Immunomodulatory Pathways and Metabolism

Immunomodulatory pathways and metabolism

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Prerna Bhargava

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ABSTRACT

Energy metabolism plays a vital role in normal physiology, adaptive responses and host defense mechanisms. Research throughout the last decade has shown evidence that immune pathways communicate with metabolic pathways to alter the metabolic status in response to physiological or pathological signals. In this thesis, I will explore how immunomodulatory molecules affect metabolic homeostasis and conversely, how metabolic sensing pathways modulate immune responses. The first part my work utilizes an immunomodulatory sugar motif to determine mechanisms by which immune cells influence metabolism. Specifically, I show in chapter 2 that lacto-N-fucopentaose III (LNFPIII), a motif used by pathogens to attenuate inflammation, is capable of improving systemic insulin sensitivity by increasing Il-10 production in macrophages and dendritic cells and subsequently improving white adipose tissue insulin sensitivity. Chapter 3 will address the observation that this same glycan is capable of directly activating Fxrα in hepatocytes. This direct effect manifests as a reduction in high-fat-diet-induced hepatic triglyceride accumulation and improvement in liver function. Lastly, in chapter 4, I will discuss the role of metabolic regulators in the macrophage and how this affects the ability of the macrophage to kill bacteria. Specifically, I will show that lipid sensing nuclear receptors, such as Pparδ and Pparγ, are critical regulators of phagosomal function and bacterial killing. Macrophage-specific deletion of these receptors prevents efficient killing of *Streptococcus pneumonia*e, the causative bacterium in many cases of respiratory pneumonia.

Ligand activation improves survival, suggesting a potential therapeutic role for Ppar activation during infection. Taken together, all the data suggest a critical role for the evolutionary interaction between metabolic and immune pathways. These interactions may be important when developing new therapeutics for complex metabolic and immunological dysfunctions.

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List of Abbreviations

Acc –Acetyl-CoA carboxylase

AIR – Autoinhibitory region

Alox5 – Arachidonate 5-lipoxygenase

ALT –Alanine AminotransferaseArg1 – Arginase 1

AP1 – Activator protein 1

aP2 – Adipocyte Protein 2/ Fatty Acid Binding Protein 4

Apoe – Apolipoprotein E

Asc – Apoptosis-associated Speck-like Protein Containing a Caspase Recruitment Domain

AST –Aspartate Aminotransferase

Atf6 – Activating Transcription Factor 6

ATM – Adipose Tissue Macrophage

ATP – Adenosine Triphosphate

AUC – Area under the curve

Balf – Bronchoalveolar Lavage Fluid

BAT – Brown adipose tissue

Bsep – Bile Salt Export Pump

Casp1 – Caspase 1

Ccl2 – Chemokine (C-C motif) Ligand 2, also known as Mcp1

Ccr2 – Chemokine (C-C motif) receptor 2

Cd36 – Cluster of Differentiation 36

C/ebpα – CCAAT enhancer binding protein a

CFU – Colony forming units

CGD – Chronic granulomatous disease

Chop – CCAAT/-enhancer-binding protein homologous protein

Chrebp – Carbohydrate response element binding protein

Ck – Creatine kinase

CLR – C type lectin receptor

CM – Conditioned medium

Cox1 – Cytochrome c oxidase subunit 1

Cox2 – Cytochrome c oxidase subunit 2

Cxcl14 – Chemokine (C-X-C motif) ligand 14

DC-SIGN – Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DHA – Docosahexanoic Acid

ECM – Extracellular Matrix

EPA – Eicosapentanoic Acid

ER – Endoplasmic Reticulum

Erk – Extracellular signal-related kinase

FACS – Fluorescence activated cell sorting

Fas –Fatty acid synthase

FITC – Fluorescein isothiocyanate

Fxr – Farnesoid X Receptor, Nr1h4

Glut4 – Glucose transporter type 4, Slc2a4

Gpcr – G-protein coupled receptor

GTT – Glucose tolerance test

HFD – High fat diet

Hif-1α – Hypoxia Inducible Factor -1a

HOMA-IR – Homeostatic model of assessment – Insulin resistance

Ifnγ – Interferon Gamma

Ikk – IkB Kinase

Il-1β – Interleukin 1b

Il-4 – Interleukin 4

Il-10 – Interleukin 10

Il-13 – Interleukin 13

Il-18 – Interleukin 18

InsR – Insulin Receptor

Ire1 – inositol-requiring enzyme 1, also known as endoplasmic reticulum to nucleus signaling 2 (Ern2)

Irs1 – Insulin receptor substrate 1

Irs2 – Insulin receptor substrate 2

ITT – Insulin tolerance test

Jnk – c-Jun N-terminal kinase, also known as mitogen-activated protein kinase 8 (Mapk8)

Ldh – Lactate dehydrogenase

LDL-c – Low-density lipoprotein cholesterol

Ldlr – Low density lipoprotein receptor

LNFPIII – lacto-N-fucopentaose III

LPS – Lipopolysaccharide

Lxrα – Liver X receptor α, Nr1h3

Lxrβ – Liver X receptor β, Nr1h2

Mcp1 – Macrophage Chemoattractant Protein 1, also known as Ccl2

MIRKO – Muscle insulin receptor knockout mice

Mgl1 – macrophage galactose-type C-type lectin 1, also known as Cd301 or Clec10a

mTOR – Mammalian Target of Rapamycin

NAD – Nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate-oxidase

NAFLD – Nonalcoholic fatty liver disease

Nf-κb – Nuclear Factor κb

Nlrp3 – NLR family, pyrin domain containing 3

NO – Nitric Oxide

Nos2 – Inducible Nitric Oxide Synthase/iNOS

Nox – NADPH Oxidase

Noxa1 – NADPH oxidase activator 1

Noxo1 – NADPH Oxidase Organizer 1

Oatp – Organic anion transporting polypeptide, also known as Slco1b2

Ox-LDL – Oxidized LDL

Perk – protein kinase RNA-like endoplasmic reticulum kinase, also known as Eif2ak3

Pfk1 – Phosphofructokinase 1

Pfkfb1 – 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1, also known as Pfk2

