgenic overexpression of lipoprotein lipase in skeletal

muscle and liver lead to tissue-specific insulin resistance.[147] However, it remains unclear whether PKCθ acts directly on the insulin signaling pathway or indirectly via its prominent interactions with the NFκB pathway. Saturated fatty acids, in particular palmitic acid, are considered pro-inflammatory with NFκB induction in vascular cells and macrophages.[49] The pro-inflammatory nature of this fatty acid may be reflected in the association of palmitic acid, but not other free fatty acids, with serum IL-6 levels in human subjects.[49]

Insulin and Leptin

Despite insulin's prominent role in glucose metabolism, it has been increasingly recognized that hyperinsulinemia associated with pre-diabetics and diabetics prior to β cell islet loss may continue to stimulate inflammation.[148,149] However, other studies suggest that early insulin treatment in diet- and streptozocin-induced diabetes in rats attenuated NFκB activity with coordinate reductions in TNFα and IL-1β mRNA expression in the liver and TNFα in muscle.[150] Whether insulin is pro- or anti-inflammatory is unclear, and likely mired in its role in metabolism, but the bulk of studies suggest that insulin does have inflammation-modulating effects.

Acutely, leptin can be increased by pro-inflammatory stimuli, such as LPS, IL-1, and TNFα.[151-153] Conversely, leptin can potentiate the acute phase response, which is attenuated in leptin-deficient (*ob/ob*) mice stimulated with LPS, resulting in diminished TNFα and IL-6 expression.[154-156] Paradoxically, leptin-deficient mice also demonstrate increased sensitivity to LPS-induced toxicity, including rapid hepatosteatosis associated

with altered cytokine expression.[156] Of interest, female rodents are more susceptible to LPS-induced toxicity and engenders the discussion of gender-specific effects that will be important in therapeutic applications.[156] Thus, similar to insulin, leptin is a double-agent likely subserving both pro- and anti-inflammatory pathways.

LIPOPOLYSACCHARIDE SIGNALING PATHWAY

Lipopolysaccharide (LPS) is a common component of gram negative bacteria, consisting of the Lipid A portion embedded in the outer wall, the core oligosaccharide, and the polysaccharide O-antigen. LPS is an amphiphilic molecule that forms aggregates and micelles in solution; thus, requiring carrier proteins such as albumin to facilitate cellular response.[157,158] LPS association with LPS binding protein (LBP) with subsequent delivery to membrane bound CD14 further enhances the cellular response, which is typified in macrophages as an inflammatory response with release of pro-inflammatory cytokines, such as TNFα, IL-1, IL-6, and IL-8. [159-165] As CD14 lacks a transmembrane signaling domain, it relies on interaction with MD2 and TLR4 to activate intracellular pathways. The cytosolic portion of TLR4 has Toll-interleukin-1 receptor (TIR) domains that can recruit different sets of adaptors that can be roughly broken down into: 1) myeloid differentiation primary response gene 88 (MyD88)-dependent pathway which utilizes the TIR domain-containing adaptor protein (TIRAP) and MyD88, and 2) MyD88-independent pathway which utilizes TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) (**Figure 1.13**). The MyD88-dependent pathway leads to nuclear translocation of NFκB and induction of Ap-1

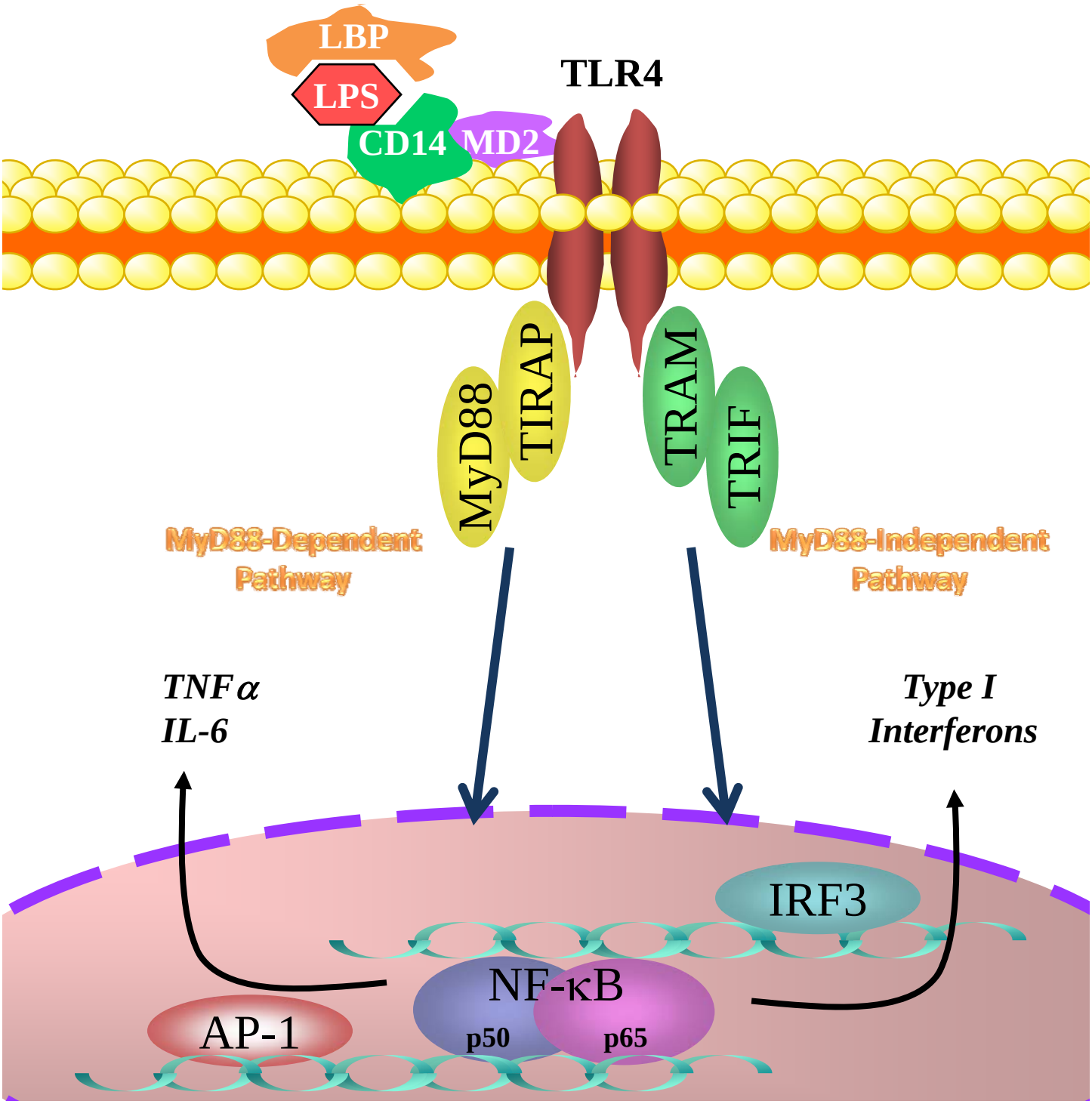


Figure 1.13: The LPS Signaling Pathway

The LPS signaling pathway can be categorized into MyD88-dependent (left side) and MyD88-independent (right side) pathways.

transcription factors, leading to pro-inflammatory secretion of TNFα and IL-6. Whereas, the MyD88-independent pathway leads to dimerization and nuclear translocation of IRF3, leading to expression of IFNα, IFNβ, and IFNω (**Figure 1.13**). Both these responses are effective and appropriate for combating bacteria; however, unimpeded LPS signaling and cytokine expression can lead to sepsis. Therefore, mechanisms of negative regulation are necessary to prevent collateral damage to the host. For the sake of our discussion we will only mention that SOCS-1 is involved in TIRAP ubiquitination and degradation, while loss of SOCS-1 leads to enhanced LPS-stimulated cytokine production in macrophages.[166]

INFLAMMATION IN INSULIN RESISTANCE – CHICKEN OR THE EGG

Clinical and experimental studies confirm the prominent role that inflammation can play with insulin resistance, obesity, and type 2 diabetes; however, whether inflammation is a causative factor, rather than merely an outcome, remains unclear. Additionally, it must be stressed that a number of *in vivo* data is derived from mice and rats that have been specifically selected for their susceptibility to developing glucose intolerance, insulin resistance, or specific microvascular or macrovascular complications. Indeed, even diet induced obesity models rely on the genetic background of the C57Bl/6J that predisposes these mice to glucose intolerance. Of interest, C57Bl/6 mice were initially chosen for their pattern of response to infection which led to primarily secretion of Th1 cytokines – a subset of cytokines implicated for their pro-inflammatory contribution to chronic inflammatory diseases, such as asthma, arthritis, and

atherosclerosis. Nonetheless, in concert with studies in humans, it remains probable that inflammation contributes to multiple etiologic theories of diabetes pathogenesis and represents a fertile area of discovery for future molecular insights and therapeutic strategies.

To add to the current body of knowledge, we will investigate the role of the LPS signaling pathway in murine models of glucose intolerance and insulin resistance. First, we will investigate whether the LPS receptor CD14 can account for the inflammatory potential of visceral adipose. Second, we will describe the metabolic phenotype of mice deficient in components of the LPS signaling cascade, including CD14, TLR4, and MyD88, to ascertain their role in whole body glucose homeostasis and as targets for systemic treatment.

Chapter II

Role of CD14 and the Lipopolysaccharide Signaling Pathway in Depot-Specific Inflammation

Chapter Contents

SUMMARY

Type 2 diabetes mellitus, known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, currently comprises 90-95% of all diagnosed diabetes, with an alarming increase in incidence among youth.[11] In the United States, current estimates approximate that one-third of children born in 2000 will develop diabetes.[11] Obesity is believed to contribute to type 2 diabetes pathogenesis in part due to enhanced secretion of adipose-derived inflammatory cytokines, such as IL-6 and TNFα.[167-170] Moreover, clinical and research oriented studies suggest that organ-associated adipose, so called visceral adipose, is a stronger predictor of diabetes incidence and concomitant macrovascular complications.[12,171] With studies by *Wiedermann et al.* suggesting a role of bacterial-derived lipopolysaccharide (LPS) in fueling systemic inflammation, we investigated whether the classical LPS receptor CD14 may be involved in determining adipose inflammatory potential.[172] Indeed, CD14 expression is enhanced on visceral adipocytes of *ob/ob* mice, which can be downregulated by *in vivo* rosiglitazone treatment. In isolated adipocytes, diminished CD14 expression in subcutaneous and rosiglitazone-treated visceral adipocytes leads to a parallel reduction in *ex vivo* IL-6 secretion. Mice deficient in components of the LPS signaling cascade, namely CD14, TLR4, and MyD88, yield adipocytes with diminished capacity to secrete IL-6. Therefore, these studies are the first to report the importance of LPS signaling in adipocyte IL-6 secretion and potentially represent a molecular explanation for differences in adipose inflammation and pathogenicity.

INTRODUCTION

The Venus of Willendorf (c. 25,000-20,000 B.C.) is one of the earliest pieces of art, depicting a plump, curvaceous form that was to represent fertility and health; it was not till later Aegean art that figures of female health encompassed slim and slight forms (**Figure 2.1**).[173] Though obesity through the millennia has maintained varying positive and negative connotations – both social and medical, it was not until 1959 that the medical insurance industry attempted to define an ideal, or in other words healthy, weight for individuals.[174] In recent years, the World Health Organization (WHO) warns that obesity has penetrated high-, low-, and middle-income countries alike, driving a "double burden" in developing countries with continuing issues of infectious disease and undernutrition now coupled with concurrent overnutrition that fuels chronic disease, such as diabetes and heart disease.[32]

Building upon compelling epidemiological evidence, scientific studies suggest that obesity contributes to pathophysiological changes that fuel Type 2 diabetes mellitus development. Empirical observations recognized that adipose distribution also contributes to risk.[175,176] Those who carried excess fat in their abdomen (so called "apple-shaped") were more prone to hypertension, cardiovascular disease, and diabetes than those who deposited excess fat in their hips and thighs (so called "pear-shaped"). The "apple-shape" occurs in part due to adipose deposits around the organs of the abdomen, which subsequently augments abdominal protrusion. Such organ-associated fat is called *visceral adipose* in contrast to *subcutaneous fat*, which is located underneath the skin. Magnetic resonance imaging (MRI) and computed tomography (CT) scanning

Venus of Willendorf
c. 25,000-20,000 B.C.
Stone Age

Idol from Amorgos
c. 2500-1100 B.C.
Aegean

Figure 2.1: Ancient Perceptions of Health and Fertility

The Venus of Willendorf isone of the earliest examples of art, reflecting a culture that saw obesity as a sign of health and fertility. It was not until roughly 20,000 years later that it is believed that lithe and slender figures were seen as comparable, if not preferable, body types.

has been used to delineate visceral from subcutaneous fat depots, reaffirming quantitatively that visceral adiposity is a strong risk factor for cardiovascular disease and diabetes.[177-179] Moreover, recent studies by Goodpaster et al. demonstrate that even in normal weight individuals, visceral adiposity was highly associated with the metabolic syndrome, a clustering of risk factors that typically precede the development of both cardiovascular disease and overt diabetes.[180]

In contrast to the previous perception of adipose as an inert lipid storehouse, adipose is in fact an important organ orchestrating metabolic, endocrine, and inflammatory systems.[181] For example, transgenic overexpression of adiponectin, a protein restricted to adipocytes, ameliorates both diabetes in the *ob/ob* mouse model and atherosclerosis in the ApoE-deficient mouse model.[182,183] Adiponectin and leptin (and potentially visfatin, resistin, and adipsin) are members of an emerging class of adipokines - secreted proteins primarily produced by adipocytes.[184] Nonetheless, adipose tissue remains an important source of classical inflammatory cytokines, including TNFα, IL-6, IL-8, IL-10, MCP-1, and IL-1 receptor antagonist.[181] It fact, it is believed that higher expression of these inflammatory cytokines account for the higher risk for disease in visceral fat.[20,175,176] Thus, in addition to its prominent role in metabolism, adipose tissues serve as an important source of secreted factors that may shape progression or regression from diseases associated with chronic subclinical inflammation, such as diabetes and atherosclerosis.

Bacterial lipopolysaccharides (LPS; also known as endotoxin) are prominent inflammatory agonists that can induce inflammation in trace amounts. LPS binds to LPS

binding protein (LBP) in the serum and is transferred to CD14 on the cell membrane where association with MD2 and TLR4 lead to intracellular signaling transduction, initiating inflammation and pathogenic response; hence, representing an important arm of innate immunity. Recently, TLR4 has been found to be increased on adipocytes with 3T3-L1 differentiation and in the *db/db* genetic model of obesity, conferring sensitivity to LPS and allowing production of inflammatory cytokines, such as TNFα and IL-6.[185] Given the potency of LPS as an inflammatory stimulus as well as early studies demonstrating the functional presence of TLR4 on adipocytes, we sought to investigate whether the classical LPS-receptor CD14, which serves in a complex with TLR4, could play a role in adipocyte inflammation as well as potentially account for the difference between visceral and subcutaneous adipose inflammation.

EXPERIMENTAL PROCEDURES

Experimental animal care

Cd14$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice have been backcrossed at least 12 generations to the C57BL/6J background. Male C57Bl/6J mice were obtained from the Jackson Laboratory at 6-11 weeks of age. Male *ob/ob* mice were obtained from the Jackson Laboratory at 4 or 10 weeks of age. All mice were housed (n=4 per cage) in ventilated polysulfone cages (Allentown Inc., Allentown, NJ) in a pathogen-free barrier facility maintained on a 12-hour light / 12-hour dark cycle. Mice had free access to autoclaved water and food. Obesity was induced by a high fat diet (HFD) consisting of ~60% of calories from fat (TD93075; Harlan Teklad, Madison, WI) starting at 11 weeks of age. Prior to 11 weeks, mice were fed the standard pellet diet (LabDiet PicoLab 5053, Purina Mills, St. Louis, MO). Control C57Bl/6J and *ob/ob* animals were maintained for their lifespan on standard diet in pellet or powder form. Rosiglitazone treatment was initiated 2 weeks prior to sacrifice by grinding Avandia™ rosiglitazone maleate tablets (SmithKline Beecham Pharmaceuticals) into powered chow or softened HFD. An effective dose of 0.375 mg/day was calculated based on average food consumption of 5g/day/mouse. During the treatment period, control animals were fed diets in the same form. Animal weight and food consumption was measured weekly for the duration of the experiment. Animal were fasted for 16-18 hours prior to sacrifice by cervical dislocation followed by bilateral pneumothorax. All experiments employed male mice in accordance with the University of Massachusetts Medical School Institutional Animal Care and Use Committee (UMMS-IACUC).

Microarray gene expression and analysis

Total RNA was extracted from isolated adipocytes (epididymal visceral, flank inguinal subcutaneous, 3T3-L1) of individual mice with a commercially available acid-phenol reagent (TRIzol; Invitrogen). RNA concentration was assessed by absorbance spectroscopy and RNA integrity confirmed by nondenaturing agarose gel electrophoresis. Total RNA was used for the preparation of cRNA from primary fat cells for use with MG-U74v2 or MOE 430 GeneChips (Affymetrix). RNA from primary fat cells was prepared from three groups of 2–4 mice for each condition. Affymetrix protocols were followed for the preparation of cRNA from the mRNA or total RNA, which was hybridized according to Affymetrix instructions. The GeneChips were washed with a GeneChip Fluidics Station 400 and were scanned with an HP GeneArrayScanner (Affymetrix). Data analysis was performed from *.cel files in the MicroArray Computational Environment 2.0 (MACE 2.0; UMass Medical School, Worcester, MA). This procedure was performed by Leanne Wilson-Fritch, Sarah Nicoloro, Alison Burkart, and My Chouinard.

Primary Adipocyte Isolation

Perigonadal (epididymal in male mice) and subcutaneous (flank inguinal) fat pads were harvested in mice and placed immediately in Krebs-Ringer solution buffered with HEPES (KRH), pH 7.4, supplemented with 2.5% BSA. Fat pads were cut into 0.3 cm^3 pieces and digested with freshly prepared collagenase type I (1mg/mL, 37°C; *Clostridium histolyticum* derived; Worthington Biochemical Corp) in previously prepared KRH, pH

7.4, 2.5% BSA – 2mL of collagenase solution was added per gram of tissue with a minimum of 5mL per container. Samples were sealed with parafilm™ and digested 30-45 minutes at 37°C in an orbital bath shaking at 100 rpm with ~2 cm excursion. Digested samples were run through chiffon material to separate isolated cells from extracellular matrix, vasculature, and undigested tissue. Adipocytes were separated by buoyancy and were washed 3 times in sterile PBS or KRH. All materials were purchased sterile and pyrogen-free and all tools were autoclaved in sterile sleeves followed by UV irradiation. Clinical-grade water was used for reagent preparation.

Ex Vivo Secretion Assay

Isolated adipocytes were placed as a monolayer in 100μL of low-glucose DMEM (Gibco) supplemented with 2.5% LPS-free, free fatty acid poor BSA (lot pre-selected for lowest endotoxin concentration and no free fatty acids; Calbiochem). Polymyxin B (5μg/mL; Sigma) was added to solution to inhibit endotoxin. Adipocytes were incubated for 1-6 hours at 37°C in an orbital bath shaking at 100 rpm. Supernatant was removed and analyzed immediately or frozen at -20°C.

ELISA

Release of IL-6 from primary adipocytes was measured by ELISA, following the recommendations of the manufacturer (Pierce Endogen). In brief, supernatants were obtained and diluted 1:10 prior to application to coated 96-well plates (2 hour, RT, orbital shaker – 600rpm, ~0.25 cm excursion). Plates were washed with supplied detergent-

based solution in automatic plate washer (3x, 300μL dispense, full aspiration) after sample addition, biotin-labeled antibody (1 hour, RT, orbital shaker – 600rpm, ~0.25 cm excursion), and streptavidin conjugated horse radish peroxidase (30 minutes, RT). Tetramethylbenzidine (TMB) solution (10-25 minutes, RT, light-protected) was used to visualize this colorimetric reaction and stopped with acidic solution prior to absorbance measurement at 450nm with subtraction of reference absorbance at 550nm (Safire 2, Tecan).

Western blot analysis

Cell extracts, equilibrated by total protein (10-25 μg total protein / lane), were separated by standard SDA-PAGE under reducing conditions and blotted to polyvinylidene difluoride membranes (PVDF; NEN Life Sciences) using a semidry blotting apparatus (0.8mA/cm^2, 30-60 minutes; Thermo Scientific Owl). Blots were blocked and first and second antibodies were diluted in 5% defatted dry milk/TBS/0.1% Tween 20. After 1 hour of incubation with respective primary antibody (CD14, 1:1000; Novocastra), blots were washed three times (TBS/0.1% Tween) and the secondary, peroxidase-conjugated goat anti-rabbit antibody (Promega) was added for an addition 1 hour. Finally, blots were washed three times (TBS/0.1% Tween) and immunoreactive proteins were visualized using chemiluminescence (NEN Life Sciences). Protocol previously described.[186]

Statistical Analysis

Statistical analysis employed general linear mixed models, including analysis of variance (ANOVA) or Student's *t* test followed by Bonferroni post hoc tests or statistically conservative Tukey/Kramer posthoc tests. Statistics were performed using Prism Software (Graphpad, San Diego, CA), Statview 5.0.1 (SAS Institute, Cary, NC), or Sigma Plot 5.0 (SPSS, Inc., Chicago, IL). Results are shown as mean±SEM unless otherwise stated. A p-value of $p<0.05$ was regarded as a significant difference.