Pgd – Phosphogluconate dehydrogenase

PIP_3 – Phosphatidylinositol (3,4,5)-triphosphate

Pkr – Double Stranded RNA-dependent Protein Kinase

Pltp – Phospholipid transfer protein

Pparα – Peroxisome Proliferator Activator Receptor α, also known as Nr1c1

Pparδ – Peroxisome Proliferator Activator Receptor δ, also known as Nr1c2

Pparγ – Peroxisome Proliferator Activator Receptor γ, also known as Nr1c3

$Pparδ^{fl/fl}$ – Macrophage specific Pparδ floxed mice

PparδKO – Pparδ knockout stable cells

$Pparδ^{LysM}$ – Macrophage specific Pparδ knockout mice

$Pparγ^{fl/fl}$ – Macrophage specific Pparγ floxed mice

PparγKO – Pparγ knockout stable cells

Pparγ^{LysM} – Macrophage specific Pparγ knockout mice

Pparδγ$^{fl/fl}$ – Macrophage specific Pparδγ floxed mice

PparδγKO – Pparδγ knockout stable cells

Pparδγ^{LysM} – Macrophage specific Pparδγ knockout mice

PPP – Pentose phosphate pathway

Ptgs1 – Prostaglandin-endoperoxide synthase 1, also known as Cox1

Ptgs2 – Prostoglandin-endoperoxide synthase 2, also known as Cox2

PX – Phosphoinositide binding domain

ROS – Reactive oxygen species

Scd –Steroyl-CoA desaturase

SEA – Soluble egg antigen

Shp – Small heterodimer partner

Sr-a – scavenger receptor type 1

Srebp – Sterol regulatory binding protein

Stat6 – Signaling transducer and activator of transcription 6

SVF – Stromal vascular fraction

Tlr – Toll-like Receptor

Tnfα – Tumor necrosis factor α

uPFK2 – 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, also known as Pfkfb3

UPR – Unfolded Protein Response

Vhl – Von-Hipple-Lindau tumor suppressor protein

WAT – White Adipose Tissue

WT – wildtype

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The contributions from publications contained within this thesis are acknowledged below:

P.Bhargava, Lee, C-H. Role and function of macrophages in the metabolic syndrome. Biochemical Journal. 2012, 442: 253-262. ©The Biochemical Society

P.Bhargava, Li, C., Stanya, K.J., Jacobi, D., Dai, L., Liu, S., Gangl, M.R., Harn, D.A., Lee, C-H. Immunomodulatory glycan LNFPIII alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways. Nature Medicine. 2012, 18(11): 1665-72.

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Chapter 1

INTRODUCTION

The innate immune system, which consists of macrophages, dendritic cells, and a variety of other effector cells, has evolved as a formidable defense against external threats to the human host. When first described by Elie Metchnikoff in the late 1800s, macrophages were thought to primarily function as phagocytes. In the next several decades, intense effort went into understanding the mechanisms that led to macrophage activation. It was not until about 100 years later that researchers first started to document the innumerable roles that macrophages play[1]. We now know that macrophages are found throughout the body and are involved in tissue homeostasis, wound healing, general surveillance of host threats, and as was first observed, killing of foreign pathogens.

Abundant sources of food that have high energy content and are enriched in saturated fats, coupled with a lack of physical activity, are responsible for the sharp increase in a collection of pathologies, such as obesity, hepatosteatosis, insulin resistance and atherosclerosis, together known as the metabolic syndrome. All these pathologies increase the chance of developing cardiovascular disease, diabetes mellitus, and premature death[2].

The observation that anti-inflammatory salicylates could improve insulin sensitivity and glucose responsiveness gave the first indication that metabolic syndrome is associated with low-grade chronic inflammation[3,4]. This was followed by reports that inflammatory cytokines, such as tumor necrosis factor α (Tnfα), inhibit insulin responsiveness in adipocytes, providing a link between inflammation and metabolic pathways[5-7]. Subsequent studies have suggested that lipid overload triggers the immune system, especially macrophages, to respond in a deleterious manner, in which a resolution state could not be achieved[8,9]. At around the same time when these studies were occurring, researchers observed that insulin resistance and hyperglycemia lead to dysregulated immune responses and increased incidences of infection in patients with the

metabolic syndrome[10-17]. Together, it has become evident that metabolic pathways are tightly intertwined with inflammatory signaling and immune responses and play an integral role in their respective functions.

As environmental sentries, it is not surprising that macrophages sense and regulate metabolic homeostasis. This link between macrophages and metabolic regulation was further strengthened with the discovery of resident macrophage populations in metabolically active tissues, such as white adipose tissue and liver[18,19]. The interplay between resident macrophages and pathologies associated with the metabolic syndrome has been studied extensively since.

AN ESSENTIAL ROLE FOR MACROPHAGES IN METABOLIC PATHOLOGIES

Resident macrophages in different tissues adapt to their local microenvironment and exhibit diverse functional and morphological phenotypes. Resident populations, such as Kupffer cells in the liver, resident macrophages in white adipose tissue (WAT) and alveolar macrophages in the lung, show expression profiles distinct from those of recruited macrophages responding to systemic cues[1]. For example, studies have demonstrated that adipose tissue resident macrophages (ATMs) are functionally distinct from infiltrating macrophages (discussed below). In the process of understanding the etiology of metabolic syndrome, we have begun to understand the general mechanisms underlying metabolic diseases, particularly the roles of macrophages in atherosclerosis and in adipose tissue insulin resistance. Due to lack of specific markers for resident and recruited macrophages and the crosstalk between tissues in the progression of metabolic diseases, it is often difficult to tease apart the precise contribution of individual macrophage populations to the onset and development of systemic metabolic dysfunction. Nonetheless, it is clear that the interaction between macrophages and metabolic cells plays a key

role in disease pathogenesis. Macrophages are recruited by proinflammatory cytokines and chemokines to deal with lipid overload-induced tissue dysfunction and are also subject to the detrimental effects of lipid influx. As such, metabolic stress initiates a feed-forward cycle of inflammatory responses resulting in a state of unresolved, chronic inflammation with macrophages as major culprits contributing to metabolic diseases (**Fig 1**).

Macrophage infiltration and activation

Recruitment: The production of chemokines, the factors that recruit immune cells to sites of trouble, is increased in states of metabolic dysfunction. Monocyte chemoattractant protein 1 [Mcp1, also known as chemokine (C-C motif) ligand 2 (Ccl2)] and its receptor Ccr2 are the most studied chemokine signaling molecules in metabolic diseases. Earlier studies have demonstrated that Mcp1 and Ccr2 gene knockout mice are protected from atherosclerosis[20-22]. Ccr2 knockout mice also display attenuated macrophage accumulation and chronic inflammation in adipose tissue[23]. In addition, mice lacking Mcp1 or expressing a dominant negative form of Mcp1 show improved insulin sensitivity[24], whereas transgenic Mcp1 overexpression in adipose tissue causes insulin resistance and hepatic steatosis[24,25]. Other chemokines, such as osteopontin, angiopoietin-like protein 2, and chemokine (C-X-C motif) ligand 14 (Cxcl14) have also been shown to play a role in obesity induced macrophage recruitment[26-29]. These studies demonstrate that macrophage infiltration is an essential step for metabolic disease pathogenesis.