RESULTS & DISCUSSION

Visceral adipocytes express more cytokines and chemokines than subcutaneous adipocytes in ob/ob mice

Previous studies of adipose-derived inflammation employed varying sample techniques, including analysis of full adipose tissue as well as isolated adipocytes. Considering the recent discovery of macrophage infiltration into adipose tissue, which serves as a prominent source of inflammatory genes, we have analyzed only isolated adipocytes that were liberated from surrounding stroma, vasculature, and cell infiltrates. Given limited subcutaneous deposits in diet-induced obesity, we first employed the genetically obese ob/ob mouse, which has an inbred defect in the leptin gene, to interrogate whether differential cytokine and chemokine expression exist between visceral- and subcutaneous-derived isolated adipocytes. Moreover, to investigate purported anti-inflammatory properties of the commonly employed type 2 diabetes medication rosiglitazone (thiazolidinedione class), we randomly stratified half our cohort to this treatment. Using a normal chow diet (ND; 5% of calories derived from fat) in the genetic model of obesity, we compared the transcriptional profile in 4-week old ob/ob mice prior to development of overt diabetes; 26-week old ob/ob mice who were diabetic; and 26-week old ob/ob whom were fed rosiglitazone (6mg/ kg body weight/ day) for 2 weeks prior to sacrifice (**Figure 2.2**).

Utilizing analyses from the MicroArray Computational Environment (MACE) developed by our Diabetes and Endocrinology Research Center (DERC), we employed gene filters based on the MOE430-2 affymetric chip descriptions of biological function.

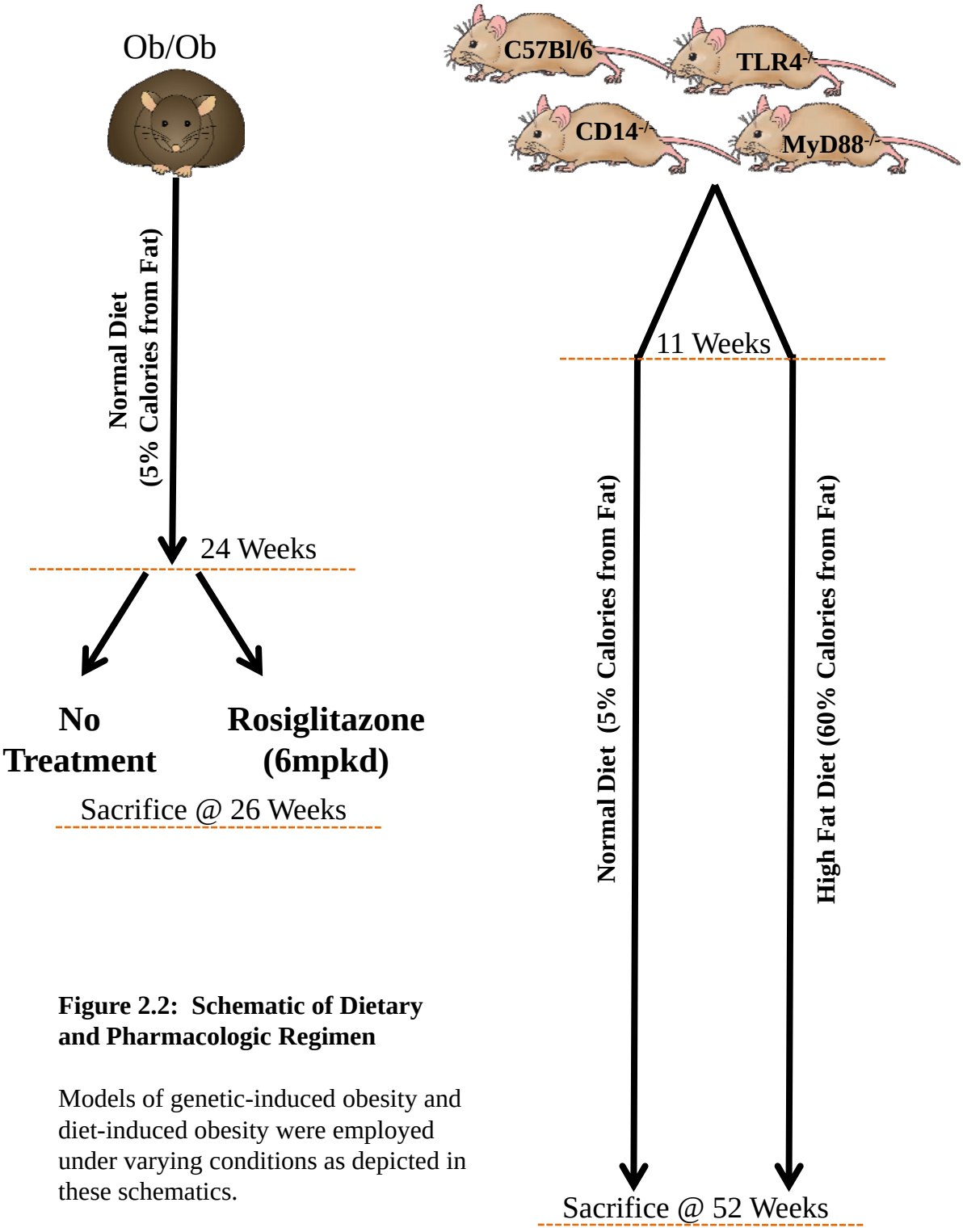


Figure 2.2: Schematic of Dietary and Pharmacologic Regimen

Models of genetic-induced obesity and diet-induced obesity were employed under varying conditions as depicted in these schematics.

A filter comprised of 902 genes with known cytokine function demonstrated that 33% of these genes were increased in visceral adipocytes in comparison to subcutaneous adipocytes, while 5% of genes were decreased, and 33% were unaltered. (**Figure 2.2**) Similarly, a filter comprised of 326 genes with known chemokine function demonstrated that 32% of these genes were increased in visceral adipocytes in comparison to subcutaneous adipocytes, while 5% of genes were decreased, and 32% were unaltered. (**Figure 2.3**). In light of the fact that macrophage infiltration and vascular components are prominent sources of inflammatory mediators, this transcriptional data from isolated adipocytes supports the theory that adipocytes themselves serve a role in determining depot-specific cytokine and chemokine expression.

Toll-Like Receptor Pathway is involved in 3 models of adiposity

Many cues can activate cascades that converge on similar inflammatory transcription factors, such as nuclear factor kappa B (NFκB), leading to similar chemokine and cytokine secretion. In order to dissect out if distinct cues and pathways may be involved, we hypothesized that differences in inflammatory capacity between subcutaneous and visceral sources may parallel inflammatory changes in visceral adipocytes that occur before and after insulin resistance, which may be reflected with adipogenesis or in adipocytes in glucose impaired animals. Employing a model of adipogenesis, namely 3T3-L1 adipocytes that form from fibroblast-like cells over 7 days, and two models of adiposity, high fat diet induced and genetic-induced, we interrogated transcriptional arrays for changes in pathways of genes according to the Kyoto

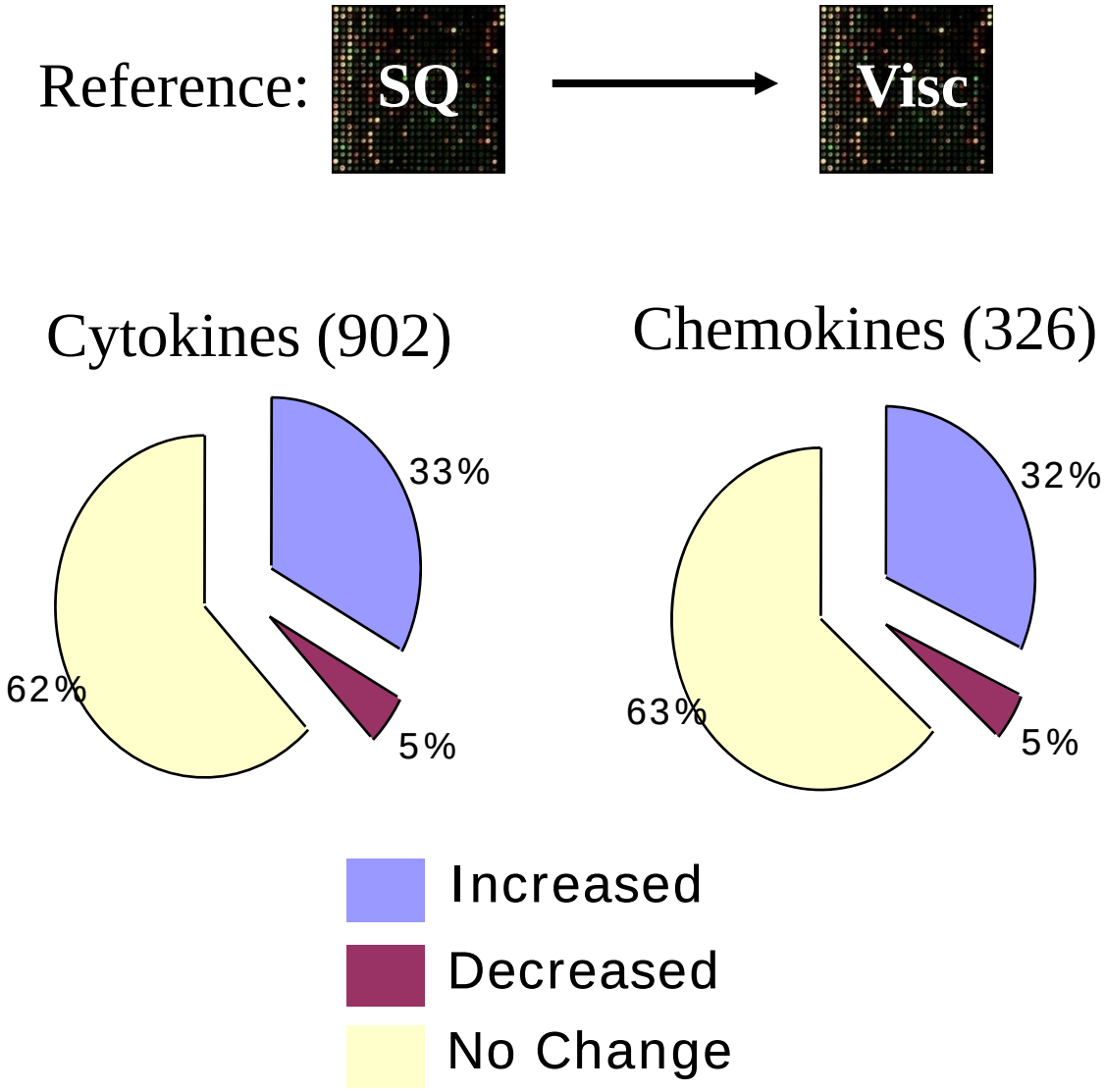


Figure 2.3: Cytokine and Chemokine Expression Pattern in Visceral Adipocytes (Subcutaneous as reference)

Transcriptional profiles from ob/ob derived subcutaneous adipocytes and visceral adipocytes were compared. The terms “cytokine” and “chemokine” were used to filter results from the Affymetrix gene chip.

Encylopedia of Genes and Genomes (KEGG). The *Toll-Like Pathway* was involved with a high degree of significance in all three pathways: 3T3-L1 differentiation, diet induced obesity, and genetic-induced obesity (**Figure 2.4**). Within the *Toll-Like Pathway* and proximal to extracellular signaling events, CD14, the classical receptor for LPS, was modulated in these models of adipogenesis and adiposity. Furthermore, previous transcriptional data in *ob/ob* mice revealed that CD14 was differentially expressed between subcutaneous and visceral adipocytes.

Adipocyte CD14 transcript expression is enhanced in visceral depots in both genetic and diet-induced obesity with subsequent downregulation by rosiglitazone treatment

In the *ob/ob* mouse, adipocyte expression of CD14 is upregulated from the 4-week old non-diabetic mouse to the 26-week old overtly diabetic mouse with a 1.8-fold increase in subcutaneous adipocytes ($p<0.05$) and a 5-fold increase in visceral adipocytes ($p<0.05$) (**Figure 2.5a**). Mice treated with rosiglitazone for 2 weeks prior to sacrifice demonstrated reduced CD14 expression in visceral adipocytes by 50% ($p<0.05$) and subcutaneous adipocytes by 50% ($p<0.05$) (**Figure 2.5a**). In the diet-induced model, we see an 80% increase in visceral adipocyte CD14 expression with HFD feeding, which can be ameliorated with rosiglitazone treatment ($p<0.05$, $p<0.05$, respectively) (**Figure 2.5b**).

Adipocyte CD14 protein expression is enhanced in visceral depots in ob/ob mice with subsequent downregulation by previous rosiglitazone treatment

Following transcriptional clues, western blotting analysis confirmed greater CD14 expression in *ob/ob*–derived visceral adipocytes over subcutaneous adipocytes (**Figure**

Kyoto Encyclopedia of Genes and Genomes (KEGG)

Toll-like Receptor Signaling Pathway

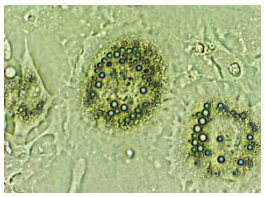
		Significance	Annotated Genes
3T3-L1 	**Day 0 vs Day 6**	<2.66e-13	25/201
C57Bl/6 	**ND vs HFD**	<6.85e-13	24/201
Ob/Ob 	**4 wk vs 26wk**	<1.03e-6	16/201

Figure 2.4: Kyoto Encylopedia of Genes and Genome (KEGG) Pathway Analysis

RNA microarrays were generated to compare different models of adiposity, including 3T3-L1 adipocytes prior to (day 0) and following differentiation (day 6), high fat diet-induced obesity in the C57BL/6J mouse, and genetic obesity in the ob/ob mouse. Algorithms from the Kyoto Encyclopedia of Genes and Genomes were applied to individual genes with a significant ($p<0.05$) change in absolute expression greater than 1.2-fold. The significance of the *Toll-Like Receptor Pathway* denotes the likelihood of the involvement of this pathway in their respective adiposity models, whereas annotated genes represent the genes in the *Toll-Like Receptor Pathway* involved in our comparison versus total genes believed to take part in this pathway.

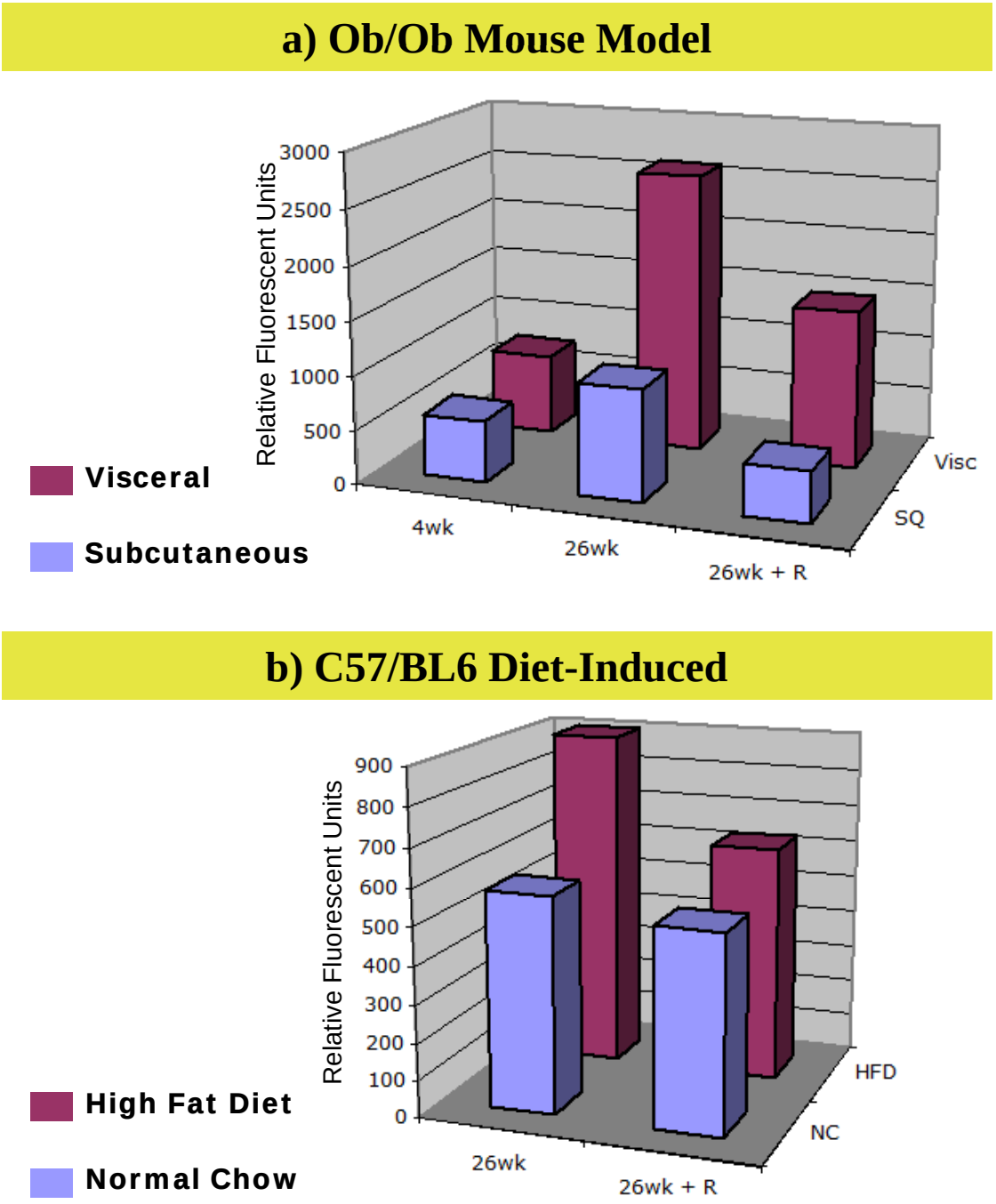


Figure 2.5: CD14 Transcriptional Profile in Genetic and Diet-Induced Obesity

(a) Visceral (Visc) and subcutaneous (SQ) were isolated from 4 week old *ob/ob* (4wk), 26 week old *ob/ob* (26wk), and 26 week old *ob/ob* mice treated with rosiglitazone (26wk+R). **(b)** Isolated visceral adipocytes were isolated from C57Bl/6J mice fed normal chow (NC) or high fat diet (HFD) without (26wk) or with rosiglitazone (26wk+R).

2.6). As a control, increased collagenase digestion time, which could potentially cleave CD14 ectodomains during tissue digestion, showed no alteration in adipocyte CD14 expression (**Figure 2.6**). Moreover, short-term (2-week) treatment of rosiglitazone in animals prior to sacrifice was sufficient to reduce CD14 expression on *ob/ob* visceral adipocytes as demonstrated by western blot analysis (**Figure 2.7**). Blots were re-probed for macrophage-specific proteins to control for potential macrophage contamination in our adipocyte preparations – no macrophage markers were visualized (Data not shown).

Enhanced ex vivo IL-6 secretion from visceral adipocytes is diminished by in vivo rosiglitazone administration

Following the hypothesis that CD14 may dictate adipocyte inflammatory potential, we investigated IL-6 secretion, a prominent adipocyte-derived inflammatory cytokine. Following isolation, adipocytes secreted IL-6 in a time dependent manner, with over 4-fold accumulation of IL-6 in media at 6 hours in comparison to at 1 hour, which is consistent with previous reports (**Figure 2.8**).[187] In contrast, adipocytes from flank subcutaneous depots secreted less IL-6 with accumulation at 6 hours similar to concentrations at 1 hour in visceral adipocytes (**Figure 2.8**). In randomized ob/ob mice fed rosiglitazone 2 weeks prior to adipocyte isolation, we see a diminished ability of visceral adipocytes to secrete IL-6 *ex vivo*. Therefore, rosiglitazone-mediated effects *in vivo* persisted in our *ex vivo* assay without direct addition of rosiglitazone (**Figure 2.8**). Therefore, our data suggest that depot-specific expression of CD14 as well as diminished CD14 expression in visceral adipocytes following rosiglitazone treatment correlate with functional IL-6 secretion *ex vivo*.

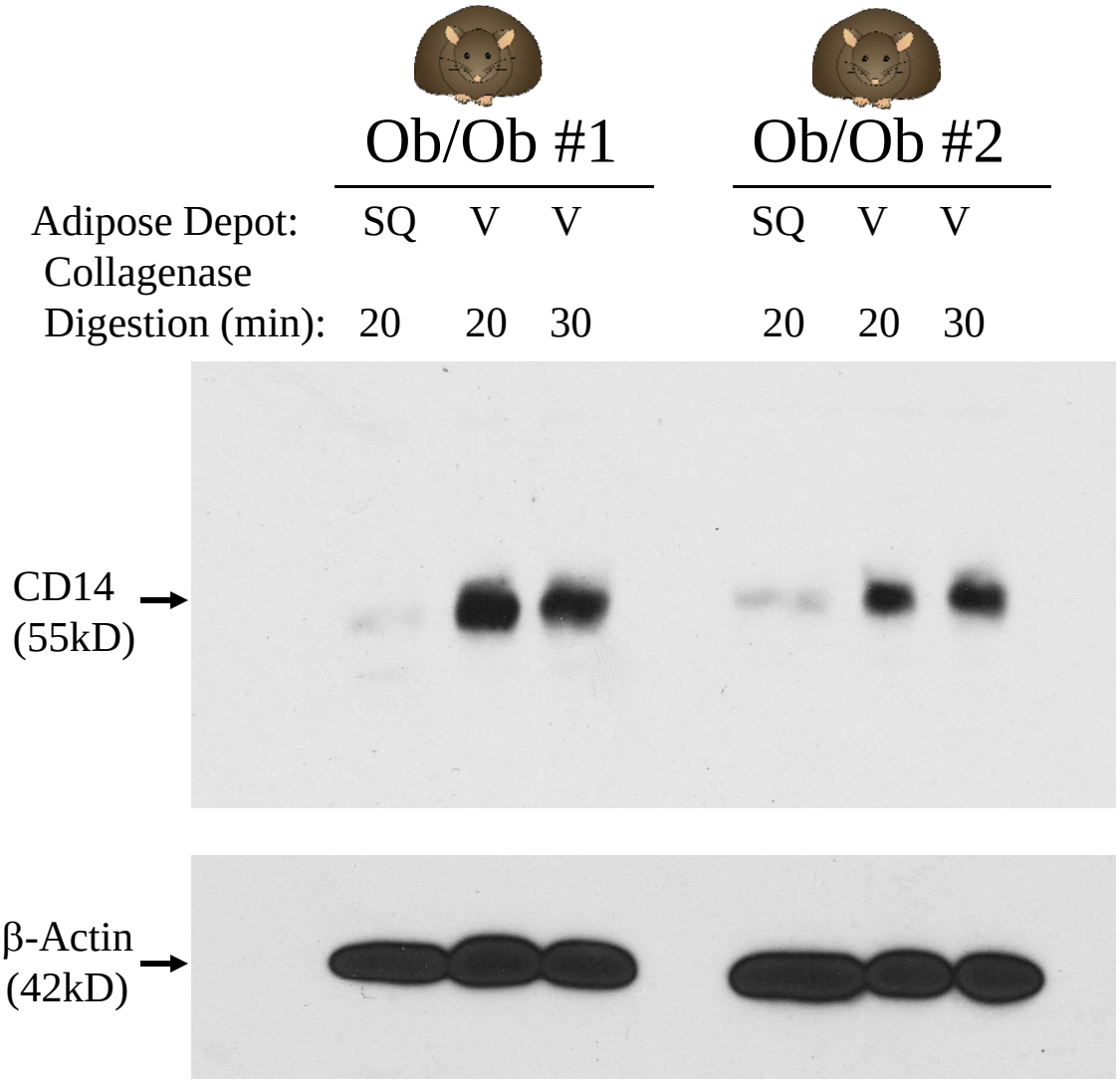


Figure 2.6: Enhanced CD14 protein expression in visceral adipocytes from *ob/ob* mice

Isolated visceral (V) and subcutaneous (SQ) adipocytes from *ob/ob* mice. Enhanced CD14 expression in visceral adipocytes was not effected by increased collagenase digestion time. β-actin controls provided. Representative blot from 3 independent experiments.

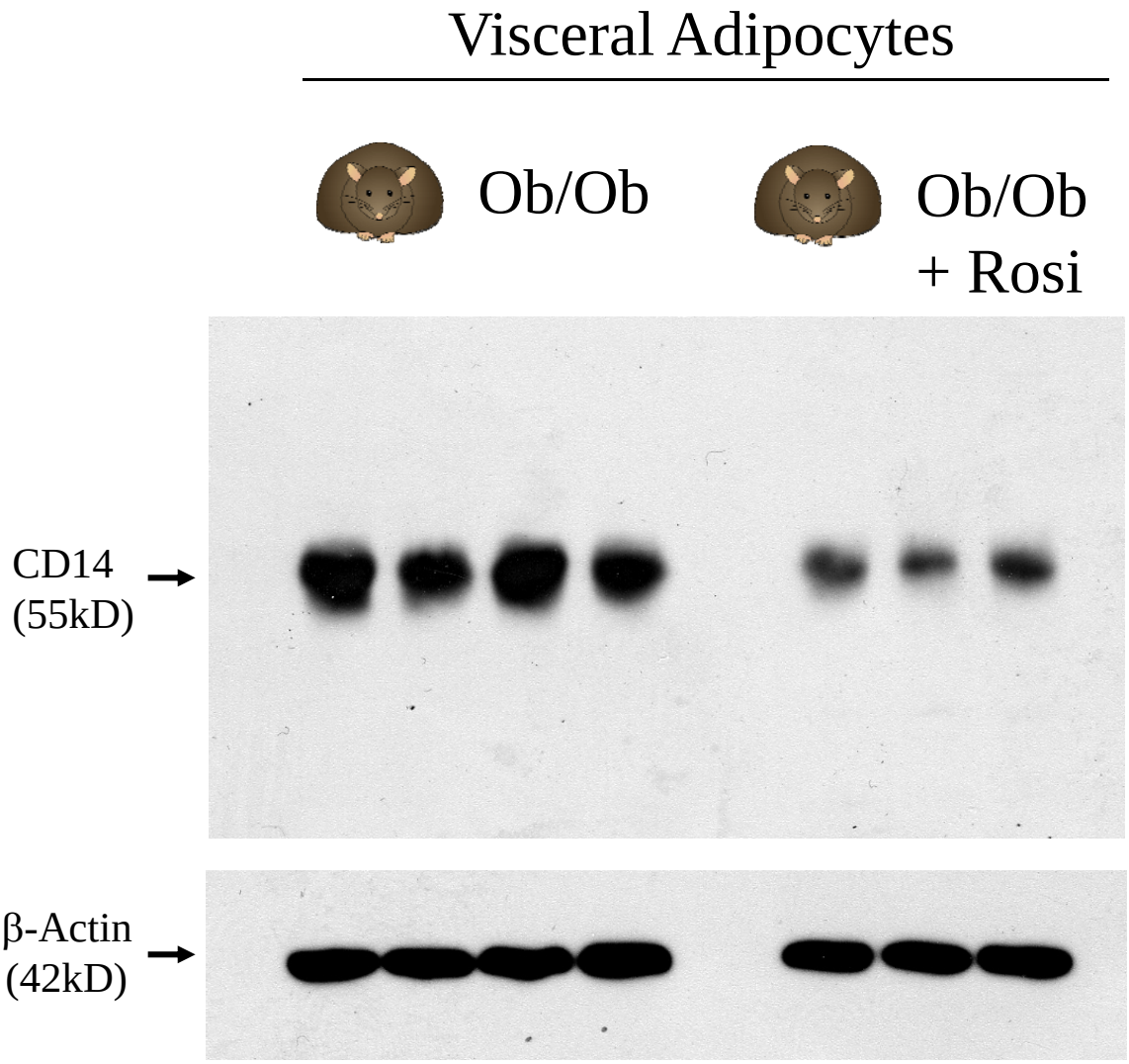


Figure 2.7: Diminished visceral adipocyte CD14 protein expression in rosiglitazone-treated *ob/ob* mice

Isolated visceral adipocytes from 26-week old *ob/ob* mice (ob/ob) and 26-week old *ob/ob* mice treated with rosiglitazone for 2 weeks (ob/ob + Rosi). β-actin controls provided. Representative blot from 3 independent experiments.

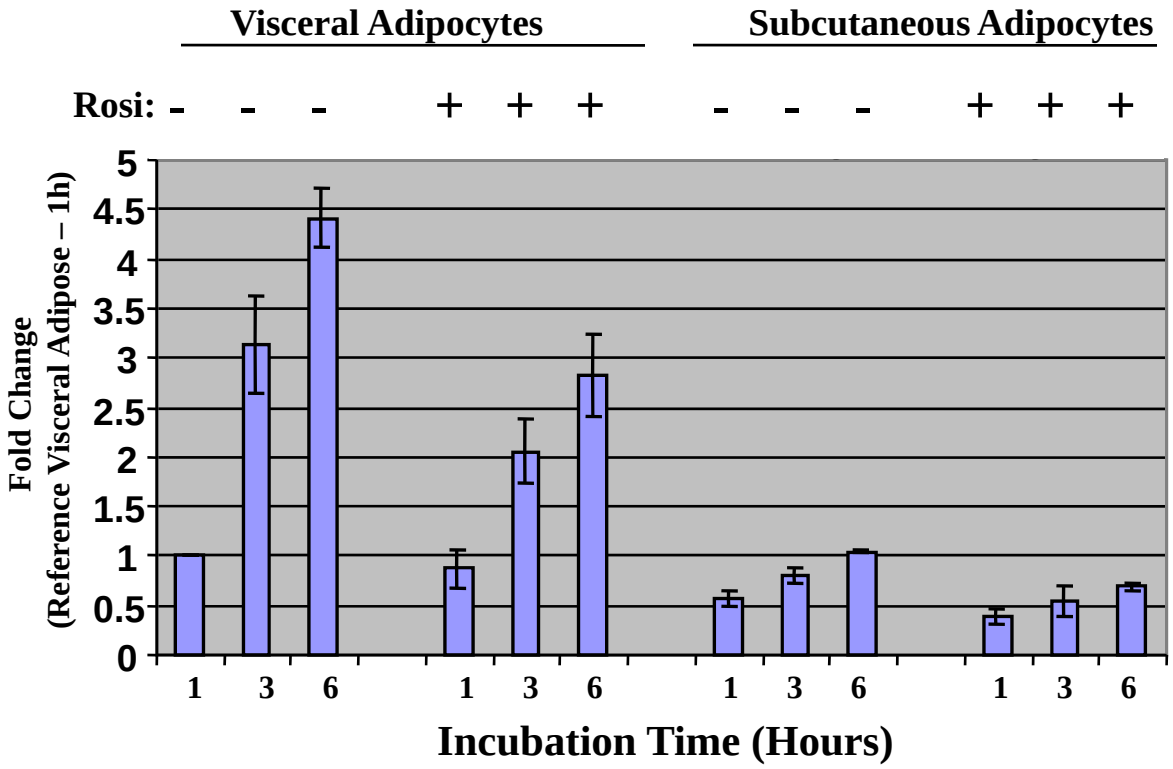


Figure 2.8: Enhanced *ex vivo* visceral IL-6 secretion diminished with rosiglitazone treatment

Isolated visceral and subcutaneous adipocytes from 26-week old *ob/ob* mice (-Rosi) and 26-week old *ob/ob* mice treated with rosiglitazone for 2 weeks (+Rosi) were incubated for 1, 3, or 6 hours in assay medium to assess IL-6 secretion. Data presented as mean±s.e.m. (n=6 independent experiments). *$p<0.05$, **$p<0.01$, ***$p<0.005$ via student t-test in comparison to WT control.

LPS Signaling Disruption Abrogates Adipocyte IL-6 secretion

To address previous data that differential CD14 expression corresponds with *ex vivo* IL-6 secretion, CD14-deficient (*cd14*$^{-/-}$), TLR4-deficient (*tlr4*$^{-/-}$), and MyD88-deficient (*myd88*$^{-/-}$) mice were stratified on to a normal diet (ND; 5% of calories derived from fat) and high fat diet (HFD; 60% of calories derived from fat) to determine if a functional LPS signaling complex played a role in: 1) adipocyte IL-6 secretion, 2) depot-dependent adipocyte IL-6 secretion, and 3) diet-induced adipocyte IL-6 secretion (**Figure 2.2**).

In lean ND fed mice, visceral adipocytes continue to secrete IL-6; however, loss of CD14, TLR4, or MyD88 resulted in reduction of IL-6 secretion (**Figure 2.9**). In HFD fed mice, we can appreciate a >4-fold increase in visceral adipocyte IL-6 secretion as would be expected in the obese, diabetic mouse versus the ND fed normoglycemic mouse (**Figure 2.9**). However, again, loss of CD14, TLR4, or MyD88 resulted in attenuation of visceral adipocyte-derived IL-6 by 74%, 84%, and 50% respectively (**Figure 2.9**). In contrast, subcutaneous adipocytes exhibit no statistical difference in IL-6 secretion in ND fed mice; however, this may be due to assay limitation as these cytokine concentrations were on the lower limit of detection (**Figure 2.10**). With HFD treatment, there is an increase in subcutaneous-derived IL-6 expression, potentially revealing the prominent role of LPS signaling in permitting IL-6 secretion as subcutaneous adipocytes from HFD fed CD14, TLR4, and MyD88 secreted 91%, 50%, and 87% less IL-6 than control mice. (**Figure 2.10**) Therefore, we see both a significant dietary effect on adipocyte IL-6

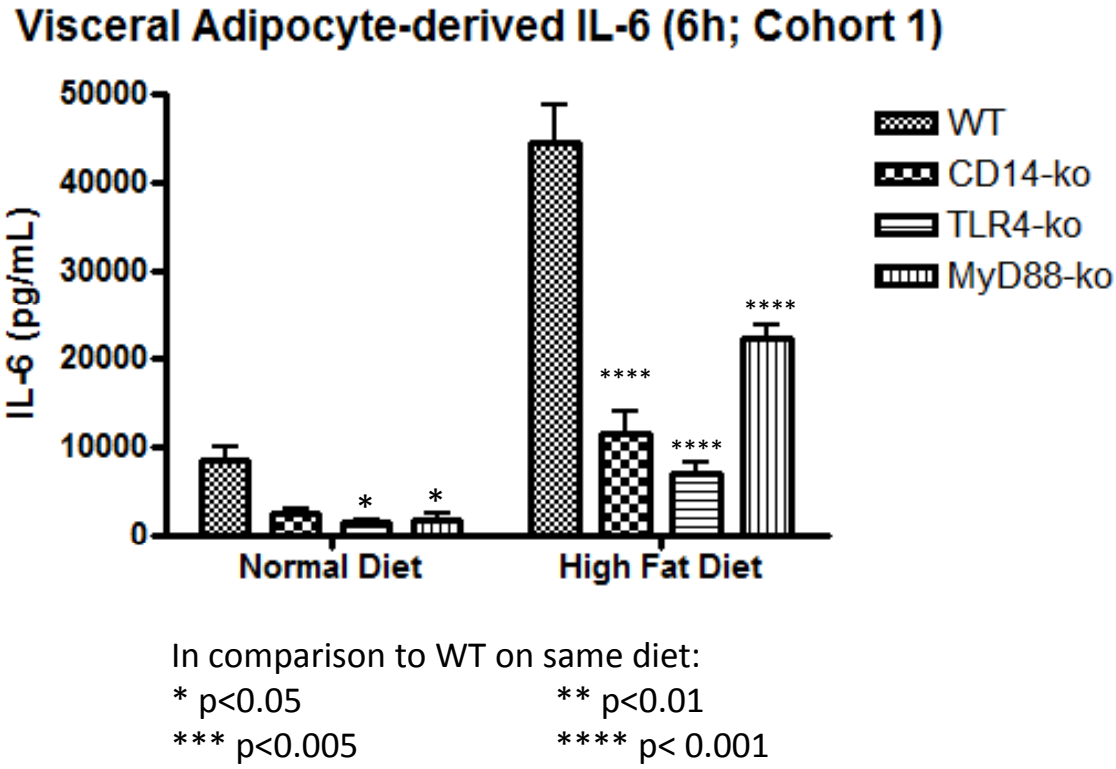


Figure 2.9: Loss of LPS signaling attenuates visceral adipocyte IL-6 expression

Visceral adipocytes were isolated from C57Bl/6J (WT), *cd14*$^{-/-}$ (CD14-ko), *tlr4*$^{-/-}$ (TLR4-ko), and *myd88*$^{-/-}$ (MyD88-ko) mice fed either normal diet (5% of calories from fat) or high fat diet (60% of calories from fat). Freshly isolated adipocytes were incubated for 6 hours in assay medium to assess IL-6 secretion. Data presented as mean±s.e.m. (n=6 independent experiments). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 via student t-test in comparison to WT control.

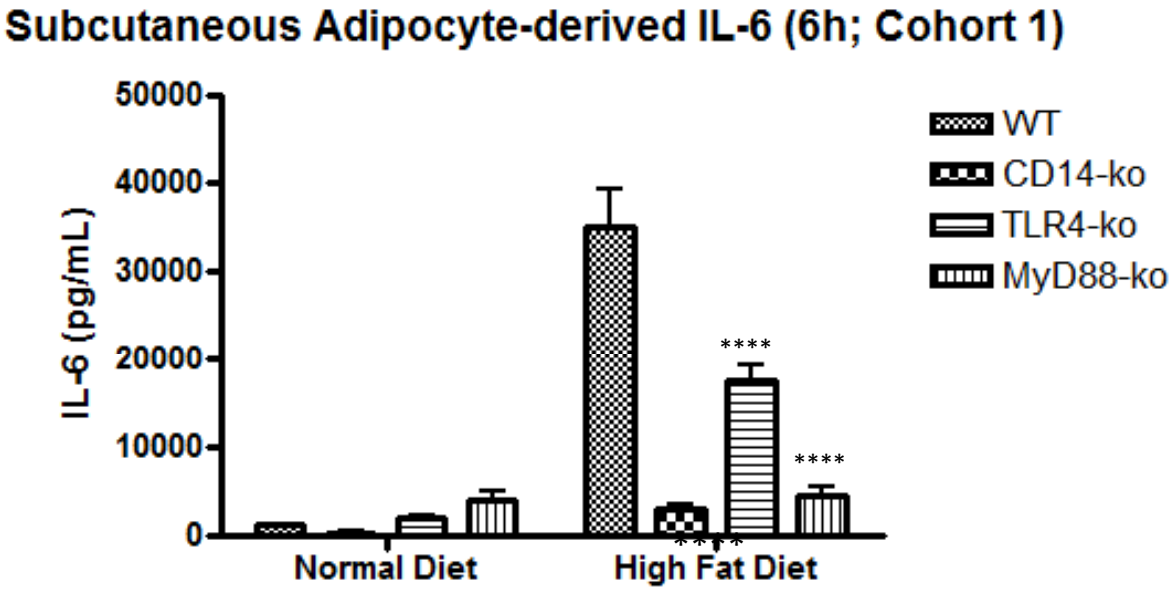


Figure 2.10: Loss of LPS signaling attenuates subcutaneous adipocyte IL-6 expression

Subcutaneous adipocytes were isolated from C57Bl/6J (WT), *cd14*$^{-/-}$ (CD14-ko), *tlr4*$^{-/-}$ (TLR4-ko), and *myd88*$^{-/-}$ (MyD88-ko) mice fed either normal diet (5% of calories from fat) or high fat diet (60% of calories from fat). Freshly isolated adipocytes were incubated for 6 hours in assay medium to assess IL-6 secretion. Data presented as mean±s.e.m. (n=6 independent experiments). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 via student t-test in comparison to WT control.

secretion (2-way ANOVA, $p<0.0001$ in visceral, $p<0.0001$ in subcutaneous) as well as a significant genotype effect on adipocyte IL-6 secretion (2-way ANOVA, $p<0.0001$ in visceral, $p<0.0001$ in subcutaneous).

Contrasting adipose depot sources taking into account all genotypes, there are significant depot and genotype effects in both ND (2-way ANOVA, $p<0.005$ depot, $p<0.0001$ genotype) and HFD fed mice (2-way ANOVA, $p<0.001$ depot, $p<0.0001$ genotype). However, looking at individual genotypes in depot-specific IL-6 secretion reveals a more complex story.

In *cd14*$^{-/-}$ mice, there is diminished IL-6 secretion in both visceral and subcutaneous adipocytes in comparison to control mice regardless of diet, supporting the hypothesis that CD14 plays a role in adipocyte IL-6 production. However, loss of CD14 fails to normalize visceral and subcutaneous contributions of IL-6, suggesting that although the loss of CD14 in visceral adipocytes can decrease IL-6 secretion to a comparable or lower level than subcutaneous adipocytes in control mice, there are other pathways dictating higher IL-6 secretion in the *cd14*$^{-/-}$ visceral depot over subcutaneous depot. Additionally, HFD treatment still leads to increased IL-6 secretion in *cd14*$^{-/-}$ mice, demonstrating that HFD maintains a pro-inflammatory effect independent of CD14.

In *tlr4*$^{-/-}$ mice, there is diminished IL-6 secretion in both visceral and subcutaneous adipocytes in comparison to control mice regardless of diet, further supporting that LPS signaling plays a role in adipocyte IL-6 production. However, in contrast to CD14, loss of TLR4 normalizes visceral and subcutaneous contributions of

IL-6 in ND fed animals, suggesting that either TLR4 plays an essential role in depot differences via either a more essential role in LPS signaling or in integration with other stimuli. TLR4 has recently been implicated as a receptor for saturated, but not unsaturated, fatty acid-induced adipocyte inflammation, including the expression of IL-6.[188] Therefore, it is possible that integration of both LPS and saturated fatty acid stimuli can more fully account for depot-specific differences in inflammation, explaining why TLR4 is able to normalize depot inflammatory potential, while *cd14*$^{-/-}$ mice with TLR4 expression maintain functional inflammatory contributions from saturated fatty acid stimuli. On the other hand, HFD fed *tlr4*$^{-/-}$ mice show a paradoxical effect where subcutaneous adipocytes produce more IL-6 than visceral adipocytes. Given that our HFD is predominantly composed of unsaturated fatty acids (~70% of fatty acid profile), this may suggest that visceral and subcutaneous adipocytes respond differently to unsaturated fatty acids in the *tlr4*$^{-/-}$ mice, which may be in part due to compensatory increases in TLR2 in *tlr4*$^{-/-}$ mice.[189-191]

In *myd88*$^{-/-}$ mice, there is only diminished IL-6 secretion in visceral adipocytes in comparison to control mice regardless of diet. However, of interest, there is no difference in IL-6 secretion in subcutaneous adipocytes in ND versus HFD fed mice, representing the only depot and genotype where IL-6 secretion does not respond to changes in diet. Potential explanations for this phenomenon may be due to the essential role of MyD88 in IL-1 signaling pathways, which regulate adipogenesis.[133,134] In accord with the hypothesis that proportional adipose expansion to accommodate lipid storage demands prevents alterations in homeostasis, it is possible that obesity and adipose

expansion that accompanies *myd88*$^{-/-}$ mice, as well as other mice with impeded IL-1 signaling, allows partitioning of exogenous lipids without alteration in inflammation. In contrast, enhanced visceral adipocyte IL-6 secretion with HFD treatment supports the belief that intrinsic differences between visceral and subcutaneous adipocytes exist, which may be exemplified by more consistent expansion of the subcutaneous versus visceral depot in IL-1 type 1 receptor knockout mice.[133]

Addition of polymyxin B, which binds and sequesters LPS via electrostatic interactions, reduces IL-6 secretion in control and CD14-deficient visceral adipocytes, but not visceral adipocytes derived from *tlr4*$^{-/-}$ or *myd88*$^{-/-}$ mice (**Figure 2.11**). Thus, implicating LPS as the pro-inflammatory stimulus in our *ex vivo* adipocyte assay system as well as further entertain the possibility of CD14-independent LPS signaling.