Activation: Macrophages respond to many different types of foreign and host derived stimuli and exhibit very precise and coordinated activation states based on the signals they receive. These states lie on a spectrum ranging from the pro-inflammatory state to the anti-inflammatory state

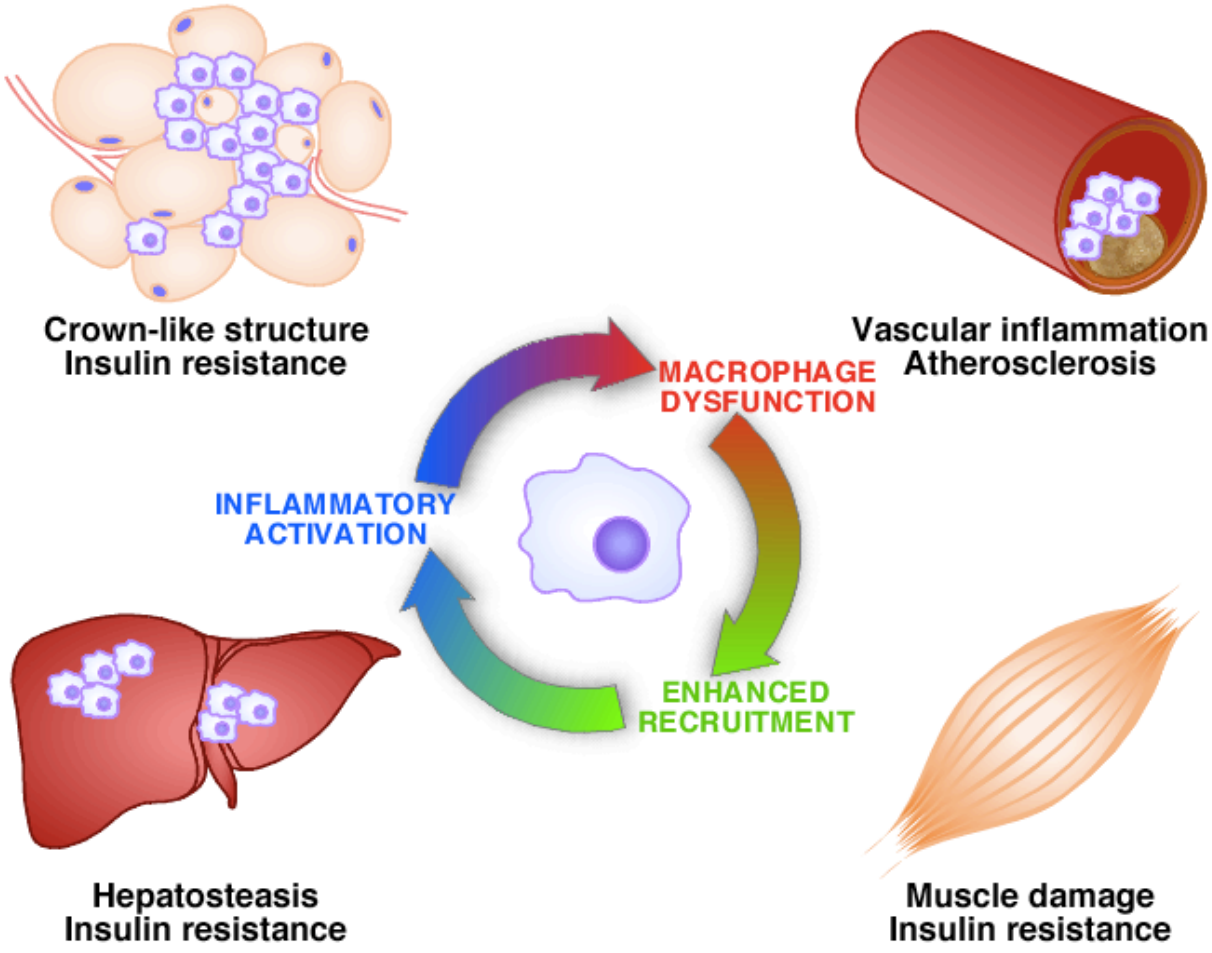


Figure 1: Metabolic dysregulation leads to a feed-forward cycle of chronic inflammation. Activation of inflammatory pathways by metabolic stress leads to macrophage recruitment to sites of affected tissues, such as vasculature, white adipose tissue, muscle and liver. There, they accumulate excess lipids and cell debris in an attempt to restore homeostasis. The presence of persistent pro-inflammatory stimulants (e.g., fatty acids and cholesterol) causes macrophage dysfunction, including defective efferocytosis and unresolved inflammation, resulting in recruitment and activation of more macrophages. In white adipose tissue, infiltrated macrophages are concentrated around the dying fat cells, known as the crown-like structure. In vasculature, macrophages are enriched in areas of fatty streaks. Inflammatory cytokines produced by activated macrophages induce insulin resistance in major metabolic tissues, promote hepatosteatosis and facilitate foam cell and plaque formation in the vessel wall.

(**Fig.2**)[30]. Foreign pathogens or T_H1 cytokines [e.g., Tnfα, interleukin 1β (Il-1β) and interferon γ (Ifnγ)] transduce signals that activate macrophages to a pro-inflammatory phenotype, typically referred to as an M1 response, characterized by the expression of inflammatory markers, such as Tnfα, Ifnγ and nitric oxide synthase 2 (Nos2, also known as iNOS)[30]. In contrast, M2 macrophages, induced by T_H2 cytokines Il-4 and Il-13, produce anti-inflammatory mediators, notably Il-10[30]. As will be discussed below, the M1/M2 paradigm also applies to metabolic regulation, with M1 inducing and M2 preventing metabolic diseases, respectively (**Fig. 3**). Interestingly, dietary fatty acids are able to polarize macrophages toward M1 or M2 activation states, depending on the signaling molecules with which they interact (**Fig. 2**), thus providing a molecular basis for the crosstalk between metabolic and inflammatory pathways.

Resolution/deactivation: The initial pro-inflammatory response is terminated by anti-inflammatory cytokines, such as Il-10, which is induced by both M1 and M2 cytokines to deactivate macrophages and promote resolution[31-34]. Lack of this termination signal leads to establishment of the inflammatory response[35-37]. Epidemiological studies have shown that polymorphisms in the IL-10 gene are associated with obesity and metabolic diseases[38-40]. Furthermore, in patients with type 2 diabetes, circulating monocytes express decreased levels of IL-10[41]. Il-10 and other deactivating signaling molecules are also induced during efferocytosis, a process by which macrophages engulf and clear apoptotic cells in an effort to prevent necrosis and suppress inflammation[42]. This process is thought to help remove oxidized lipids and cholesterol and to activate Akt and Nf-κb survival pathways[43]. Several studies have shown that efficient efferocytosis is necessary to inhibit necrotic plaque formation and defects in efferocytosis lead to the formation of unstable lesions and an increase in systemic inflammation[44-47]. Faulty efferocytosis has also been observed in diabetic mouse models and is