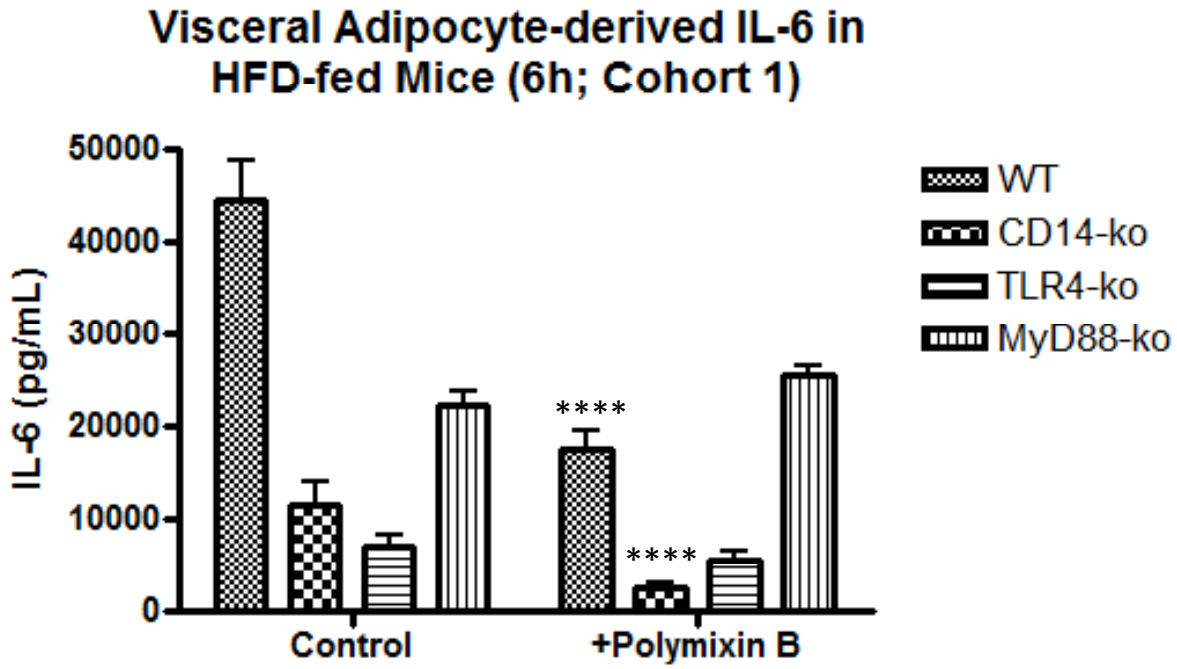


Figure 2.11: Polymixin B treatment diminished *ex vivo* secretion of IL-6 in WT- and *cd14*$^{-/-}$ – derived visceral adipocytes

Visceral adipocytes were isolated from C57Bl/6J (WT), *cd14*$^{-/-}$ (CD14-ko), *tlr4*$^{-/-}$ (TLR4-ko), and *myd88*$^{-/-}$ (MyD88-ko) mice fed high fat diet (60% of calories from fat). Freshly isolated adipocytes were incubated for 6 hours in assay medium without (Control) or with 5μg/ml Polymyxin B (+Polymyxin B) to assess IL-6 secretion. Data presented as mean±s.e.m. (n=6 independent experiments). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 via student t-test in comparison to genotype-matched untreated controls.

CONCLUDING THOUGHTS

CD14 plays an essential role in adipocyte-derived IL-6 and may account for depot-specific inflammation in both models of genetic and diet induced obesity, representing a potential molecular target for the commonly employed type 2 diabetes medication rosiglitazone. Although the anti-inflammatory effects of rosiglitazone are touted as a potentially beneficial property in combating cardiovascular disease, recent data suggests that rosiglitazone may have neutral, if not adverse, effects on mortality and cardiovascular events.[192-200] This clinical correlate highlights the complexity of inflammation, suggesting that inflammation and their agents, i.e., cytokines, can not be simply considered equivalent to pathogenesis. Although the acute phase reactant IL-6 is a classical marker of inflammation, it is a double-agent in metabolism subserving both beneficial and detrimental processes. The detrimental role of IL-6 is suggested by population studies linking elevated serum IL-6 concentration and glucose intolerance with or without adjustment for obesity.[80,81] Furthermore, addition of exogenous IL-6 has been shown *in vivo* and *in vitro* to impair insulin signaling components.[96,97,123,201,202] However, skeletal muscle produces IL-6 in response to exercise and temporally correlates with AMPK activation - a master switch to catabolic pathways that is involved in muscle-contraction stimulated glucose uptake. [203-206] This potential beneficial role of IL-6 is supported by IL-6 infusion in human subjects that enhances glucose disposal and fatty acid oxidation.[203] Conversely, global loss of IL-6 in *il6*$^{-/-}$ mice reduces AMPK activity in skeletal muscle and adipose at rest and diminishes absolute increases in response to

exercise.[207] With advancing age, mice lacking IL-6 show impaired glucose tolerance, obesity, and dyslipidemia, suggesting that absolute deficiency of IL-6 may also be detrimental to systemic glucose homeostasis.[207] Therefore, absence or excess of IL-6 may be detrimental, suggesting a therapeutic level of IL-6 and likely tissue-specific benefits that underscores the importance of targeted therapeutics. With emphasis on tissue specificity and local delivery strategies, our observation that LPS signaling components (CD14, TLR4, and MyD88) are involved in adipose cytokine elucidation and depot-specific inflammatory potential serves as a new approach in adjusting pathophysiological pathways to our own therapeutic goals.

Chapter III

Lipopolysaccharide Signaling Disruption Fails to Ameliorate High Fat Diet Induced Glucose Intolerance and Alters Sympathoadrenal Response to Hypoglycemia

SUMMARY

Chronic low-grade inflammation has been suggested to perpetuate glucose intolerance and insulin resistance. As a function of its potency and wide distribution, bacterial endotoxin or lipopolysaccharide (LPS) is a likely candidate to fuel chronic inflammation associated with diabetes. To test this hypothesis, we employed mice deficient in components of the LPS signaling complex, namely CD14, TLR4 and MyD88, to investigate glucose homeostasis and insulin sensitivity following high fat diet challenge. Contrary to our hypothesis, LPS signaling disruption impairs glucose tolerance and insulin sensitivity in *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice without alteration in body mass or impairment of insulin secretion. In contrast, high fat diet fed *myd88*$^{-/-}$ mice gained ~20% more mass, but developed a mild glucose intolerance and insulin resistance. In *cd14*$^{-/-}$ and *tlr4*$^{-/-}$, but not *myd88*$^{-/-}$ mice, an exaggerated rebound phase in the insulin tolerance test was associated with enhanced catecholamine response to hypoglycemia, which could be abrogated by the adrenergic β-blocker propranolol. The overlay of these mouse models reveals a divergence of phenotypes that demonstrate LPS signaling disruption may lead to glucose intolerance and insulin resistance in part due to enhanced sympathoadrenal tone, uncovering an essential role of innate immunity in physiological stress and its impact upon glucose homeostasis.

INTRODUCTION

Glucose intolerance and insulin resistance are hallmarks of the type 2 diabetes population which is estimated to afflict 180 million people globally and anticipated to double by the year 2030.[28] Despite a predominance of obesity in the type 2 diabetic patient population, it is estimated that the majority of obese individuals are not overtly diabetic, suggesting that nutrient excess alone is a strong but not singular risk factor for glucometabolic dysfunction.[31,32] Recent studies over the last decade reveal chronic inflammation's involvement in feeding behavior, activity, stress, insulin sensitivity, hypertension, truncal obesity, coagulation, and lipid metabolism – classical susceptibility factors in type 2 diabetes and hence potentially a common link in glucose intolerance and insulin resistance.

We have previously shown that components of the LPS signaling pathway, namely CD14, TLR4, and MyD88, play a prominent role in adipocyte inflammation marked by its ability to produce IL-6 *ex vivo*. Expression of the classical LPS receptor CD14 mirrors purported *in vivo* patterns of cytokine secretion with enhanced cytokine and chemokine secretion in visceral adipocytes, suggesting that it may play a role in the pathogenic potential ascribed to this adipose depot in clinical medicine. Indeed, inflammatory responses to LPS stimulation is well documented in cell culture models, procedures eliciting acute insulin resistance in animals, and in clinical scenarios of severe infection that lead to sepsis with acute insulin resistance. Therefore, LPS serves as an

attractive candidate for a causative factor in systemic inflammation and may play a role in fueling metabolic disturbance associated with type 2 diabetes.

LPS binding to CD14 requires co-signaling partners MD2 and TLR4 to recruit either TIRAP/Mal to activate NFκB via MyD88 (MyD88-dependent pathway) or to recruit TRAM to activate IRF3 (MyD88-independent pathway). LPS activates NFκB in multiple cell types, contributing to local and systemic elevation of inflammatory cytokines such as IL-6, TNFα, CRP, and MIF which have been associated with human diabetes.[201] Moreover, randomly selected individuals in the Bruneck Study cohort demonstrated an association between subclinical endotoxemia > 50 pg/mL and carotid atherosclerosis, furthering an intriguing hypothesis that LPS may play a role in chronic inflammatory diseases.[172] Given the importance of the MyD88-dependent pathway and NFκB in inflammation, we employed CD14-deficient (*cd14*$^{-/-}$) and TLR4-deficient (*tlr4*$^{-/-}$) mice to block LPS binding and membrane signaling as well as MyD88-deficient (*myd88*$^{-/-}$) mice to block the MyD88-dependent arm of LPS response in order to dissect their roles in glucose homeostasis and insulin sensitivity when challenged with high fat diet (HFD).

EXPERIMENTAL PROCEDURES

Microarray gene expression and analysis

Total RNA was extracted from isolated epididymal adipocytes or 3T3-L1 cells (ATCC, Manassas, VA) of individual mice with a commercially available acid-phenol reagent (TRIzol, Invitrogen Inc, Carlsbad, CA). RNA concentration was assessed by absorbance spectroscopy and RNA integrity confirmed by non-denaturing agarose gel electrophoresis. Preparation of cRNA, Affymetrix procedures, and data analysis via proprietary MicroArray Computational Environment 2.0 (MACE 2.0, Worcester, MA) were performed as previously described in Chapter II.

Experimental animal care

Cd14$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice have been backcrossed at least 12 generations to the C57BL/6J background. C57Bl/6J mice were obtained from the Jackson Laboratory at 6-11 weeks of age. All mice were housed in ventilated polysulfone cages (Allentown Inc., Allentown, NJ) in a pathogen-free barrier facility maintained on a 12-hour light / 12-hour dark cycle. Mice had free access to autoclaved water and food. Obesity was induced by a high fat diet consisting of ~60% of calories from fat (TD93075; Harlan Teklad, Madison, WI) starting at 11 weeks of age. Prior to 11 weeks, mice were fed the standard pellet diet (LabDiet PicoLab 5053, Purina Mills, St. Louis, MO). Animal weight and food consumption was measured weekly for the duration of the experiment. Animal were fasted for 16-18 hours prior to sacrifice by cervical dislocation followed by bilateral pneumothorax. Harvested tissues were immediately frozen in liquid nitrogen and stored

at -80°C or fixed in formalin for chromagenic or immunohistochemical analysis. All experiments employed male mice in accordance with the University of Massachusetts Medical School Institutional Animal Care and Use Committee (UMMS-IACUC).

Activity Measurements

Mouse movement was measured in custom-built activity monitors using 880 nm near-infrared light emitting diodes with a scan frequency of 125 Hz and a minimum detection interval of 0.040 seconds (**Appendix 1**). Ambulatory activity was calculated by sequential beam breaks, while stereotypic activity was calculated by multiple breaks of the same beam. Mice were housed individually in their native cages in parallel with controls with an initial 24-hour acclimatization period and were monitored for at least 96 hours.

Body Fat Analysis

Fat tissue, lean tissue, and free fluid were measured by time domain nuclear magnetic resonance (TD-NMR) using the Bruker LF50 (Bruker Optics Inc, Billerica, MA) which, in brief, utilizes relative relaxation amplitude and duration to provide tissue contrast.

Analysis of metabolic parameters

Metabolic testing (Glucose Tolerance Test and Insulin Tolerance Test)

Glucose tolerance test was performed with intraperitoneal injection of 10% w/v D-glucose in sterile water (2g glucose/kg body weight) following a 16-18 hour overnight

fast. Whole blood glucose values were measured using Ascencia Breeze (Bayer Healthcare Diabetes Care Division, Tarrytown, NJ) or BD Logic (Becton, Dickinson and Co, Franklin Lakes, NJ) before and after 15, 30, 45, 60, 90, 120, 180, and 240 minutes post challenge/injection. Insulin tolerance tests were similarly performed with i.p. injection of recombinant human insulin (0.75U insulin / kg body weight; Novolin R™, Novo Nordisk Inc, Princeton, NJ) and blood glucose measurements before and after 15, 30, 45, 60, 90, 120, 180, and 240 minutes post challenge/injection. Propranolol (2mg/kg body weight; Sigma, St. Louis, MO) or saline was injected 30 minutes prior to insulin tolerance test protocol initiation.

Insulin-Induced Hypoglycemia and Norepinephrine measurement

Hypoglycemia was induced in conjuction with insulin tolerance tests with i.p. injection of recombinant human insulin (1-1.5U insulin / kg body weight; Novolin R™, Novo Nordisk Inc, Princeton, NJ) and blood was collected in EDTA-treated capillary tubes (Sarstedt) before and after 45 and 90 minutes post injection.

Norepinephrine was measured according to manufacturer's protocol. In short, plasma noradrenaline was extracted with a cis-diol affinity gel, acylated to *N*-acylnoradrenaline, converted enzymatically to *N*-acylnormetanephrine, and quantified in a competitive immunoassay (Alpco Diagnostics, Salem, NH).

Insulin

Serum insulin was measured in the fed state or following a 14-16 hour fast using ELISA (Millipore, Billerica, MA) as previously described in Chapter II.

Serum Lipid Analyses

Serum was collected following 9 hour fast via retro-orbital bleeding. Colorimetric analyses were employed to measure total cholesterol (Wako Diagnostics, Richmond, VA), triglyceride (Sigma, St. Louis, MO), and non-esterified fatty acid (Wako Diagnostics) according to manufacturer's protocol.

Adrenal Gland dissection and size analysis

Ventro-medial adrenal surface was marked with surgical ink to maintain orientation. Adrenal glands were removed under a dissecting microscope with liberal margins to prevent tissue distortion and were immediately fixed in Bouin's solution for 8 hours followed by a circulated wash overnight. Tissue was embedded in paraffin and sliced in serial horizontal, longitudinal sections. Images were captured with a Zeiss Axiovert 200 inverted microscope (Thornwood, NY) and morphometric analyses were conducted by Axiovision 4.0. Four largest sections by area were measured to approximate total size, while medullary area was measured and cortical area was calculated.

Statistical Analysis

Statistical analysis employed general linear mixed models, including analysis of variance (ANOVA) or Student's *t* test followed by Bonferroni post hoc tests or statistically conservative Tukey/Kramer posthoc tests. Statistics were performed using Prism Software (Graphpad, San Diego, CA), Statview 5.0.1 (SAS Institute, Cary, NC), or Sigma Plot 5.0 (SPSS, Inc., Chicago, IL). Results are shown as mean±SEM unless otherwise stated. A p-value of $p<0.05$ was regarded as a significant difference.

RESULTS & DISCUSSION

Models of adiposity implicate the Toll-Like Receptor Pathway

To investigate inflammatory pathways involved in type 2 diabetes, we employed three models of insulin resistance, namely 3T3-L1 model of adipocyte differentiation, high fat diet-induced obesity, and genetic model of obesity in the ob/ob mouse. Utilizing the Kyoto Encyclopedia of Genes and Genomes to analyze gene pathways altered, we identified the *Toll-Like Receptor Pathway* to be commonly involved with development of insulin resistance in 3T3-L1 adipogenesis (25 out of 201 annotated genes, $p<2.66e-13$), high fat diet-induced obesity (24 out of 201 annotated genes, $p<6.85e-13$), and genetic obesity (16 out of 201 annotated genes, $p<1.03e-6$) (**Figure 2.4**). Comparison of these three models revealed similar expression patterns of CD14 and Toll-like receptor 4 (TLR4) – classical cosignaling molecules responsible for LPS binding and transmembrane signaling.

Progressive obesity in myd88$^{-/-}$, but not cd14$^{-/-}$ and tlr4$^{-/-}$ mice

Cd14$^{-/-}$ and *tlr4*$^{-/-}$ mice fed either normal diet (ND) or HFD gained weight at a similar rate to the C57Bl/6J control cohort (**Figure 3.1a, b**). *Myd88*$^{-/-}$ mice on ND demonstrated a trend of weight gain but failed to meet consistent statistical significance; however, *myd88*$^{-/-}$ littermates fed HFD were 14% heavier at 6 months of age (40.5g vs 35.6g ; $p<0.05$) and 22% heavier at 12 months of age (50.9g vs 41.6g; $p<0.05$) (**Figure 3.1c**). NMR analysis of lean mass and adipose mass demonstrated no differences

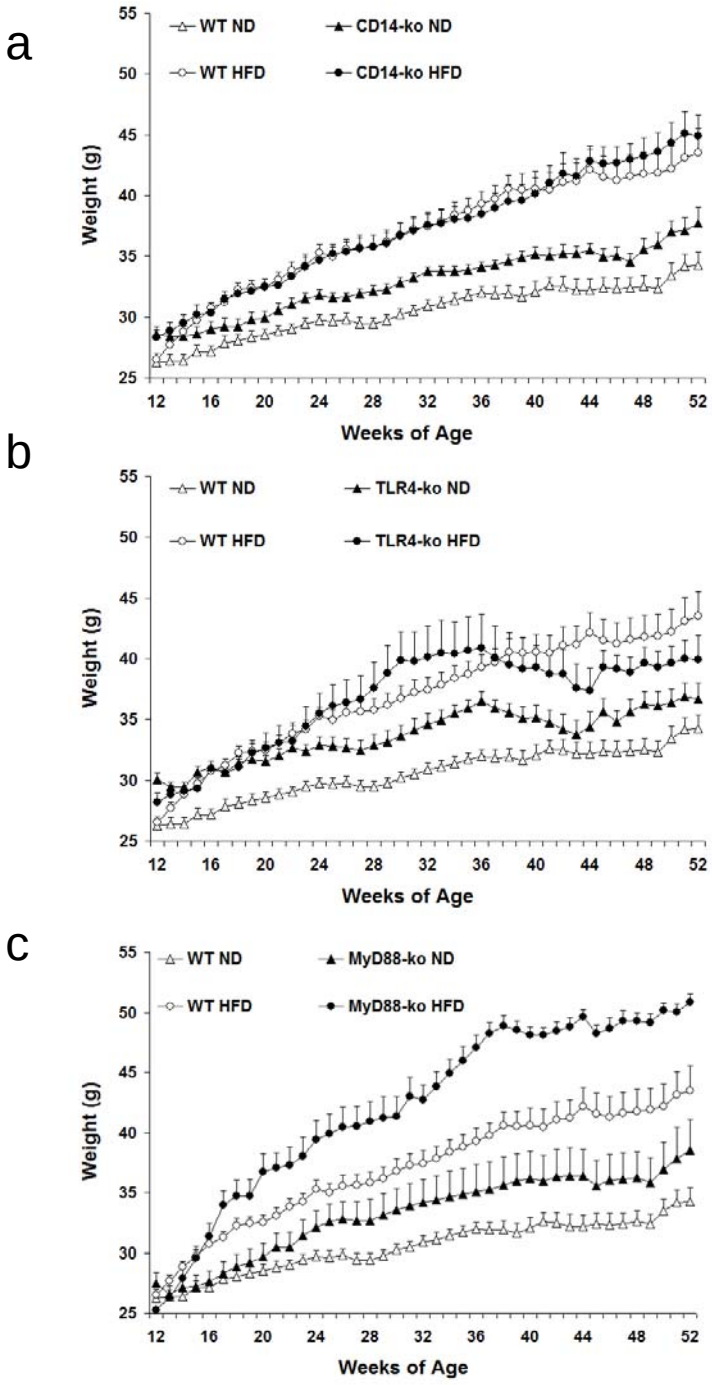


Figure 3.1: Progressive obesity in myd88-deficient, but not cd14- and tlr4-deficient animals

Growth curves of (a) *cd14*$^{-/-}$, (b) *tlr4*$^{-/-}$, (c) *myd88*$^{-/-}$ mice starting at 11 weeks of age when animals were randomized to normal diet (ND; black triangle) and high fat diet (HFD; black circle) cohorts. Data presented as mean±s.e.m. (n=8 mice per group for weight analyses)

between 12-month old *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice from controls, while *myd88*$^{-/-}$ mice had enhanced adipose mass employing either ND (28.8±3.0% in *myd88*$^{-/-}$ vs 15.5±5.6% in control mice; $p<0.05$) or HFD (42.1±1.7% in *myd88*$^{-/-}$ vs 29.4±4.2% in control mice; $p<0.05$) (**Figure 3.2a, b**). This data suggests that the majority of weight gain in *myd88*$^{-/-}$ mice is attributable to adipose mass, which may be due to the role of MyD88 in IL-1 and IL-18 signaling. Previous studies demonstrated mature onset obesity in IL-1 receptor type I knockout mice, whereas unimpeded IL-1 signaling in IL-1 receptor antagonist knockout mice resulted in leaness.[133,134] Furthermore, *Netea et al*. described obesity in mice with impaired IL-18 signaling due to either deletion of the IL-18 cytokine or the α-chain of the IL-18 receptor.[202] Therefore, this divergent phenotype on weight gain and adipose expansion may be in part due to the additional role of MyD88 in non-LPS signaling pathways, such as IL-1 and IL-18.

Cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice have no alteration in total ambulatory or circadian activity

To assess energy expenditure, *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice were individually housed in activity cages, demonstrating no differences in spontaneous ambulatory movements from age- and gender-matched controls (**Figure 3.3a**). Alteration in circadian rhythm is a prominent source of metabolic variations when considering whole-body knockouts; however, we observed no significant alteration in circadian activity patterns [203] (**Figure 3.3b**).

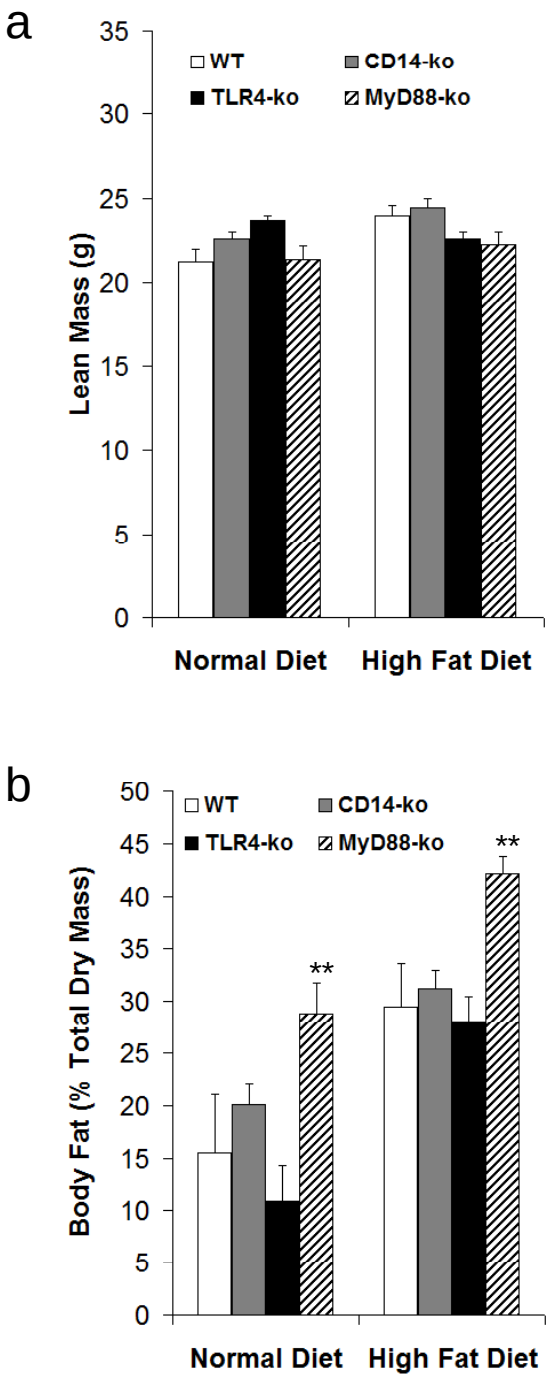


Figure 3.2: NMR-based Analysis of Lean Mass and Adipose Mass

(a) NMR analysis of absolute lean mass and (b) fat mass as a percentage of total dry mass. Data presented as mean±s.e.m. (n=8). *p<0.05, **p<0.01, ***p<0.005 via student t-test in comparison to WT control.

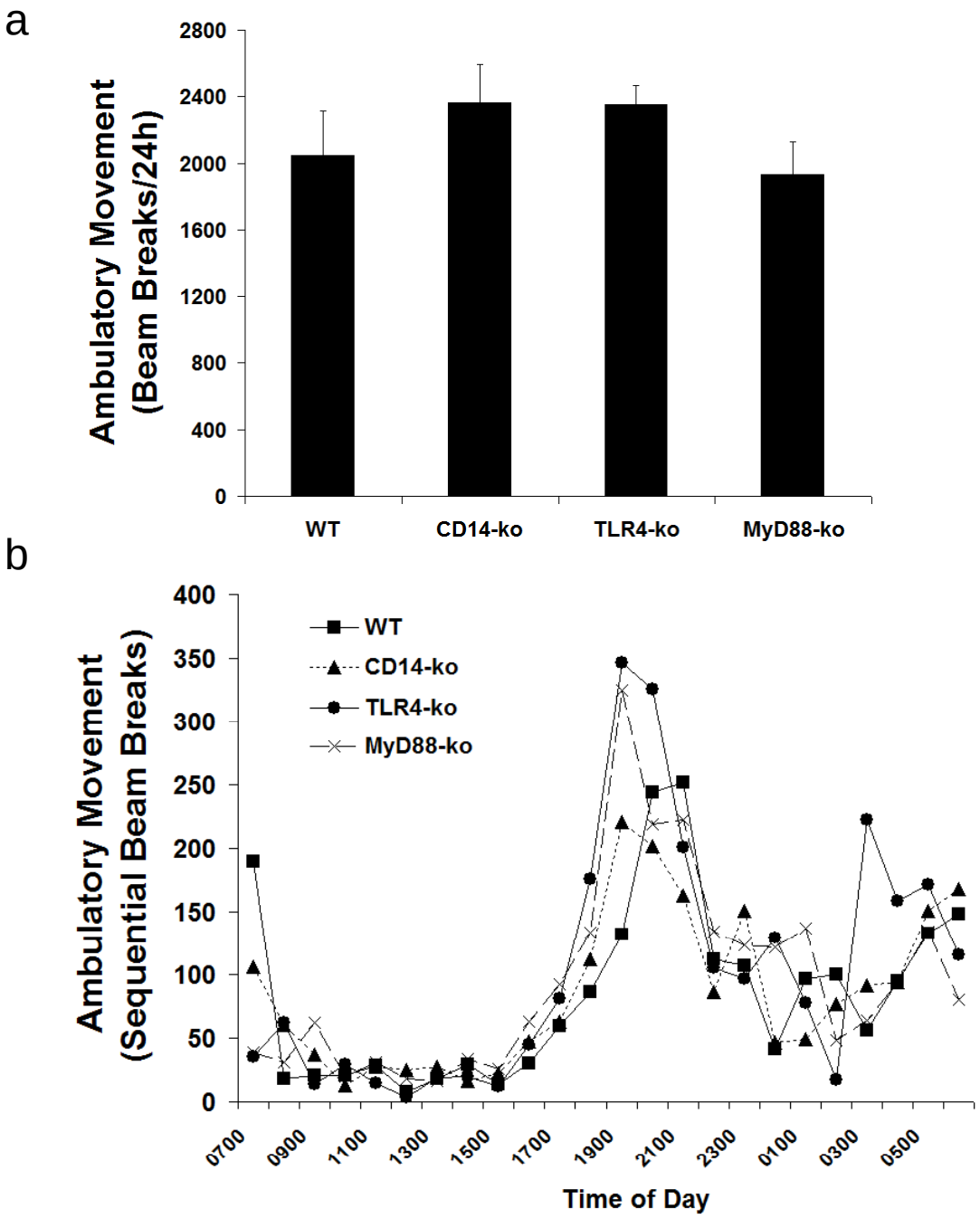


Figure 3.3: Ambulatory Activity and Circadian Rhythm

(a) After 24-hour acclimatization, *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice demonstrate similar overall ambulatory activity as approximated by sequential beam breaks in the x-y axis over a 72-hour period. (b) Analysis of movement data demonstrated no alterations in circadian rhythm in comparison to WT mice. Data presented as mean±s.e.m. (n=6 mice per group). *p<0.05, **p<0.01, ***p<0.005 via student t-test in comparison to WT control.

CD14 and TLR4 genotype impairs glucose tolerance and alters insulin response in middle-aged animals

Despite the lack of genotype-specific changes in overall body composition in *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice, glucose tolerance tests (GTT) uncovered glucose intolerance in 6-month old mice fed either ND or HFD as demonstrated by greater peak glucose values that persists through later timepoints (**Figure 3.4a**). However, *myd88*$^{-/-}$ mice demonstrated no alteration in glucose tolerance when fed ND as reported previously by Netea et al. (**Figure 3.4a**).[202] Conversely, when challenged with HFD, *myd88*$^{-/-}$ reveals minor glucose intolerance shown at 120 minutes post-injection but fails to be significant to net glucose flux as assessed by area under the curve (AUC) (**Figure 3.4a**).

In insulin tolerance tests, *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice show no alteration in the initial downward phase in either ND- or HFD-fed cohorts, but unexpectedly rebound beyond baseline glucose in *tlr4*$^{-/-}$ mice, suggesting either impaired glucose uptake and/or inhibition of hepatic gluconeogenesis (**Figure 3.4b**). On the other hand, *myd88*$^{-/-}$ mice display minimal insulin resistance in either ND or HFD fed cohorts (**Figure 3.4b**).

Advanced-aged cd14*$^{-/-}$ *and tlr4*$^{-/-}$ *mice have marked glucose regardless of diet, whereas myd88*$^{-/-}$ *mice show minor HFD-specific effects

To assess these phenotypes in the context of advancing age, which was necessary to uncover effects in IL-1 signaling knockouts, we followed these mice cohorts to 12 months of age. Similar to trends at 6 months of age, *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice exhibited marked glucose intolerance fed either ND (AUC 49,191±3852 mg-min/dL in *cd14*$^{-/-}$ and AUC 46,423±2610 mg-min/dL in *tlr4*$^{-/-}$ vs AUC 29,256±1211 mg-min/dL in control

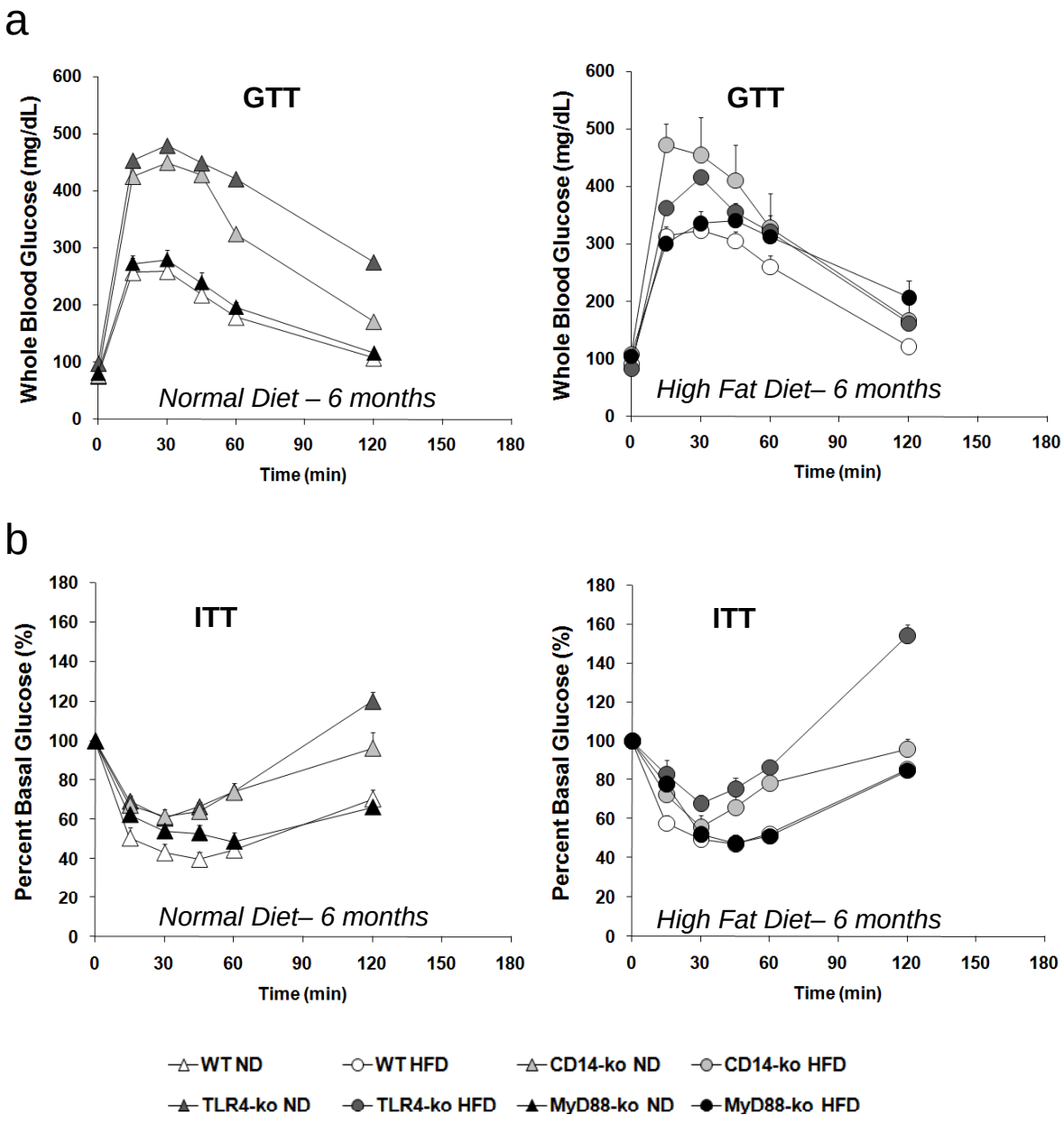


Figure 3.4: Glucose Homeostasis in Middle-Aged (6 month) *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice

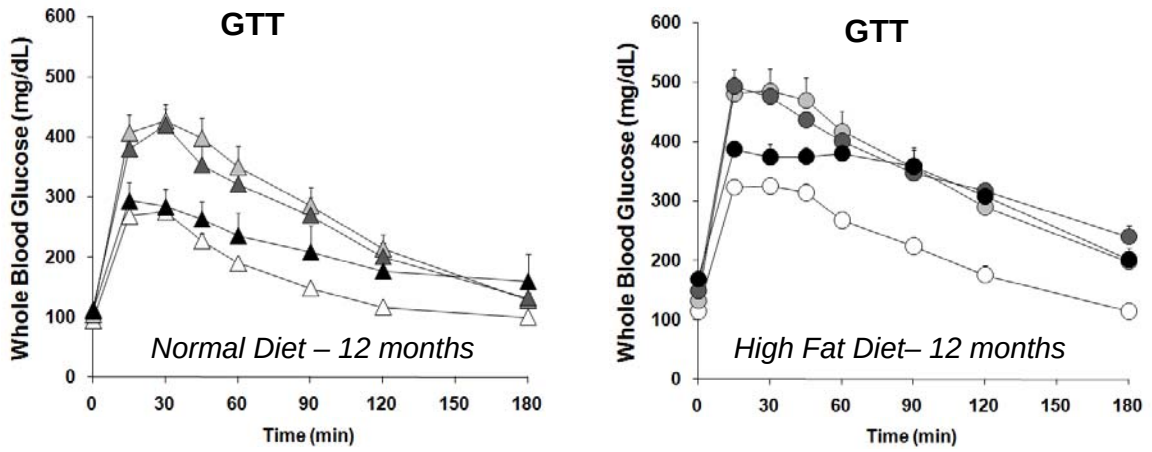
(a) Glucose tolerance test (GTT) of *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice fed a normal diet (ND; left side) or high fat diet (HFD; right side). (b) Insulin tolerance test (ITT) of *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice fed a normal diet (ND; left side) or high fat diet (HFD; right side) Data presented as mean±s.e.m. (n=8 mice per group).

mice; $p<0.0005$, $p<0.00005$ respectively) or HFD (AUC 61,636±5318 mg-min/dL in *cd14*$^{-/-}$ and AUC 63,126±2335 mg-min/dL in *tlr4*$^{-/-}$ vs AUC 39,399±2129 mg-min/dL in control mice; $p<0.001$, $p<0.00001$ respectively) (**Figure 3.5a**). *Myd88*$^{-/-}$ mice maintained glucose homeostasis when fed ND; however, HFD challenge uncovered glucose intolerance (AUC 57,626±2700 mg-min/dL in *myd88*$^{-/-}$ vs AUC 39,399±2129 mg-min/dL in control mice; $p<0.00005$) that was hinted at 6 months of age (**Figure 3.5a**).

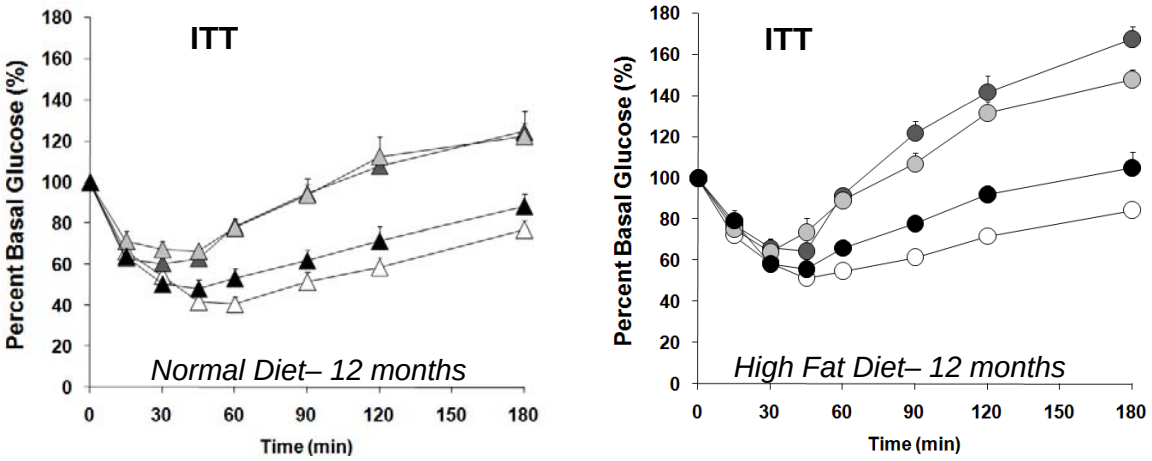
Enhanced glucose-induced insulin secretion in cd14$^{-/-}$, tlr4$^{-/-}$, and myd88$^{-/-}$ mice

As insufficient insulin secretion and peripheral insulin resistance are two major origins of glucose intolerance, we first investigated whether glucose intolerance seen in these mice may be explained by a defect in insulin production. Fasting insulin levels were 2-3-fold higher in *myd88*$^{-/-}$ mice with respect to control mice on the same diet; however, *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ demonstrated no difference with controls on either ND or HFD (**Table 3.1**). The secretion of insulin following an i.p. bolus of glucose (2g/kg body weight) demonstrated a time-weighted average of insulin in *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice that exceeded that of wildtype controls (3213±344 ng-min/mL in *cd14*$^{-/-}$; 2598±325 ng-min/mL in *tlr4*$^{-/-}$; and 5522±489 ng-min/mL in *myd88*$^{-/-}$ vs 1187±82 ng-min/mL in control mice; $p<0.005$, $p<0.05$, $p<0.0005$ respectively). Although more insulin was being produced in *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice, glucose intolerance may still arise from insulin resistance, specifically the inability of insulin to promote glucose uptake in peripheral tissues and/or inhibit hepatic gluconeogenesis and glycogenolysis.

a



b



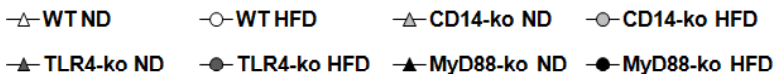


Figure 3.5: Glucose Homeostasis in Advanced-Aged (12 month) *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice

(a) Glucose tolerance test (GTT) of *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice fed a normal diet (ND; left side) or high fat diet (HFD; right side). (b) Insulin tolerance test (ITT) of *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice fed a normal diet (ND; left side) or high fat diet (HFD; right side) Data presented as mean±s.e.m. (n=8 mice per group).

Genotype	Diet	Cholesterol (mg/dL)	Triglyceride (mmol/L)	NEFA (mEq/L)	Fasting Insulin (mEq/L)
WT	Normal Chow	133.7 ± 12.1	1.61 ± 0.11	0.688±0.16	0.76±0.15
CD14$^{-/-}$	Normal Chow	127.2 ± 8.6	1.62 ± 0.02	0.804 ±0.05	1.10±0.16
TLR4$^{-/-}$	Normal Chow	93.9 ± 1.9^{A}	1.42 ± 0.05	0.778 ±0.11	0.67±0.11
MyD88$^{-/-}$	Normal Chow	148.5 ± 7.7	1.65 ± 0.13	0.752 ±0.12	2.60±0.72^{C}
WT	High Fat Chow	152.9±14.7	1.52 ± 0.13	0.530±0.08	1.52±0.27
CD14$^{-/-}$	High Fat Chow	140.1 ± 10.9	1.54 ± 0.03	0.536 ±0.01	2.72±0.55
TLR4$^{-/-}$	High Fat Chow	137.3 ± 1.9	1.39 ± 0.04	0.536 ±0.03	1.27±0.02
MyD88$^{-/-}$	High Fat Chow	239.2 ± 13.1^{B}	1.35 ± 0.06	0.586 ±0.09	3.15±0.42^{C}

Table 3.1 Lipid Profile and Fasting Insulin of *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice

Cd14$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ at 52 weeks of age following 41 weeks of dietary treatment demonstrated no genotype-specific lipid abnormalities with the exception of reduced serum triglyceride in normal diet fed (ND) *tlr4*$^{-/-}$ mice ($^{A}p<0.05$) and increased total cholesterol in high fat diet fed (HFD) *myd88*$^{-/-}$ mice ($^{B}p<0.005$). *Myd88*$^{-/-}$ mice fed either a ND or HFD demonstrated elevated fasting serum insulin ($^{C}p<0.001$). Values are expressed as mean ± s.e.m. NEFA, non-esterified fatty acid.

Enhanced hypoglycemic rebound in cd14$^{-/-}$ and tlr4$^{-/-}$ mice

The ability of *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$ mice to respond to insulin was tested with an insulin tolerance test (ITT) that demonstrated no alteration in the initial downward phase in either ND- or HFD-fed cohorts, suggesting normal insulin sensitivity to an i.p. bolus of insulin (**Figure 3.5b**). However, the rebound phase of the ITT was dramatic with *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice fed ND exceeding basal glucose by 22% ($p<0.005$) and 25% ($p<0.0001$), respectively, at 180 minutes. Whereas, HFD challenged *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice surpassed basal glucose by 48% ($p<0.0001$) and 68% ($p<0.0001$), respectively, at 180 minutes (**Figure 3.5b**). Whereas *myd88*$^{-/-}$ mice display minimal insulin resistance on ND, HFD-fed cohorts unmask impairment in glucose rebound after 60 minutes in the 12-month old animals (**Figure 3.5b**). The lack of insulin resistance or glucose intolerance in *myd88*$^{-/-}$ mice fed ND has been previously reported.[202] Our data recapitulate this observation, but expose a minor glucose intolerance and insulin resistance in these mice when challenged by HFD. Of note, the lack of changes in metabolism was unanticipated by both groups which hypothesized that loss of inflammatory players would dampen metabolic dysregulation. Similar to the IL-18 knockout mouse, frank obesity in *myd88*$^{-/-}$ mice was compensated with enhanced serum insulin in contrast to *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice that show no alteration in fasting serum insulin (**Table 3.1**). Paradoxically, *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice demonstrate comparable cholesterol, triglyceride, and non-esterified fatty acid (NEFA) serum concentrations to control mouse cohorts despite marked glucose intolerance, whereas *myd88*$^{-/-}$ mice are hypercholesterolemic with normal triglyceride and NEFA with relatively minor

alterations in glucose homeostasis, supporting concepts that obesity and excess adipose by itself may not correlated to diabetes pathogenesis (**Table 3.1**). In this spirit, we sought to investigate additional pathways that could impair glucose homeostasis in *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice independent of adipose accumulation.

Norepinephrine drives hypoglycemic rebound in cd14*$^{-/-}$ *and tlr4*$^{-/-}$ *mice

Acute counter-regulatory pathways to hypoglycemia rely on adrenal-derived bioactive catecholamines to inhibit insulin secretion and peripheral glucose utilization, while promoting hepatic glycogenolysis and stimulate lipolysis. The importance of the adrenal gland in glucose homeostasis is exemplified in human maladies where adrenal excess in Cushing's disease is marked by hyperglycemia, whereas adrenal insufficiency in Addison's disease is marked by hypoglycemia. Secreted adrenal factors play an essential role of the hypothalamic-pituitary-adrenal (HPA) axis in stress reactivity, fat deposition, and hyperphagic responses, which may account for strain differences in rodents.[211] Main contributions from the adrenal gland include: 1) corticosterone from the adrenal cortex that is involved in long-term glucose maintenance through gluconeogenesis and 2) catecholamines from the adrenal medulla that is essential for short-term glucose maintenance through glycogenolysis. Mouse models of diabetes, such as the genetically obese *ob/ob* mouse, corroborate the contribution of adrenal glands as seen with normalization of insulin sensitivity and glucose uptake in adrenalectomized *ob/ob* mice in part due to normalization of hypothalamic melanocortin tone, corticosterone levels, and functional β-adrenergic signaling.[212-218] Studies by

Zacharowski et al. found adrenal hypertrophy in *tlr4*$^{-/-}$ mice with functional implications in sepsis patients; however, did not address alterations in bioactive catecholamines in the context of glucose homeostasis.[219] To determine if adrenal catecholamines may explain this rebound phase in the ITT, we measured norepinephrine at baseline, at 45 min post-injection to coincide with the inflection in circulating glucose, and at 90 min post-injection when basal glucose levels are re-established. In *cd14*$^{-/-}$ mice, there is a 3.3- and 2.5-fold increase in norepinephrine at 45 minutes and 90 minutes post-injection, respective to control mice (**Figure 3.6a**). Similarly *tlr4*$^{-/-}$ mice show a 3.5- and 3.4-fold increase in norepinephrine at 45 minutes and 90 minutes post-injection, respective to control mice (**Figure 3.6a**). In contrast, *myd88*$^{-/-}$ mice shows a norepinephrine response similar to control mice (**Figure 3.6a**). Following the hypothesis that a hyperactive adrenal response to hypoglycemia may account for this alteration in glucose homeostasis, we preconditioned mice with the non-specific β-blocker propranolol prior to insulin administration. Sham saline injection prior to insulin-induced hypoglycemia recapitulated enhanced glucose flux previously demonstrated in *cd14*$^{-/-}$ (AUC 39,205±1092 mg-min/dL in *cd14*$^{-/-}$ vs AUC 31,188±964 mg-min/dL in control mice; $p<0.002$) and *tlr4*$^{-/-}$ mice (AUC 41,276±1827 mg-min/dL in *tlr4*$^{-/-}$ vs AUC 31,291±1070 mg-min/dL in control mice; $p<0.0001$) (**Figure 3.6b,c**). Indeed, pretreatment with propranolol mitigated this excess flux in both *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice ($p<0.005$, $p<0.0005$, respectively) (**Figure 3.6b,c**).

Expansion of total and medullary adrenal size in tlr4$^{-/-}$ and myd88$^{-/-}$ mice

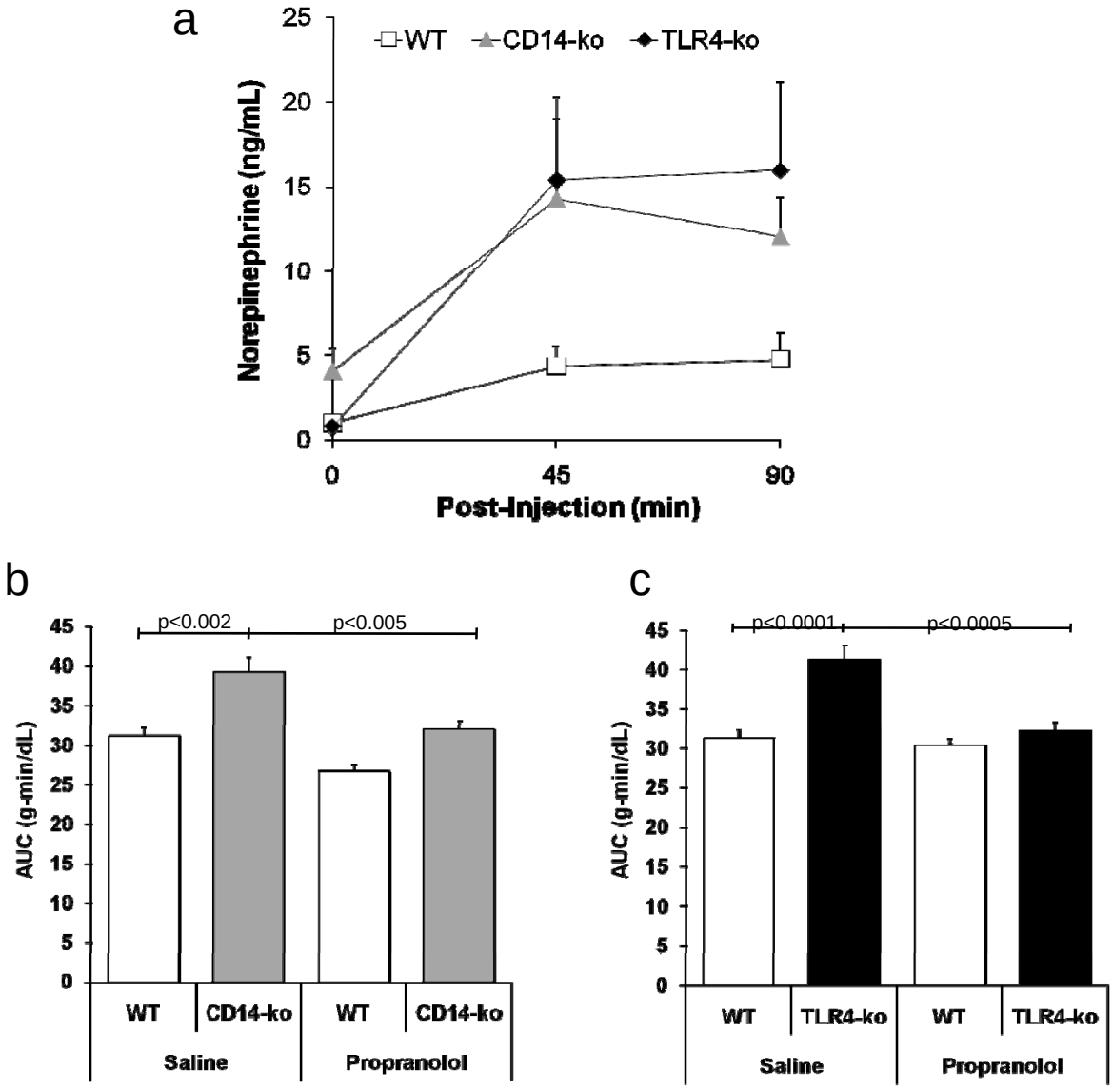
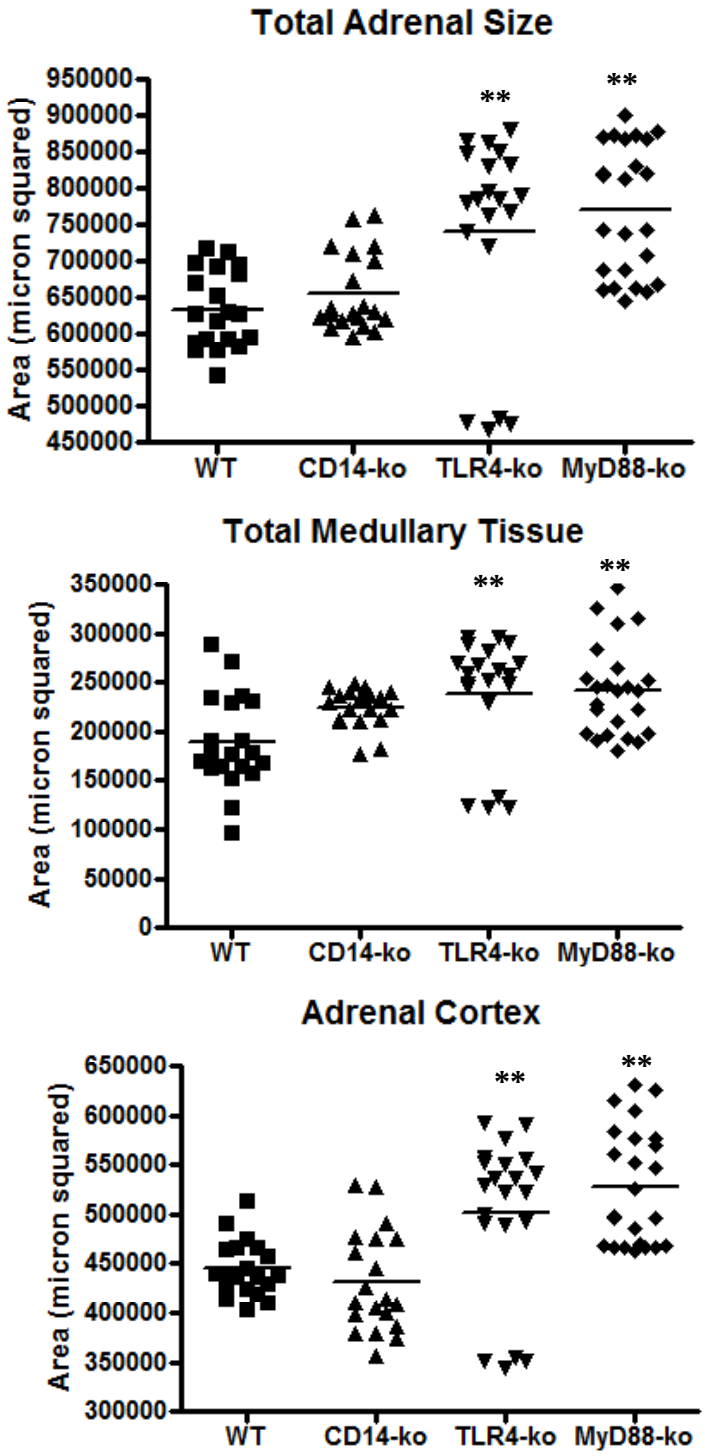


Figure 3.6: Norepinephrine drives hypoglycemic rebound in *cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice

(a) *Cd14*$^{-/-}$ and *tlr4*$^{-/-}$ mice were injected i.p. with insulin (1-1.5U / kg body weight) to elicit a counter-regulatory norepinephrine response to hypoglycemia. Blood was taken before and after 45 and 90 minutes for norepinephrine measurements. Propranolol (2mg/kg of body weight) or saline was injected 30 minutes prior to insulin tolerance tests to demonstrate the role of β-adrenergic blockade on glucose flux, represented as area under the curve (AUC), in (b) *cd14*$^{-/-}$ and (c) *tlr4*$^{-/-}$ mice. Data presented as mean±s.e.m (n=8).

Adrenal gland dissection revealed increased overall, calculated cortical, and medullary size in *tlr4*$^{-/-}$ and *myd88*$^{-/-}$ mice (**Figure 3.7**). *Cd14*$^{-/-}$ mice fit a trend of medullary expansion, the adrenal region responsible for catecholamine synthesis, but failed to meet statistical significance (**Figure 3.7**). Of interest, strain analysis by Jackson Laboratories demonstrate that male C57Bl/6J mice demonstrate 100% more adrenal mass than gender-matched sv129 mice and 40% more than strain-matched female littermates.[220,221] In fact, adrenal mass in C57Bl/6J mice is considered exceptional in comparison to 13 other common strains of mice with greater than 2.5 standard deviations above mean adrenal mass.[220,221] Coupled with our data demonstrating excess adrenal counter-regulation to hypoglycemia in male mice, this gender-specific difference in adrenal physiology may partially explain the lack of insulin sensitization seen in male mice by *Shi et al.* in contrast to their female littermates. Moreover, additional impairment of glucose tolerance and insulin sensitivity in our studies may be attributed to further backcrossing (12+) into the C57Bl/6J background, potentially allowing more complete penetration of the adrenal contribution, which appears to be an "exceptional" characteristic of the C57Bl/6J background.



comparison to WT
p<0.05
p<0.01
* p<0.005
** p< 0.001

Figure 3.7: Adrenal Gland Size Analysis

Four largest sections by area (horizontal, longitudinal) were measured to approximate total size, while medullary area was measured and cortical area was calculated. Images quantification were conducted by Axiovision 4.0 at1.5x magnification. Data analyzed with a 1-way ANOVA with Dunnet Post-Hoc Test with a $p<0.05$ considered significant. Data presented as mean±s.e.m (n=5-6).

CONCLUDING THOUGHTS

Despite data suggesting insulin sensitization in the C2C12 skeletal muscle model with TLR4 abrogation *in vitro* and insulin resistance of the 3T3-L1 adipocyte cell model with TLR4 activation *in vitro*, human population studies of TLR4 and CD14 polymorphisms have failed to demonstrate a clear association between functional changes in LPS response and predisposition to diabetes or metabolic syndrome.[222-232] Furthermore, human serum studies of soluble CD14 (sCD14) show a positive correlation with insulin sensitivity, supporting our observation that whole body loss of CD14 may lead to insulin resistance.[233] In concert with previous experimental and population studies, our data support a hypothesis that whole body alteration of LPS signaling may involve glucose homeostasis beyond insulin-sensitive peripheral tissues and hence systemic therapeutic strategies to attenuate LPS signaling may have unclear benefits in glucose lowering.

The novel employment of three genetic knockouts, namely *cd14*$^{-/-}$, *tlr4*$^{-/-}$, and *myd88*$^{-/-}$, localizes metabolic observations to these genes shared role in LPS signaling *in lieu* of alternate biological functions. The overlay of these mouse models suggests an intimate association of CD14 and TLR4 with sympathoadrenal tone and impaired glucose homeostasis. While, less severe metabolic disturbance in the MyD88 mouse, even in the context of augmented obesity, suggests the importance of MyD88 in non-LPS pathways and supports the hypothesis that obesity alone is not a singular cofactor for glucose intolerance and insulin resistance.

Our results further support that multiple etiologies are involved in glucometabolic derangements and invites inquiry into the role that multiple organs play in the pathogenesis of glucose intolerance and insulin resistance. Moreover, these data demonstrate that whole body disruption of LPS signaling, despite its potential anti-inflammatory sequellae, leads to metabolic derangement and thus tissue-specific effects must be carefully considered with the development of future therapeutic strategies.

Discussion and Final Thoughts

DISCUSSION

CHAPTER II

Differential expression of CD14 between visceral and subcutaneous depots coupled with the prominent role CD14, TLR4, and MyD88 plays in *ex vivo* IL-6 secretion provides an attractive explanation for depot-specific inflammatory potential. Moreover, the CD14 ligand LPS is an omnipresent pro-inflammatory stimulus in the human body with well-established pro-inflammatory functions that might contribute to insulin resistance. Indeed, bacterial-derived LPS can drive sepsis (also classified as the systemic inflammatory response syndrome (SIRS) of infectious origin), promoting inflammatory cascades that culminate in a "cytokine storm." Of interest, hyperglycemia is seen in many septic patients, but diabetic patients are particularly predisposed, which would match our observations that CD14 transcriptional expression increases in both genetic and diet-induced models of obesity with concurrent diabetes. Our observation of enhanced CD14 expression in visceral adipocytes has recently been validated by *Poulain-Godefrey et al.* who took paired adipose tissue biopsies from omental (visceral type) and subcutaneous depots.[222] Although the pattern of enhanced omental expression of CD14 persisted in all volunteers, this was independent of obesity as well as glycemic state.[222]

Preferential CD14 expression in visceral adipocytes may be sufficient to account for the pro-inflammatory potential of this depot; however, as predicted by receptor-ligand kinetics, the availability of the LPS stimulus may also be responsible. Although LPS can be found in circulating blood, the majority of the body's LPS is likely associated with

bacterial populations. In particular, it is popular belief that bacterial populations that co-exist with gut mucosa numerically outnumber the cells of the human body; thus, representing a significant reservoir of LPS. Disruption of the integrity of the gastrointestinal tract may leach LPS into the peritoneum, fueling chronic inflammation or, depending on the severity, initiate acute sepsis. Moreover, chronic gastrointestinal inflammation may also uniquely contribute, as a function of anatomy, multiple inflammatory stimuli to visceral, but not subcutaneous adipose. Indeed, diverticulitis has been associated both with obesity and diabetes as well as radiologic findings termed "fat stranding" that commonly represent active infection or inflammation of adipose tissues.[223,224] Additionally, the gastrointestinal system from the lower esophagus to upper anal canal drain into the hepatic portal venous system, which shares drainage with visceral adipose, but not subcutaneous adipose; thus, by proximity, may alter LPS bioavailability. Therefore, anatomic juxtaposition of visceral adipose with the major reservoir of LPS in the gastrointestinal tract may play a pathophysiological role in visceral adipose inflammation and supports our observation of attenuated IL-6 secretion from visceral adipocytes isolated from mice deficient in the LPS signaling pathway, i.e., CD14, TLR4, and MyD88.

Though differential expression of CD14 and varying exposure of LPS may contribute to depot-specific inflammation, what accounts for enhanced subcutaneous and visceral adipocyte IL-6 secretion in WT mice fed a high fat diet? Likewise, how can this dietary effect be attenuated by the loss of CD14, TLR4, and MyD88? Studies by *Cani et al.* has promoted a theory of metabolic endotoxemia with the posit that HFD feeding: 1)

enhances intestinal permeability with downregulation of genes encoding tight junction proteins, and 2) skew bacterial populations in the gut mucosa to LPS-containing species; therefore, leading to acute elevation of circulating LPS that was sustainable by continued high fat dietary intake.[225,226] Correlates in humans are preliminary with one study demonstrating that in apparently healthy men, 3-day food diaries reveal a positive association of serum LPS levels with fat intake; however, total energy intake was yet a better predictor of LPS levels.[227]

Our data demonstrates for the first time both differential expression of CD14 between visceral and subcutaneous adipose depots as well as the central importance of members of the LPS signaling cascade (CD14, TLR4, MyD88) in mediating depot-specific and high fat diet-induced adipocyte inflammation. With anatomic susceptibility to LPS exposure, visceral adipose is predisposed to a prominent inflammatory cue that is mediated by the LPS signaling cascade. Early evidence that high fat diet may elevate circulating LPS levels lends credence to our observation that LPS signaling disruption can mitigate HFD-induced adipocyte inflammation. Enhanced expression of inflammatory cytokines by visceral, but not subcutaneous, adipocytes drains directly into the portal circulation where it may have direct impacts on hepatic contributions to systemic inflammation and metabolism.[228] Therefore, enhanced CD14 expression on visceral adipocytes integrates both inputs of inflammatory stimuli with outputs to relevant target organs, providing explanation for enhanced inflammatory potential of the visceral depot as well as afford new strategies for therapeutic interventions.

CHAPTER 3

We present for the first time that whole-body LPS signaling disruption in CD14- or TLR4-deficient mice leads to marked impairment of glucose tolerance and insulin sensitivity with an enhanced norepinephrine counter-regulatory response to insulin-induced hypoglycemia. We have previously shown that loss of CD14 and TLR4 limits production of IL-6 from visceral and subcutaneous adipocytes; thus, attenuating depot-specific and high fat diet induced inflammation. Following the hypothesis that inflammation drives metabolic dysfunction with glucose intolerance and insulin resistance, we expected that CD14- and TLR4-deficient mice with demonstrated reduction in *ex vivo* IL-6 secretion capability would improve glucose homeostasis. However, our surprising result of impaired glucose homeostasis in these mice contradicts our hypothesis, yet elucidates the importance of global LPS signaling in glucose homeostasis and point to new avenues of inquiry.