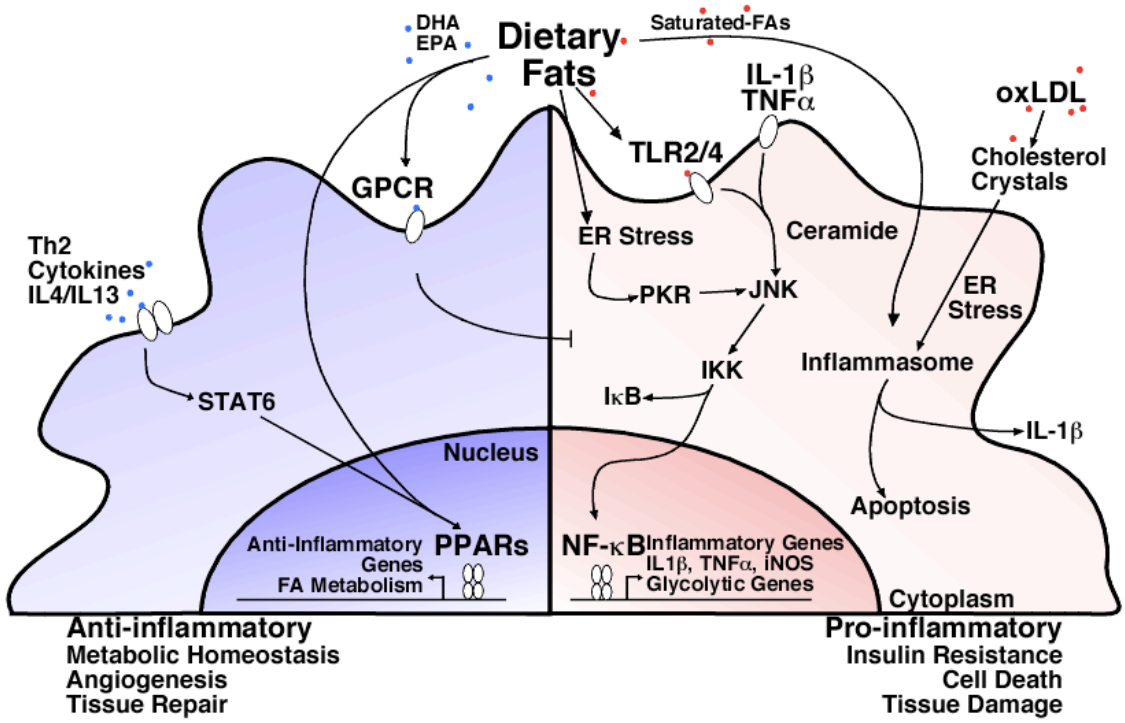


Figure 2: Dietary lipids and inflammatory mediators share common signaling pathways in the control of macrophage activation. The T_H2 (M2) type response, elicited by T_H2 cytokines, notably Il-4 and Il-13, and parasitic worm infection, induces an anti-inflammatory phenotype in the macrophage and promotes metabolic homeostasis, tissue repair, wound healing and angiogenesis. Stat6 is activated by T_H2 cytokines to control the expression of Pparδ and Pparγ. Together, they regulate mitochondrial oxidative metabolism and macrophage alternative activation. ω3-Fatty acids DHA and EPA are known to ligate the G protein coupled receptor Gpr120 to promote anti-inflammatory responses. These fatty acids can also mediate Ppar activation. In contrast, saturated fatty acids, ceramides (metabolites of fatty acids) and cholesterol crystals can induce pro-inflammatory activation of the macrophage through pathogen-sensing proteins Tlr2/4, which activate the c-Jun N terminal kinase (Jnk) cascade leading to Nf-κb relocation to the nucleus and induction of inflammatory cytokines and glycolytic pathways. Excess lipids have also been shown to induce ER stress and inflammasome activation. Pro-inflammatory cytokines produced by these macrophages, including Tnfα and Il-1β, cause tissue damage and inhibit insulin signaling, which contribute to the pathogenesis of metabolic diseases.

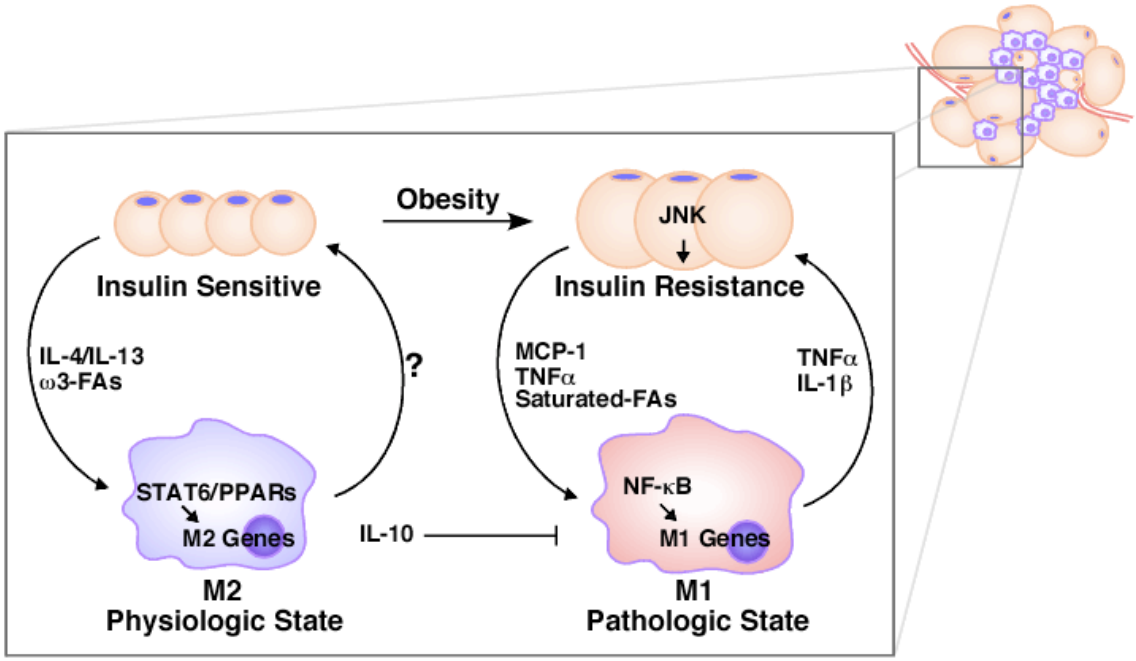


Figure 3: Crosstalk between macrophages and adipocytes plays an important role in adipose tissue homeostasis. In the physiologic state, adipose tissue resident macrophages exhibit an M2 phenotype, mediated by T_H2 cytokines, Il-4 and Il-13, and downstream transcription factors, Stat6 and Pparδ/Pparγ. Several cell types within white adipose tissue have been reported to be sources of T_H2 cytokines, including T lymphocytes, eosinophils and adipocytes. ω3-Fatty acids are also able to induce an anti-inflammatory response through Gpr120 and possibly PPARs. Il-10 is one of the well-characterized anti-inflammatory cytokines produced by these macrophages. In obesity, stressed adipocytes produce inflammatory mediators (e.g., Mcp-1, Tnfα and saturated fatty acids) to induce M1 activation through inflammatory transcription factors, such as Nf-κb. Macrophages respond by up-regulating inflammatory cytokines, which activate Jnk and inhibit insulin signaling pathways in the adipocyte.

thought to promote inflammatory signaling[48]. Oxidative stress and macrophage insulin resistance are two of the potential mechanisms that cause defective efferocytosis[42,49]. Therefore, the combined effects of persistent pro-inflammatory stimulation from increased lipid influx, decreased deactivating signals and compromised efferocytosis lead to the unresolved inflammation in metabolic dysregulation.