The role of CD14 in organisms can serve both pro- and anti-inflammatory functions. In addition to membrane bound forms, CD14 can be released as a soluble protein, typically from the liver, that circulates throughout the vasculature. Soluble CD14 (sCD14) can confer LPS sensitivity to cells that do not normally express CD14, such as endothelial cells. Therefore, sCD14 enhances the pool of LPS-sensitive cell populations, increasing sources of cytokine release, which may be seen generally as pro-inflammatory. However, free sCD14 maintains its ability to bind and sequester LPS in the circulation. LPS-sCD14 complexes are able to donate LPS molecules to high density lipoproteins (HDL) via a reaction catalyzed by LPS binding protein (LBP). It is

estimated that sCD14 amplifies transfer of LPS to HDL by 30-fold, resulting in LPS-laden HDL that can be transported to the liver for elimination.[229] Therefore, sCD14 is a double agent - expanding the population of LPS-sensitive cells as well as sequestering and targeting circulating LPS for elimination.[157,230]

With elimination of both sCD14 as well as membrane-bound (mCD14) in CD14-deficient mice, it is unclear whether a net anti-inflammatory effect prevails. In accord, it is difficult to ascertain the relationship of inflammation with whole body glucose homeostasis in these mice. In our adipocyte studies, it is clear that loss of membrane-bound (mCD14) function results in attenuated IL-6 secretion. However, with an appreciation of various target cells and organs that LPS may affect, an isolated metabolic phenotype would be surprising. Nonetheless, population data in insulin-resistant individuals suggests that serum sCD14 is inversely correlated with fasting insulin – a surrogate for insulin resistance. Therefore, this data suggests that on the complexity level of an organism, it appears that greater sCD14 concentration is associated with improved whole-body insulin sensitivity. This association, though opposite of our experimental animal setup, lends support to a hypothesis that global loss of CD14 may impair insulin sensitivity, inviting future inquiries in the importance of CD14 function in other organs that may shape whole body metabolism.

The adrenal gland is one such example of an organ that may not be a classical insulin-sensitive tissue, but maintains LPS as a potential input and mediates whole body glucose homeostasis as an output. In fact, adrenalectomy in genetically obese *ob/ob* mice

is sufficient to improve hyperglycemia, weight gain, and insulin-induced glucose uptake.[204-206,231-233] Adrenalectomy in insulin-resistant lipoatrophic mice corrects hepatic insulin resistance and enhances muscle glucose uptake, highlighting the contribution of adrenal glands to tissue-specific as well as systemic glucose homeostasis.[234]

Our data is the first to demonstrate that CD14- and TLR4-deficient mice have an enhanced adrenal counter-regulatory response to hypoglycemia – marked by a dramatic increase in the bioactive catecholamine norepinephrine. Consistent with compensatory adrenal hypertrophy seen in previous studies of TLR4-deficient mice, our studies demonstrate adrenal hypertrophy in both CD14- and TLR4-deficient mice.[207] Excess norepinephrine can direct many metabolic factors, including lipolysis, insulin secretion, glucagon secretion, glucose uptake, and hepatic glucose production.[235] It is possible to speculate that excess norepinephrine may mimic pheochromocytoma, which is a neuroendocrine tumor of the adrenal medulla marked by excess production of bioactive catecholamines. In this malady, excess norepinephrine contributes to insulin resistance and hyperglycemia, stemming from increased lipolysis, gluconeogenesis, and glycogenolysis.[236-238] The role of catecholamine-driven counter-regulatory responses in physiological glucose maintenance provides a basis for pathophysiological metabolic changes with alteration in norepinephrine regulation that is seen in our CD14- and TLR4-deficient mice.

It is important to note that adrenal medullary catecholamine secretion is a salient response to reduced glucose availability to the brain, initiating a hyperglycemic response

aimed at restoring glucose supply – the preferential energy source for cells of the central nervous system.[239] Local destruction of spinally-projecting norepinephrine and epinephrine neurons abrogates the hyperglycemic response, suggesting that adrenal secretion is both centrally controlled and essential for elevation of systemic glucose.[239] In addition to reduced glucose supply to the brain, LPS is a prominent stimulus during septic shock, elevating adrenal catecholamines to lower insulin and enhance glucose production – processes interrupted by either adrenal demedullation or guanethidine-induced sympathectomy.[240] Hence, both hypoglycemia and LPS employ central mechanisms to release catecholamines in order to elevate glucose availability. Therefore, it remains possible that exaggerated norepinephrine response to hypoglycemia seen in our CD14- and TLR4-deficient mice may be due to central defects; in particular as brain/neuron insulin receptor specific knockout (NIRKO) mice show diminished norepinephrine responses to insulin-induced hypoglycemia; thus, localizing these responses to central pathways.[241]

As CD14 has additional functions outside LPS signaling (i.e., LPS sequestration and elimination), so is TLR4 purported to mediate other inflammatory signals, such as saturated, but not unsaturated, free fatty acids – an intriguing hypothesis that validates the danger of high saturated fat consumption in the western diet. However, in our *in vitro* studies performed by Melvin Chan, application of the commonly employed saturated free fatty acid palmitate had no stimulatory effect on IL-6 secretion in macrophage or microglial cells (**Appendix 2**). In contrast, IL-6 secretion was observed with activation of both the LPS signaling pathway (CD14/TLR4/MyD88) and the Poly I:C pathway

(TLR2) (**Appendix 2**). In accord, macrophages and microglial cells derived from TLR2-deficient animals lost responsiveness to Poly I:C, while cells derived from CD14-, TLR4-, or MyD88-deficient animals lost responsiveness to LPS (**Appendix 2**). As proper solubilization of free fatty acids in aqueous solutions is challenging, free fatty acid concentration was validated with commercial colorimetric assays, while free fatty acid availability to cells was validated with enhanced oxygen consumption (**Appendix 3**). It is important to note that careful reagent selection is paramount in these studies as acceptable endotoxin levels in bovine serum albumin (BSA) and fetal bovine serum (FBS) routinely used in cell culture maintenance are sufficient for inflammatory activation of these cell types. Although our data do not support saturated free fatty acid signaling through TLR4, we must assume that differences in cytokine production with saturated and unsaturated free fatty acids persist in the context of similar levels of LPS contamination from reagents. Thus, this suggests that saturated free fatty acids may yet potentiate LPS-driven inflammation through the CD14-TLR4 signaling complex, potentially taking advantage of the role of CD14 in phospholipid and LPS cellular influx.[242-246]

It is clear from molecular and nutritional studies that not all fatty acids are created equal; hence, high fat diet selection based on fatty acid profile rather than total calories from fat should be considered. In both our studies, we utilize a diet with a predominance of *cis* unsaturated and polyunsaturated fatty acids (~68% of total fatty acids) in line with a relative fatty acid profile recommended by the USDA Dietary Reference Intake (DRI) guidelines.[247] Furthermore, based on results from the Continuing Survey of Food Intakes

by Individuals (CSFII), ~34% of fat calories consumed by individuals in the United States are derived from saturated fat in contrast to previous mouse studies employing diets with an unrealistic predominance of saturated fatty acids (>90%).[188,247] In order to achieve diets with high saturated fatty acid content, animal sources such as lard, are typically employed. In conversations with industry researchers supplying fats and oils for food manufacture, it is a common belief that animal sources have higher levels of bacteria and bacterial products than vegetable sources. Although in line with government bacteria/endotoxin standards, this provides a significant confounding effect in LPS-related research in animals. Therefore, we must take into account that diets rich in saturated fat may employ animal-based fat sources that could represent higher exogenous sources of LPS/endotoxin. This difference may be reflected in personal communications that suggest that lard-based diets induce glucose intolerance faster in C57Bl/6J mice.

Higher exogenous LPS in saturated fat based diets may explain why *Shi et al.* failed to see changes in glucose tolerance in his male mice. With HFD harboring LPS, HFD fed control mice should become glucose intolerant and insulin resistant. However, TLR4-deficient mice should be protected from this HFD/LPS effect, suggesting either a relative improvement in glucose homeostasis or masking of genotype-specific glucose intolerance as suggested by our studies.[188] This comparison may be further exaggerated by saturated free fatty acid signaling through TLR4. In sum, this may yet have important implications for one's food selection, but the proper correlate should employ more realistic free fatty acid profiles.

As discussed previously in Chapter III, there was improvement in insulin sensitivity in female TLR4-deficient mice; however, this again may be due to high LPS contamination in diets. However, a relative gender effect persists and may be explained by gender differences in adrenal mass. The male C57Bl/6J mouse has enhanced adrenal mass in comparison to female C57Bl/6J, male sv129, and female sv129 mice.[208,209] With our results suggesting altered homeostasis with adrenal excess, we may speculate that female mice may rely less on adrenal contributions. Furthermore, reduced adrenal mass in the sv129 background may be a confounder, suggesting that knockout mice be more extensively backcrossed on to the C57Bl6/J background. In fact, well documented sexual dimorphism in neuroendocrine and inflammatory signaling supports our hypothesis that the intersection of adrenal and LPS signaling pathways with glucose homeostasis provides a strong basis for gender-specific and strain-specific differences in metabolic phenotypes.[248]

FINAL THOUGHTS

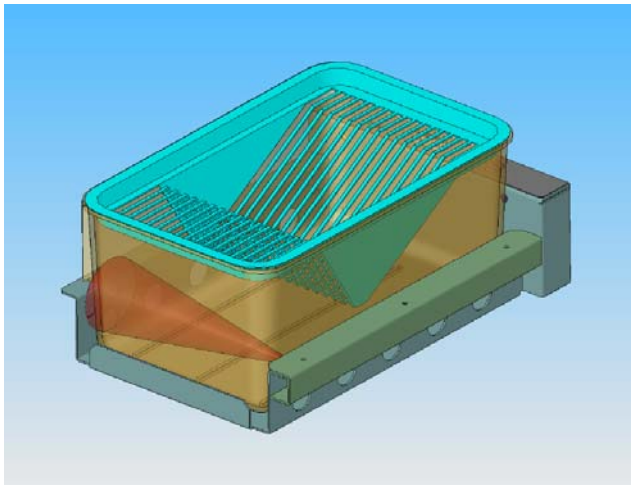
Recent data from the Action to Control Cardiovascular Risk in Diabetes (ACCORD) and the Action in Diabetes and Vascular Disease: Preterex and Diamicron Modified Release Controlled Evaluation (ADVANCE) studies suggest that intensive reduction of glucose (glycated hemoglobin < 6.0% and 6.5%, respectively) failed to reduce major macrovascular events (death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke).[249,250] As the majority of our diabetes patients will eventually succumb to macrovascular complications, this data underscores the need to understand how diabetes beyond hyperglycemia contributes to atherosclerosis and vascular dysfunction. Moreover, emerging data in countries with the highest rate of diabetes development, namely India and China, suggest that ethnic differences in insulin secretion and inflammatory contribution predispose even lean individuals in these populations, calling for a high degree of clinical suspicion as these countries adopt western dietary practices.[251-256] In the United States, October 2007 marked the first of the estimated 78 million baby boomers to be qualified for social security, representing the aging of our population and calling for an expansion of our therapeutic armamentarium. Therefore, as the global burden of diabetes, obesity, and their concomitant macrovascular sequellae intensifies, multiple avenues of therapy must be investigated.

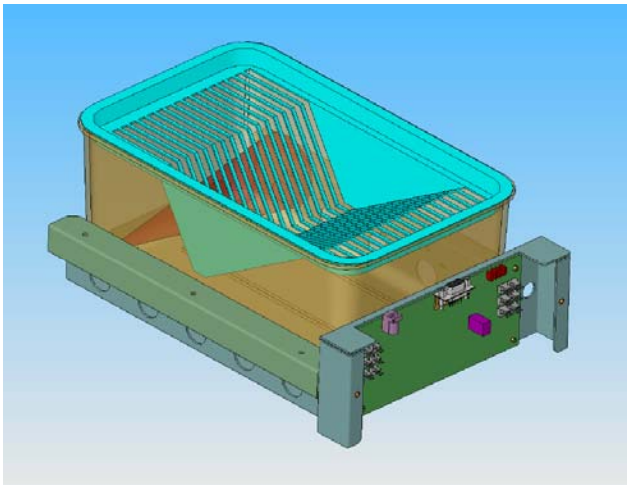
Further understanding and dissection of the complex crosstalk between parenchymal cells, vascular cells, circulating cells, and the central nervous system will continue to represent a challenge in this orchestra of interactions, but also ensures that

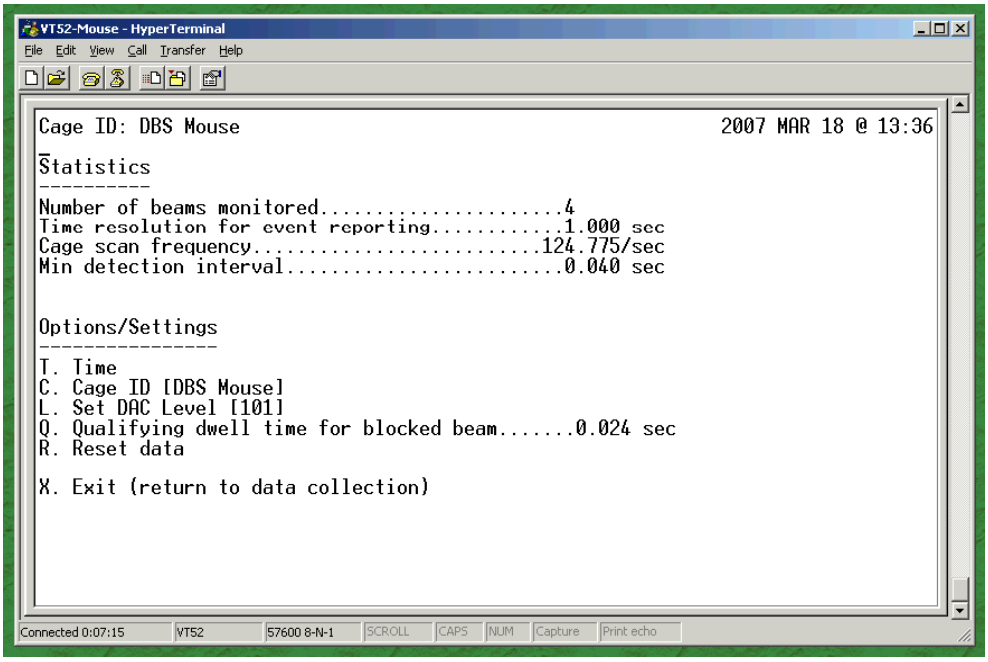
scientific inquiry no matter how narrowly defined contributes to our base of knowledge and is readily applicable to multiple fields. The complex interplay of the human body also serves as opportunities where properly targeted therapeutic strategies will one day be able to modify human disease and we can truly go from bench to bedside. It is always with the small pebble in a pond that science is both daunting and exhilarating, questions multiply faster than answers, and a thesis is never finished.

Appendices

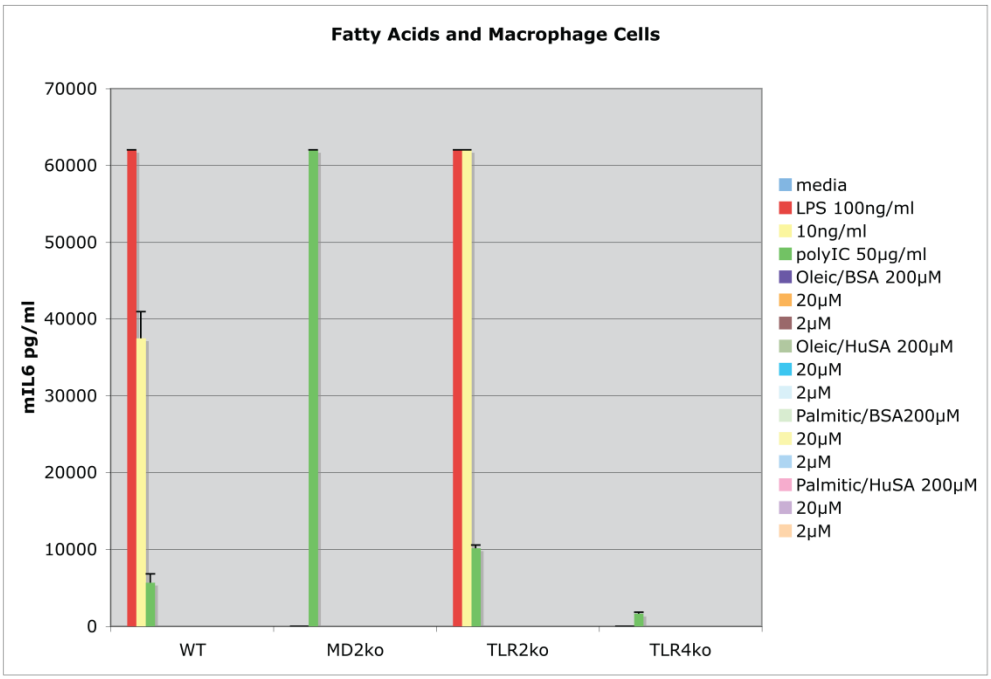
Mouse Activity Monitor







Appendix 1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10ng/ml
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20µM
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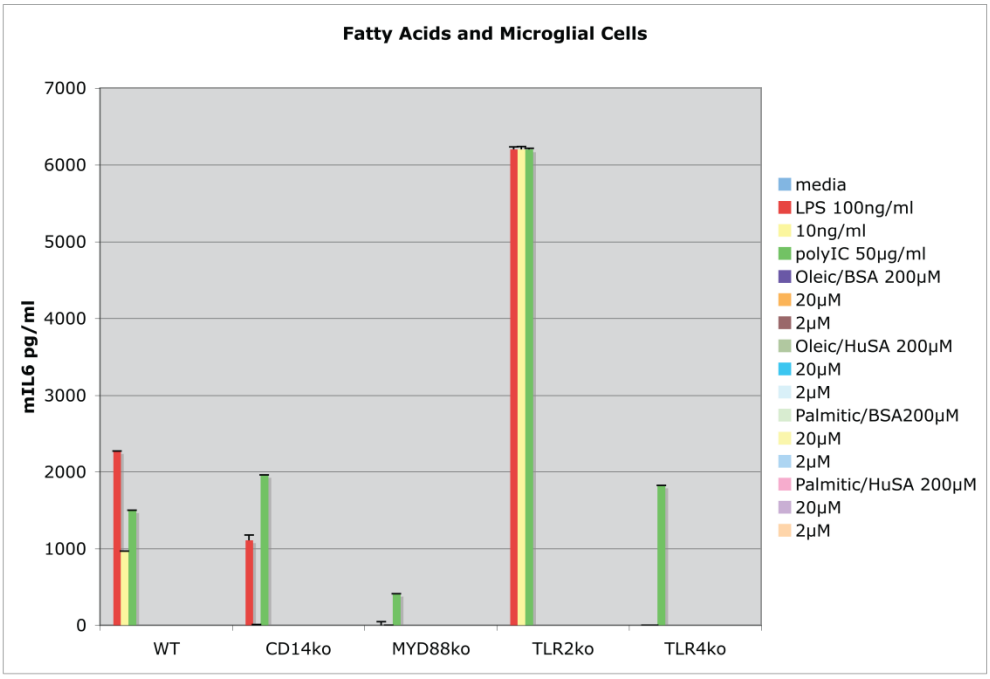
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20µM
2µM
Oleic/HuSA 200µM
20µM
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Palmitic/HuSA 200µM
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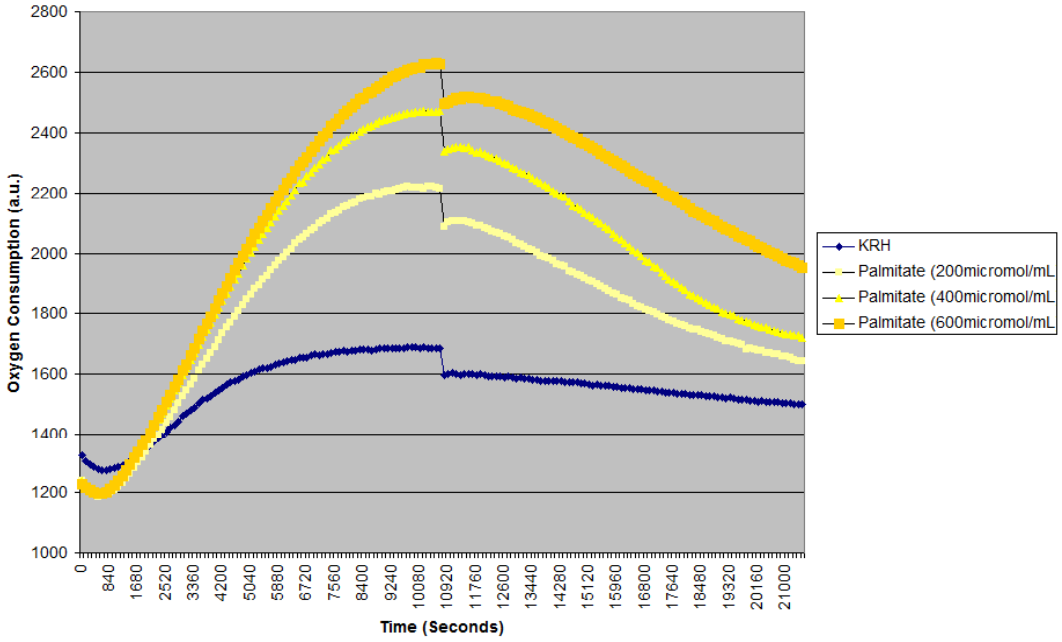
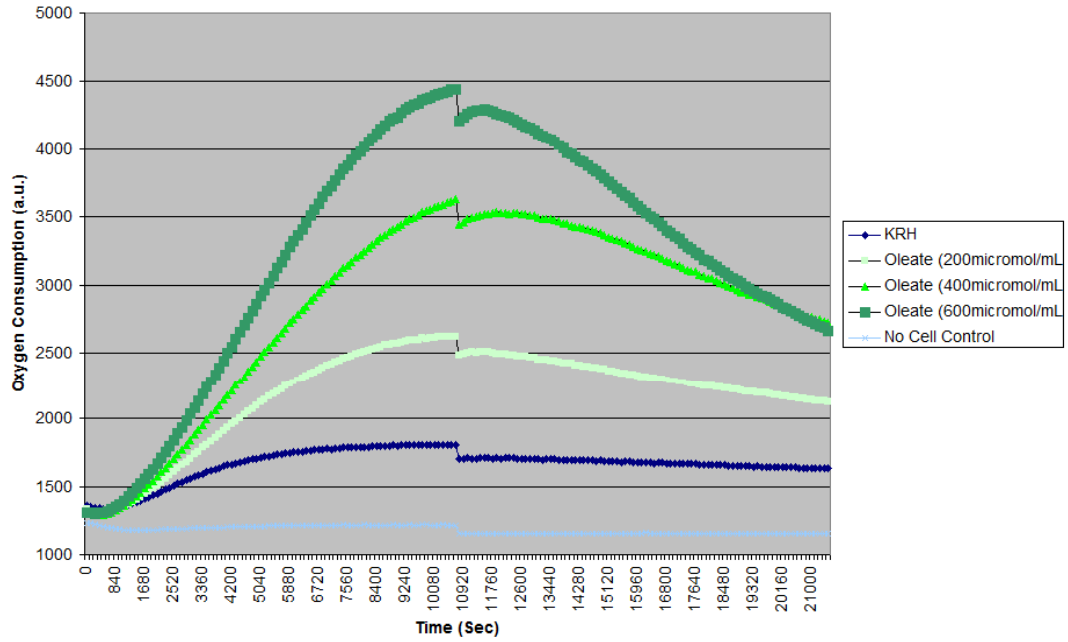
2µM