Macrophages and metabolic pathologies

Macrophage infiltration and adipose tissue insulin resistance: The stromal vascular fraction (SVF) of white adipose tissue (WAT) has been extensively studied in the progression of obesity-induced insulin resistance. Adipose tissues from lean animals and humans contain a resident macrophage population exhibiting the M2 phenotype[50] (**Fig. 3**). In contrast, the obese state is characterized by infiltration of M1 macrophages that accumulate around apoptotic fat cells (referred to as crown-like structures) and express the Cd11c marker[50]. It has been suggested that macrophages are recruited in response to adipocyte hypertrophy or to signals from dying adipocytes in an attempt to restore homeostasis[51-53]. There, they can be further activated by free fatty acids released by dysfunctional fat cells (discussed below), thus amplifying the inflammatory response. These macrophages contain lipid droplets derived from direct lipid intake or ingestion of dead fat cells and account for up to 40% of the SVF in severe cases of obesity[23]. They secrete pro-inflammatory factors, such as Mcp1, Tnfα and Il-6, which both amplify inflammatory responses and inhibit adipocyte insulin signaling[18,19]. Similarly, monocytes from type 2 diabetic patients or obese individuals express more M1 markers[41]. Conditional depletion of Cd11c-expressing macrophages or inhibition of macrophage recruitment (e.g., Mcp1 knockout) in obese mice resulted in a significant reduction in systemic

inflammation and an improvement in insulin sensitivity[24,25,54]. It should also be noted that adipocytes also produce inflammatory mediators; due to similarities between adipocytes and macrophages, the source of pro-inflammatory cytokines/chemokines within WAT is not clear. In contrast to WAT, the role of macrophages in brown adipose tissue (BAT) is unclear. A recent study suggested that alternatively activated macrophages in WAT produce catecholamines that signal to the BAT and promote cold-induced thermogenic responses[55]. However, a direct role for macrophage infiltration into brown adipose tissue (BAT) seems to be limited[56,57].

Vascular inflammation and atherosclerosis: The best-characterized macrophage population in metabolic syndrome is that of recruited macrophages to sites of endothelial dysfunction in atherosclerosis[58,59]. Elevated low-density lipoprotein cholesterol (LDL-c) levels result in accumulation of LDL particles in the sub-endothelial matrix. Careful analyses of temporal changes leading to lesion formation revealed that this lipid deposition is the crucial initiating event in macrophage recruitment[59]; the accumulated LDL becomes oxidized (ox-LDL) and locally produced cytokines and chemokines, in response to ox-LDL, recruit monocytes/macrophages into the sub-endothelial space. Lipid particles are taken up by macrophages through specialized receptors Sr-a and Cd36, among other mechanisms[60]. These processes evoke a characteristic inflammatory response by releasing inflammatory molecules, such as Mcp1, to the extracellular matrix (ECM), which recruit more macrophages. The lipid-laden macrophages become foam cells and undergo necrotic cell death releasing intracellular components leading to a vicious cycle of chronic inflammation[37]. Subsequently, smooth muscle cells migrate to the lesion in response to inflammatory mediators and contribute to fibrotic plaque formation and rupture[59].

Hepatosteatosis: Liver insulin resistance and hepatic steatosis, referred to as nonalcoholic fatty liver diseases (NAFLD), are major components of the metabolic syndrome. Lipidomic analyses of liver tissue during various stages of NAFLD show a strong correlation between abnormal fat composition and disease pathogenesis[61] with excess fat accumulation leading to the recruitment of inflammatory cells[62]. Together with Kupffer cells, these immune cells are responsible for the development of hepatic inflammation in both humans and various animal models. Recent studies have shown that depletion of Kupffer cells or inhibition of pro-inflammatory mediators protect against the development of NAFLD[63-65]. During high fat diet treatment, depletion of Kupffer cells by $GdCl_3$ or clodronate-encapsulated liposomes has been shown to improve insulin sensitivity and glucose tolerance[66-68]. A single dose of $GdCl_3$ in mice on normal chow enhances insulin signaling and reduces glucose production in the liver[67]. In contrast, macrophage-specific *Ppard*$^{-/-}$ mice, whose macrophages/Kupffer cells exhibit a predominantly M1 phenotype, develop insulin resistance and hepatic steatosis[69].

Muscle Dysfunction: The resident macrophage population in muscle has not been characterized. Macrophages do, however, play a significant role in muscle repair during exercise and tissue damage[70]. Studies have shown that macrophages infiltrate fat depots formed around the muscle in obesity[18]. Additionally, muscle is a target for inflammatory cytokines from other tissues, such as liver and adipose tissue, even though it may not be a site of significant production of inflammatory mediators[71].

INFLAMMATORY SIGNALING AND METABOLIC DISEASES

Although the role of inflammation in atherogenesis is well documented[37,58,59], the underlying mechanisms through which pro-inflammatory signaling pathways cause insulin resistance are still under investigation. One of the better-defined mechanisms is through c-Jun N-terminal kinase (Jnk) activation, which phosphorylates insulin receptor substrate-1 (Irs1) to block insulin signal transduction[8,72]. Similarly, our knowledge regarding how anti-inflammatory pathways improve metabolic homeostasis is limited, apart from their ability to antagonize M1 activation[73]. In this section, I will summarize various signaling pathways mediating macrophage activation during the pathogenesis of the metabolic syndrome.

Pro-inflammatory signaling

Jnk and Pro-inflammatory cytokines: Jnk is an important regulatory node for inflammatory mediators, including pro-inflammatory cytokines, toll-like receptors and endoplasmic reticulum (ER) stress (**Fig. 2**). In obesity-induced insulin resistance and hepatic steatosis, deletion of Jnk1 in the hematopoietic compartment by bone marrow transplantation is beneficial[74]. Tnfα was one of the first pro-inflammatory cytokines to be linked to Jnk activation[5,72]. Tnfα receptor knockout mice show improved metabolic homeostasis and insulin sensitivity on both normal chow and high fat diet[75]. Additionally, $Tnfa^{+/+}$ bone marrow donated to a $Tnfa^{-/-}$ mouse induces insulin resistance in an otherwise, insulin sensitive mouse[76]. Recently, double-stranded RNA-dependent protein kinase (Pkr), which senses viral infection, was shown to interact with Jnk and Irs1[77]. Interestingly, Pkr is able to directly phosphorylate Irs1, suggesting that Jnk integrates different inflammatory signals through a multi-component inflammatory complex.