Appendix 2

Palmitic Acid – BSA Conjugated



Oleic Acid – BSA Conjugated



Appendix 3

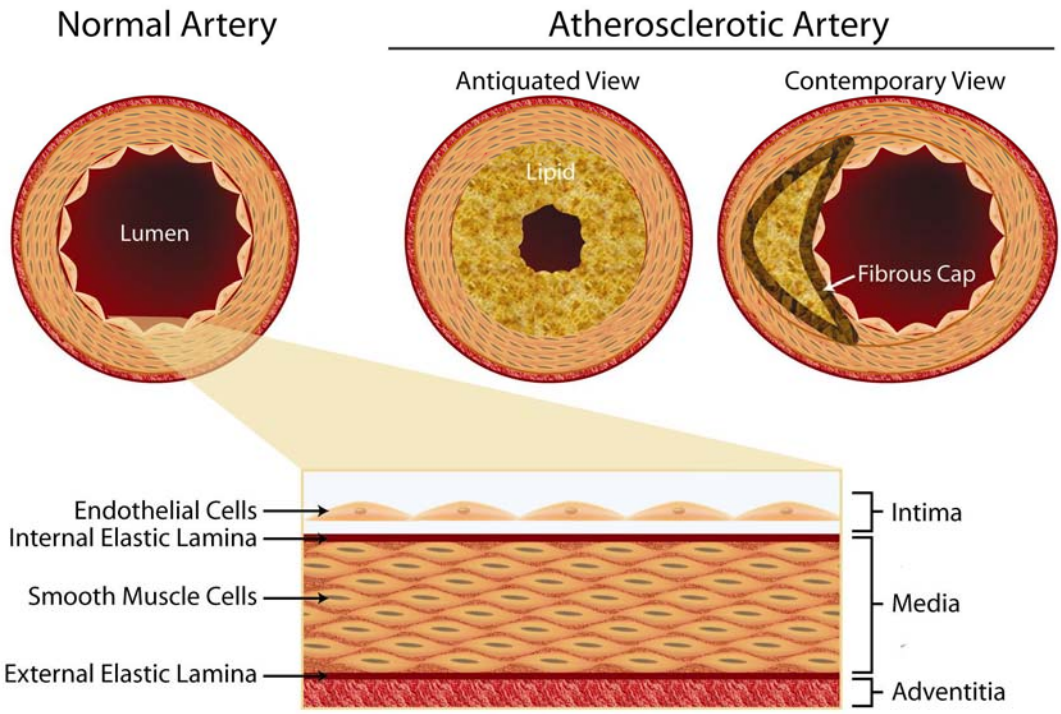
Supplement

Cells of the Vasculature – Lessons from Atherosclerosis

Using atherosclerosis as a framework to discuss vascular inflammation and injury, we will draw on mechanisms of vascular dysfunction with immune cell chemotaxis to explain likely correlates with the vasculature of insulin-sensitive peripheral tissues. In brief, the antiquated view of atherosclerosis as a gradual process of lipid accumulation is no longer favored. *In lieu*, atherosclerosis is seen as an active cellular process that may progress to form advanced plaques, which are composed of a highly thrombotic core shielded by a fibrous cap, hence protecting the circulation from acute thrombotic events (**Supplement, Figure 1**). This degree of plaque formation is not currently believed to take place in the larger vessels supplying insulin-sensitive peripheral tissues, but the principle of vascular dysfunction leading to endothelial damage, increase in cellular adhesion molecules, and ensuing recruitment of leukocytes are emerging concepts in adipose inflammation

Normal Arterial Wall

Far from an inert conduit, the living arterial wall hosts dynamic interchange between its cellular residents, most importantly endothelial cells, vascular smooth muscle cells, and their surrounding extracellular matrix (**Supplement, Figure 1** ***inset***).



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Supplement, Figure 1: The Vascular Wall

The arterial wall consists of three layers (**Fig. 1.12, *inset***): the **intima,** closest to the arterial lumen and therefore most "intimate" with the blood; the **media,** which is the middle layer; and the outer **adventitia.** The intima consists of a single layer of endothelial cells that acts as a metabolically active barrier between circulating blood and the vessel. The media is the thickest layer of the normal arterial wall. Boundaries of elastin, known as the internal and external elastic laminae, separate this middle layer from the intima and adventitia, respectively. The media, composed of smooth muscle cells and extracellular matrix, subserves the contractile and elastic functions of the vessel. The adventitia, the outer lining of the artery, contains the nerves, lymphatics, and blood vessels (vasa vasorum) that nourish the cells of the arterial wall.

Understanding the dysfunction that leads to disease requires knowledge of the normal function of these components.

Endothelial Cells

In a healthy artery, the endothelium serves structural, metabolic, and signaling functions that maintain the homeostasis of the vessel wall. The tightly adjoined endothelial cells form a barrier that contains circulating blood within the lumen of the vessel and limits the passage of large molecules from the circulation into the subendothelial space.

The endothelium expresses cell-surface and circulating antithrombotic molecules (e.g., heparan sulfate, thrombomodulin, and plasminogen activators, prostacyclin and nitric oxide [NO]), favoring a net anticoagulant state; however, the endothelium can also produce prothrombotic molecules when stressed (see below).

Endothelial cells in post-capillary venules respond to local injury or infection by secreting immune cell recruiting chemicals known as chemokines, while enhancing expression of cell-surface adhesion molecules that serve to anchor mononuclear cells to the endothelium and facilitate migration to the site of tissue injury. In the absence of such pathologic stimulation, healthy arterial endothelial cells *resist* leukocyte adhesion. However, under adverse influences, endothelial cells actually *recruit* leukocytes to the vessel wall. In sum, the normal endothelial layer provides a protective non-thrombogenic surface with homeostatic vasodilator and anti-inflammatory properties (**Figure 1.12A**).

Vascular Smooth Muscle Cells

Smooth muscle cells within the vessel wall is best known for their contractile function, responding to various vasoactive substance derived from circulating agents, nerve terminals, or overlying endothelium. However, vascular smooth muscle cells also synthesize the collagen, elastin, and proteoglycans that form the vascular extracellular matrix (**Figure 1.12A**). Similar to a normal injury response (e.g. scar formation), the synthetic functions of smooth muscle cells become more prominent and may contribute to its pathogenesis as will be outline below in the *Activated Arterial Wall* .

Activated Arterial Wall

As the normal arterial wall is a dynamic and regulated system, noxious elements such as inflammatory mediators can corrupt normal homeostasis, and pave the way for local inflammation. Vascular cells, such as endothelial and smooth muscle cells, respond readily to the prototypical inflammatory mediators interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα). Surprisingly, these inflammatory agents could also *activate* vascular cells to produce IL-1 and TNFα themselves, in contrast with past dogma that solely cells of the immune system produced these cytokines. Without a reliance on immune cells as a source of pro-inflammatory agents, investigation into the role of "activated" endothelial and smooth muscle cells in atherogenesis burgeoned similar to the current direction that adipocyte-derived inflammation in diabetes pathogenesis has progressed (**Fig 1.12B**).

This fundamental research identified several key stimuli and components that cultivated the vascular inflammatory process and has potential significance in moderating adipose inflammation, including concepts such as: 1) endothelial dysfunction, 2) accumulation of modfied lipids within the intima, 3) recruitment of leukocytes to the vessel wall, 4) and macrophage accumulation and activation.

Endothelial Dysfunction

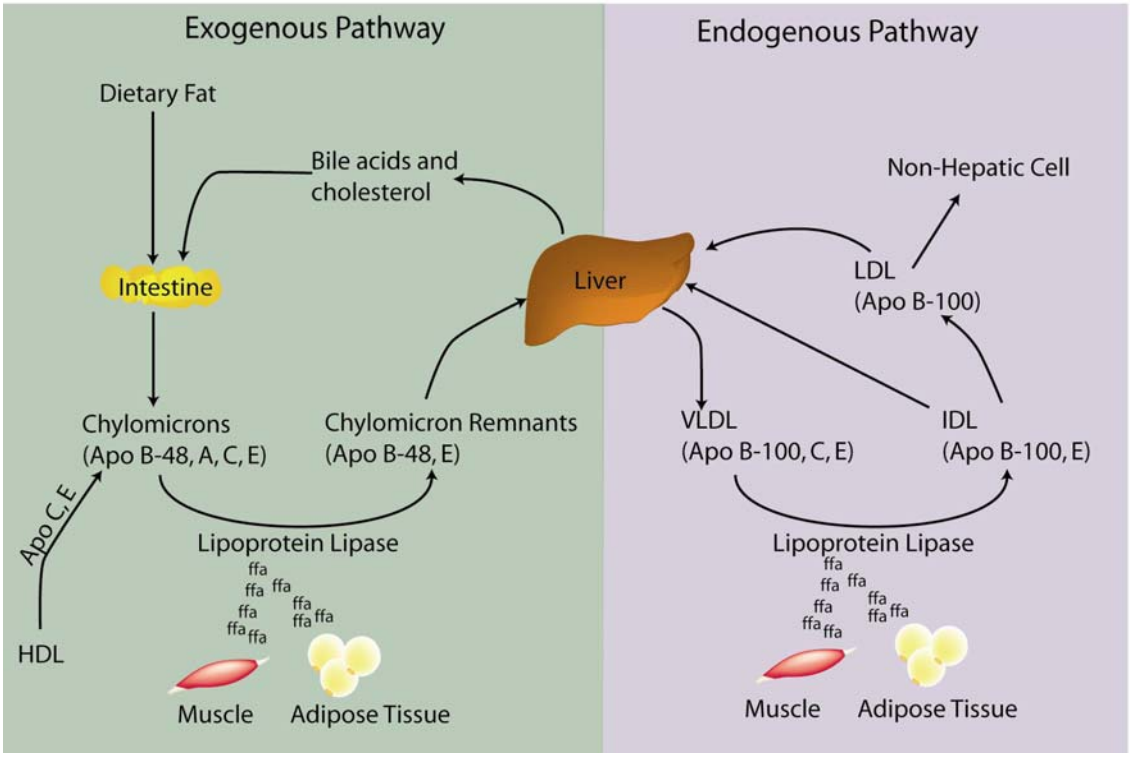
Endothelial dysfunction may also result from exposure to a "toxic" chemical environment. For example, cigarette smoking, abnormal circulating lipid levels, or diabetes—all known risk factors for atherosclerosis—can promote endothelial dysfunction. Recent studies indicate that each of these states increases endothelial production of reactive oxygen species. These substances, notably superoxide anion, interact with other intracellular molecules to influence the metabolic and synthetic functions of the endothelial cell, skewing the cell to pro-inflammatory processes.

Physical and chemical *stressors* corrupt normal endothelial homeostasis, creating an activated state manifested by 1) impairment of the endothelium's role as a permeability barrier, 2) release of inflammatory cytokines, 3) increased production of cell-surface adhesion molecules that recruit leukocytes, 4) altered release of vasoactive substances (e.g., prostacyclin and NO), and 5) interference with normal antithrombotic properties. These undesired effects of endothelial dysfunction lay the groundwork for subsequent events in the development of local inflammation (Fig 1.12B).

Lipoprotein Entry and Modification

The activated endothelium no longer serves as an effective barrier to the passage of circulating lipoproteins into the arterial wall (**Supplement, Figure 2** summarizes the major lipoprotein pathways). Increased endothelial permeability allows the entry of low density lipoprotein (LDL) into the intima where accumulation is enhanced by binding to proteoglycans. This "trapping" increases the residence time of LDL within the vessel wall, where the lipoprotein may undergo pro-inflammatory chemical modifications. Hypertension, a major risk factor associated with diabetes and a component of the metabolic syndrome, promotes retention of lipoproteins in the intima by accentuating the production of LDL-binding proteoglycans by smooth muscle cells.

Oxidation is one type of modification that befalls LDL trapped in the subendothelial space. It can result from the local action of reactive oxygen species and pro-oxidant enzymes derived from activated endothelial or smooth muscle cells, or from macrophages that penetrate the vessel wall. In diabetic patients with sustained hyperglycemia, *glycation* of LDL may also occur, a derivatization that may ultimately render LDL antigenic and pro-inflammatory. These biochemical modifications of LDL act early with initial endothelial dysfunction, but likely promote the persistence of inflammation throughout diabetes. In early endothelial dysfunction and likely throughout vascular inflammation, modified LDL (mLDL) promotes **leukocyte recruitment** and **macrophage accumulation and activation**.



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Supplement, Figure 2: Lipoprotein Transport System

Lipoproteins ferry water-insoluble fats through the bloodstream. These particles consist of a lipid core surrounded by more hydrophilic phospholipid, free cholesterol, and apolipoproteins (also called apoproteins). The apoproteins present on different classes of lipoprotein molecules are the "conductors" of the system, directing their lipoprotein payload to specific tissue receptors and also mediating enzymatic reactions essential for proper distribution.

Five major classes of lipoproteins exist, distinguished by their densities, lipid constituents, and associated apoproteins: chylomicrons, very–low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The exogenous and endogenous lipoprotein pathways are shown above.

Leukocyte Recruitment

Recruitment of leukocytes, primarily monocytes and T lymphocytes, to the vessel wall requires: 1) luminal expression of leukocyte adhesion molecules (LAM) to the normally non-adherent endothelial surface, and 2) chemoattractant signals (e.g., MCP-1, IL-8, IP-10) to direct diapedesis into the subintima. Two major subsets of LAM persist in the inflamed vessels: the immunoglobulin gene superfamily (particularly vascular cell adhesion molecule-1 [VCAM-1] and intercellular adhesion molecules-1 [ICAM-1]) and the selectins (particularly E- and P-selectin).

Indeed, *ex vivo* monocyte adhesion studies using aortic endothelial cells from the db/db mouse demonstrated enhanced monocyte adhesion via ICAM-1 and VCAM-1, suggesting that diabetic endothelium may be pre-activated.[257] Many relevant agonists related to diabetic physiology have been shown to upregulated leukocyte adhesion molecules, including high glucose exposure, high fat diet, and AGE.[258-260] Beyond these agonists that may work through common ROS reactions, adipokines may also play a direct role as resistin induces EC activation (as gauged by endothelin release) with coordinate expression of VCAM-1 adhesion molecules and the MCP-1 chemokine. [261] Whereas, adiponectin attenuates both resistin- and TNFα- mediated expression of endothelial ICAM-1, VCAM-1, and E-Selectin.[262,263] Furthermore, adiponectin-deficient animals demonstrate enhanced *in vivo* leukocyte adhesion visualized by intravital microscopy.[264] Modified LDL (mLDL) and pro-inflammatory cytokines (e.g., IL-1, TNFα) can induce LAM and chemokine expression independently; however, mLDL also

potently stimulates endothelial- and smooth muscle-derived pro-inflammatory cytokines, thus reinforcing any direct action. This dual ability of mLDL to promote inflammation directly or indirectly via cytokines is likely a persistent concept in the diabetic patient, thus making it difficult to parse out lipid and non-lipid effects.

Macrophage Accumulation and Activation

Identification that macrophages accumulate in adipose tissue commensurate with weight gain generated new interest in the macrophage as a potent source of inflammatory stimuli.[265-267] Engendering interest in macrophage-derived inflammatory signals in adipose, it has been found that macrophage chemokine signaling is enhanced with adiposity, while interruption in chemokine signaling, such as in the CCR2-MCP-1 receptor-ligand pair, results in diminished macrophage infiltration and improved insulin sensitivity.[148,268,269] Demonstrating enhanced MCP-1 content in high fat diet fed mice, *Kanda et al.* further observed that loss of MCP-1 through genetic knockouts and expression of dominant-negative mutants were able to reduce insulin resistance and hepatic steatosis in both diet and genetic models of obesity.[268] Conversely, overexpression of MCP-1 in adipose tissue under the aP2 promoter was sufficient to induce insulin resistance with enhanced hepatic triglyceride content.[268]

However, presence alone may not predict pathogenicity as adipose-resident macrophages can be “alternatively activated” and “classically activated”, which differ in their pro-inflammatory potential [270,271] In fact, it is believed that macrophage recruitment to atherosclerotic sites is intended to be beneficial with the ability of macrophage to

imbibe excess lipids. However, lipid overload leads to macrophage conversion to pro-inflammatory foam cells. Foam cells cannot form from uptake of LDL through the "classical" LDL-receptor, as intracellular cholesterol exquisitely suppresses the expression of this pathway. Also, the "classical" LDL-receptor does not recognize mLDL. Therefore, macrophages rely on a family of "scavenger" receptors that preferentially bind and internalize mLDL. However, unlike classical LDL receptors, macrophages that ingest mLDL through scavenger receptors evade negative feedback inhibition and permit engorgement of the cell as it fills with the cholesterol-rich lipid. Such lipid-laden scavenger cells, also known as "foam cells," may initially serve a salutary role by sequestering potentially pro-inflammatory mLDL constituents and removing them from the plaque. However, impaired efflux of foam cells may lead to local accumulation and persistence, mitigating the foam cells' protective role as the "clean-up crew" and instead becoming active sources of pro-inflammatory cytokines. The formation of foam cells in adipose tissue has not been identified; however, given the surplus of mLDL either from excess oxidation or glycation in the diabetic patient, it should be an attractive area of study in macrophage activation and its role in adipose inflammation.

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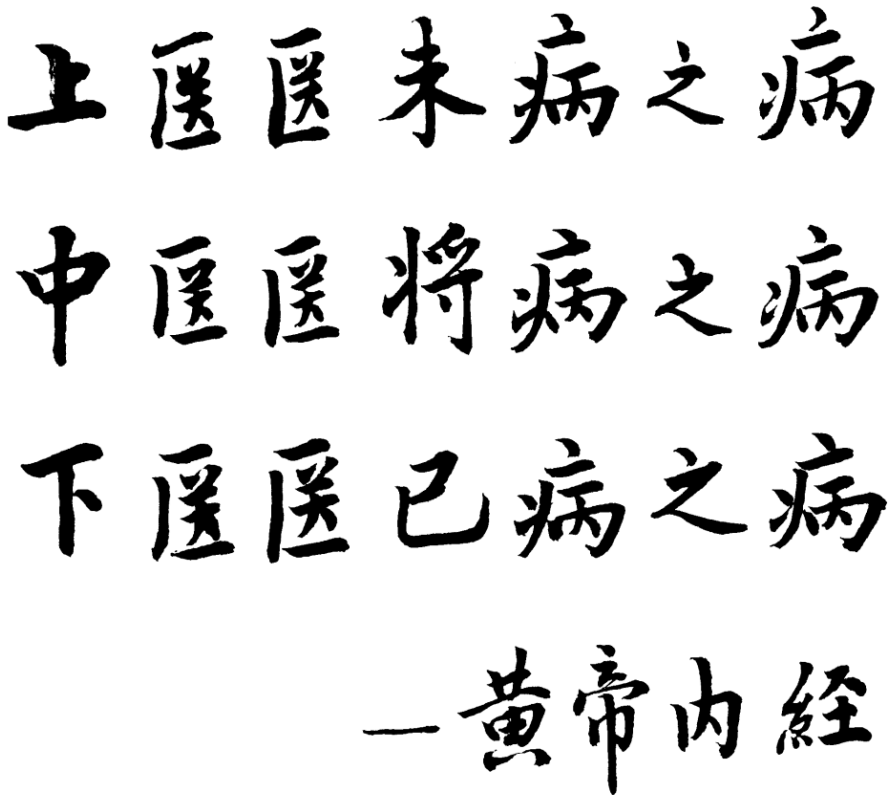

The superior doctor prevents disease;
The mediocre doctor attends to impending disease;
The inferior doctor treats full-blown diseases.

- Huang Di Neijin

Calligraphy by Ge Jiyun, wife of Han Xu, former Chinese ambassador to the US