Toll-like receptors and Nf-κb: TLRs are pattern recognition receptors that respond to pathogenic antigens and propagate inflammatory signaling[78]. *In vitro*, free fatty acids (saturated fatty acids, e.g. palmitic acid) or ceramides can signal through Tlr2 and Tlr4 on macrophages and induce pro-inflammatory gene expression[79-81]. *In vivo*, whole body Tlr4 gene deletion improves insulin sensitivity in a lipid infusion model of transient insulin resistance. Similarly, Tlr4 loss of function mutation or Tlr2 deficiency protects against diet-induced obesity and insulin resistance[82-84]. However, Tlr2/4 are expressed on most tissue types including adipocytes and hepatocytes, in addition to immune cells. The contribution of macrophage Tlr4 was examined through hematopoietic cell-specific Tlr4 deletion, which ameliorates high-fat diet induced hepatic and adipose tissue insulin resistance[85].

Tlr4 ligation promotes Ikk activation, followed by phosphorylation and nuclear translocation of Nf-κb to activate inflammatory gene transcription. Systemic deletions of Nf-κb or various Ikk isoforms (e.g., Ikkβ and Ikkε) prevent obesity induced insulin resistance[37,86,87]. Myeloid specific Ikkβ deletion also leads to improved systemic insulin sensitivity, increased glucose disposal rate and suppressed hepatic glucose production[86]. In line with these observations, salicylate treatment, known to attenuate Nf-κb activation, increases insulin sensitivity in humans[88-90].

In the setting of atherosclerosis, many studies demonstrated that a complete deletion of various TLR family members reduces the lesion size in *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice[91,92]. *Ldlr*$^{-/-}$ mice transplanted with *Tlr2*$^{-/-}$ bone marrow are protected from atherosclerosis[91]. Similarly, mice deficient in myeloid specific NF-κB subunit p50 have decreased atherosclerotic lesions in *Ldlr*$^{-/-}$ mice[93]. Lesions in these animals exhibit near complete loss of foam cells, supporting previous findings that Nf-κb regulates genes involved in lipid uptake and foam cell formation[94].

Inflammasome: The Nlr family, pyrin domain containing 3 (Nlrp3) inflammasome senses endogenous danger signals and generates the mature, secreted forms of Il-1β and Il-18 through caspase 1 (Casp1) activation[95-98]. *In vitro*, palmitic acid and ceramide induce Il-1β and Casp1 processing in the macrophage[99]. Mice deficient in Nlrp3 or Asc (an adaptor protein of the Nlrp3 inflammasome) exhibit increased insulin sensitivity in liver, WAT and muscle[99-101]. In addition, Casp1 and Il-1β have been shown to mediate inflammasome induced insulin resistance[100]. Along these lines, lack of Il-1R, the receptor for Il-1β, confers protection against insulin resistance induced by a high-fat diet[102]. Inflammasome activation can also be detected in atherslerotic lesions[103,104]. Cultured macrophages exposed to crystalline cholesterol secrete Il-1β and Il-18[103,104]. In concert, *Ldlr*$^{-/-}$ mice transplanted with bone marrow from mice lacking Nlrp3 or Asc show a reduction in the lesion area and Il-18 level[103].

ER Stress: The endoplasmic reticulum (ER) is the site of protein folding, vesicle transport and lipid synthesis. When ER capacity is overburdened, the unfolded protein response (UPR) is activated, as characterized by increased activities of Prkr-like endoplasmic reticulum kinase (Perk, also known as Eif2ak3), inositol-requiring 1 (Ire1, also known as Ern2), and activating transcription factor 6 (Atf6)[105]. Activation of the UPR through these signaling molecules leads to downstream CCAAT/enhancer-binding protein homologous protein (Chop, also known as C/ebpε) activation and subsequent induction of apoptosis. The initiation and propagation of the UPR and ER stress by obesity and metabolic stress have been reviewed in detail[105]. ER stress in adipose tissue or liver leads to insulin resistance, which is associated with activation of Jnk and Ikk, suggesting that ER stress is linked to inflammatory pathways[105-107]. In atherosclerosis, chronic activation of the ER stress pathway contributes to macrophage death and subsequent

plaque necrosis[43]. *Chop* expression and macrophage apoptosis have been correlated with advanced lesions[108,109] and deletion of Chop suppresses lesional macrophage death/necrosis[110]. Lastly, it has been shown that the ER stress in the macrophage is caused by increased fatty acid binding protein 4 (FABP4, also known as aP2) lipid chaperone activity, which promotes atherogenesis[111].

Hypoxia: Activation of macrophages to a proinflammatory state increases glucose influx for glycolysis, which is controlled by hypoxia inducible factor (Hif)-1α[112-115]. Loss of Hif-1α in the macrophage leads to decreased pro-inflammatory cytokine production, while deletion of von Hipple-Lindau tumor-suppressor protein (Vhl), a repressor of Hif-1α, results in chronic activation of Hif-1α and uncontrolled inflammation[114,116]. In the context of metabolic dysregulation, adipose tissues in obese mice are hypoxic, which may cause macrophage Hif-1α activation[52,53,117,118]. Systemic hypoxia has been shown to induce insulin resistance and NAFLD[52,119,120]. Furthermore, high levels of hypoxia during adipose expansion may lead to necrotic death of both adipocytes and macrophages, since Hif-1α activation blocks physiological apoptotic cell death[53,118,121]. Similarly, hypoxia promotes macrophage necrosis in mouse models of atherosclerosis[121-123].

Anti-inflammatory signaling

T_H2 cytokines: The other end of the macrophage activity spectrum is the T_H2 cytokine-induced alternative activation, characterized by the expression of M2 markers, such as *Arg1, Mgl1,* and *Ym1*[30,73]. These macrophages function to repair damages elicited by pro-inflammatory M1 macrophages. As discussed earlier, in the lean state, ATMs display an M2 phenotype, suggesting

that at the physiological level, M2 macrophages may play an important role in maintaining metabolic homeostasis[50]. In line with this notion, T_H2 cytokines, notably Il-13, have been detected in WAT and liver[124]. Treatment of diet-induced obese mice with Il-4 significantly improves glucose homeostasis and insulin sensitivity, whereas deletion of signal transducer and activator of transcription 6 (Stat6), a T_H2 effector, worsens insulin resistance[125]. In addition, mice infected with helminth worms, which induce an M2 response, exhibit improved insulin sensitivity[126].

Nuclear Receptors: Nuclear receptors are ligand activated transcription factors that control important biological processes[127]. Several lipid sensing nuclear receptors, such as the peroxisome proliferator activator receptors [Pparα, also known as Nr1c1; Pparδ, also known as Nr1c2; and Pparγ, also known as Nr1c3; activated by dietary fatty acids][128,129] and the liver X receptors [Lxrα, also known as Nr1h3 and Lxrβ, also known as Nr1h2; activated by cholesterol metabolites] are drug targets to treat metabolic diseases[130,131]. In the macrophage, Il-4/Il-13-induced alternative macrophage activation is associated with increased fatty acid b-oxidation and oxidative metabolism[124,132], pathways regulated by PPARs[133]. Accordingly, Pparδ and Pparγ have been shown to be induced by T_H2 cytokines and control M2 activation[124,134]. Mice with myeloid specific deletion of Pparδ or Pparγ show increased M1 and decreased M2 markers in WAT and liver and develop systemic insulin resistance[124,134-137]. The M1/M2 paradigm is also relevant in Kupffer cells in the liver. Myeloid Pparδ deletion worsens hepatic steatosis in mice fed a high fat diet[124,137]. It has been shown that macrophages from these mice produce factors that promote adipocyte and hepatocyte dysfunction *in vitro*[124]. Earlier studies also demonstrated

an anti-inflammatory role for PPARs and LXRs, which correlates well with the athero-protective roles for these nuclear receptors[138-140].

GPCRs: Although saturated fatty acids are known to induce inflammatory signaling pathways, epidemiological studies show that Mediterranean diets high in polyunsaturated ω-3 (n-3) fatty acids actually reduce the incidence of metabolic diseases[141-144]. ω-3 fatty acid derivatives, such as docosahexanoic acid (DHA), eicosapentanoic acid (EPA) and resolvins, exhibit anti-inflammatory activities[145]. DHA and EPA have been shown to bind to G-protein coupled receptor Gpr120 on macrophage surfaces. Gpr120 ligation inhibits the actions of Tnfα and reverses insulin resistance brought on by a high-fat diet[146]. Gpr120 and PPARs share several fatty acid ligands, suggesting potential crosstalk between these two signaling pathways[147].

METABOLIC CONTROL OF MACROPHAGE FUNCTION

One of the main functions of the macrophage is to integrate a broad variety of stimuli to sense and eliminate those pathogenic signals that are either excessive or harmful to the host. Metabolites within the macrophage are critical for the ability of the macrophage to travel to sites of stimulation, respond efficiently and restore homeostasis. In fact, the host undergoes a state of transient insulin resistance during an infection, presumably in an effort to sequester energy for immune cell utilization, suggesting a critical role for metabolites in the immune cell response to pathogens[148]. Phagocytosis of a pathogen by the immune cell is a tightly regulated process in which macrophages can respond to and activate energy demanding cellular remodeling and signaling cascades (**Fig. 4**). Upon recognition, the pathogen is taken up into an invagination of the macrophage membrane[149,150]. This invagination detaches from the membrane by a series of

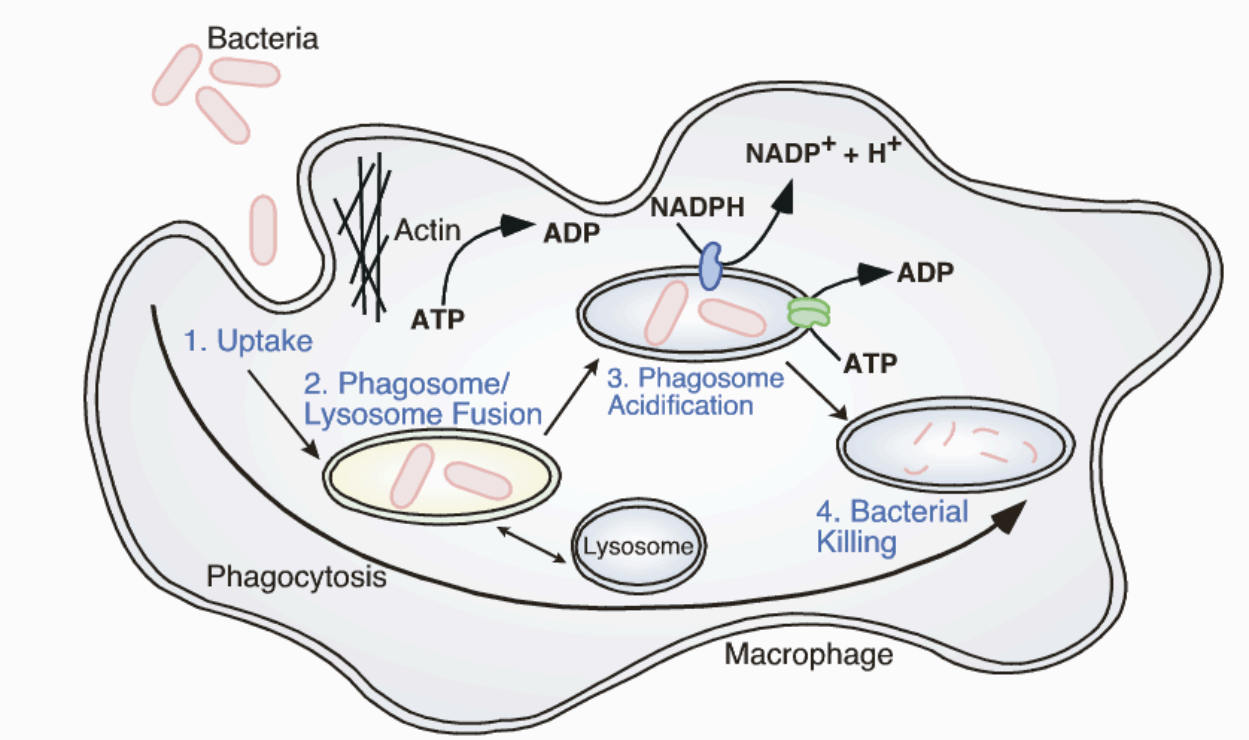


Figure 4: Phagocytosis and bacterial killing rapidly utilize ATP and NADPH. Phagocytosis of a pathogen requires several critical steps including recognition and uptake, fusion of the lysosome to the phagosome, phagosome acidification, and bacterial killing. Actin polymerization and hydrolysis of ATP aid in vesicular trafficking and membrane modification. NADPH oxidases and vacuolar ATPases utilize NADPH and ATP, respectively to generate protons and electrons that can be used to acidify the phagosome and generate ROS. Both of these processes contribute to efficiency of bacterial killing. Deficiencies in any of the processes involved in phagocytosis lead to increased susceptibility to infection.

actin polymerization events to become its own organelle known as the phagosome. The phagosome then goes through a series of fusion/fission events with the lysosome to acquire enzymes and proteins necessary for acidification and bacterial killing. These proteins cooperatively acidify and produce reactive oxygen species (ROS) within the phagosome[151]. Fusion and acidification processes are necessary to activate several enzymes that are only functional in an acidic environment. Through the activation of these enzymes and production of ROS, the macrophage is able to kill the ingested pathogen and break it down into small peptides that are presented to the adaptive immune system for development of memory against future invasions[152-154]. Phagocytosis therefore enables the macrophage to kill pathogens and clear debris within the organism in an effort to maintain systemic homeostasis.

The maturation and acidification of the phagosome leads to the eventual degradation of the pathogen. Phagosomal ROS production by the NADPH oxidase 2 (Nox2) complex is required for efficient and complete bacterial killing[155]. The Nox2 complex consists of the membrane localized gp91phox (Nox2, also known as Cybb) protein and an associated protein cytochrome b-245, alpha polypeptide (Cyba, also known as p22phox) (**Fig. 5a**). Activation of the Nox2 complex is tightly regulated by spatial separation of the membrane associated catalytic Nox2/p22phox complex and its cytosolic activator complex that consists of an activator protein (neutrophil cytosolic factor 2 (Ncf2), also known as p67phox), organizer protein (Ncf1, also known as p47phox), Ncf4 (also known as p40phox), and the small GTPase Rac1. Association of the cytosolic complex with the membrane bound proteins creates a functional Nox complex that oxidizes NADPH and shuttles electrons across the membrane generating ROS within the phagosome[156]. Defects in any of the components of the Nox2 complex results in chronic

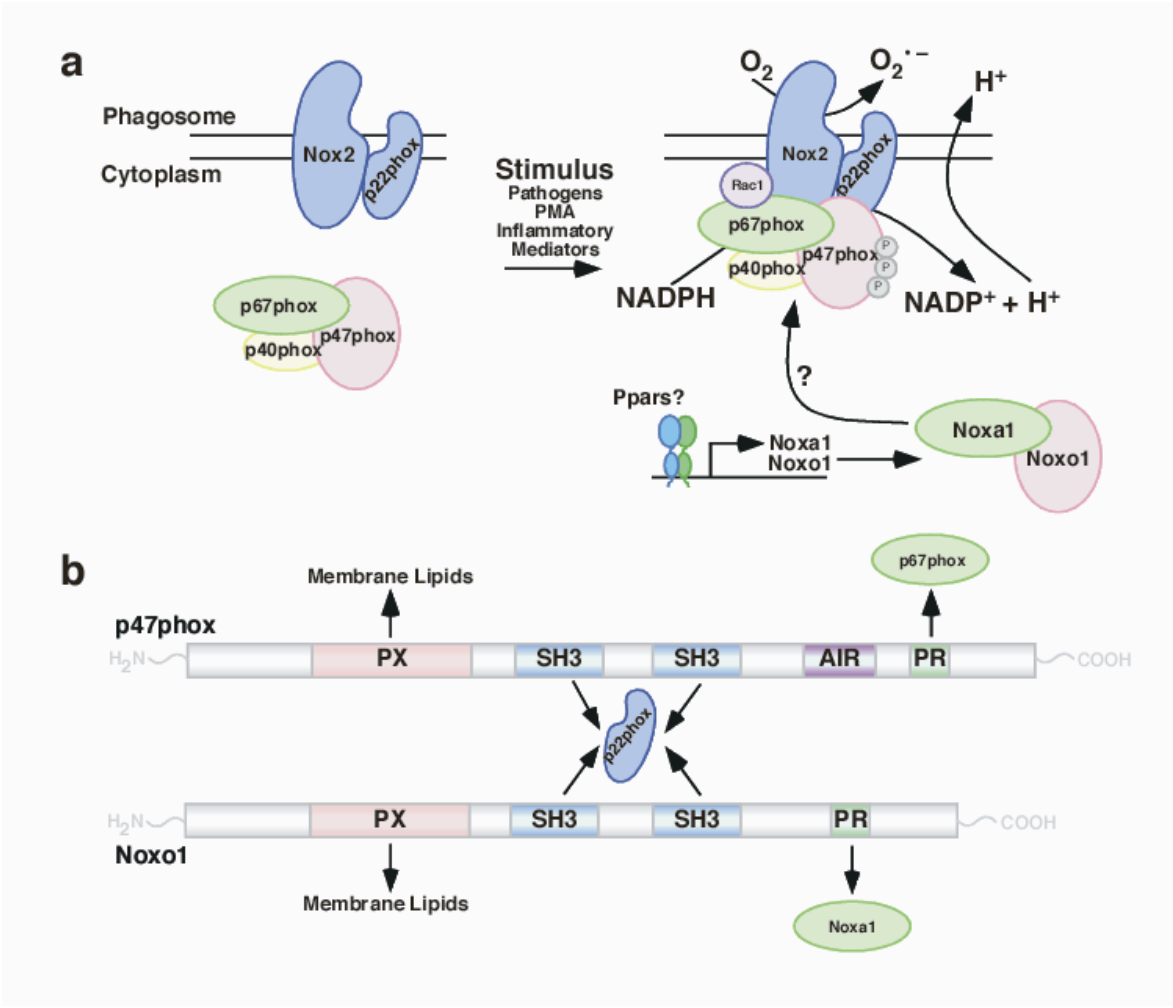


Figure 5: NADPH oxidase 2 regulation by its cytosolic activator complex. Nox2 is the main oxidase responsible for ROS production in the phagosome. Because of ROS cellular toxicity, ROS production by Nox2 is tightly regulated through temporal and spatial activation of activator complexes in the cytoplasm. (**a**) In the unstimulated state, Nox2 (gp91phox) and p22phox reside on the cellular membrane. The activator complex, consisting of p47phox, p67phox, and p40phox, remains spatially separated in the cytoplasm. Upon pathogenic stimulation, the AIR domain of p47phox becomes phosphorylated and the PX associates with membrane bound lipids. These changes lead to association of the activator complex to the membrane bound catalytic complex and subsequent production of phagoomal ROS. The lack of an AIR domain and differential lipid specificities of the PX domain compared to p47phox suggest an alternate role for Noxo1 in ROS production. As such, the reason for transcriptional activation of the Noxo1/Noxa1 complex in response to pathogenic stimuli is unclear. In chapter 4, I show that Ppars can regulate Noxo1 transcriptional activation. The significance of this regulation is unclear. (**b**) Comparison of protein domains contained within p47phox and Noxo1.

granulomatous disease (CGD) and renders patients susceptible to bacterial and fungal infections[157,158].

High-energy molecules, such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NAD(P)H), are crucial for the substrate driven activities of enzymes involved in various processes such as phagocytosis, movement, and cytokine production. In addition to the catabolism of glucose for the production of ATP, significant changes occur in the production and utilization of other metabolites, such as lipids and amino acids (**Fig. 6**). The many roles that metabolites play in macrophage activation and function are detailed below.

ATP and NADPH: Perhaps the most apparent metabolic alteration between proinflammatory and anti-inflammatory macrophages is the switch between anaerobic and aerobic catabolism of glucose for generation of high-energy molecules. During proinflammatory activation, production of ATP occurs in the macrophage primarily through anaerobic respiration in the cytoplasm[159]. This is controlled by a switch from the liver type 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (Pfkfb1, also known as Pfk2) to the more ubiquitous and active form Pfkfb3 (also known as uPFK2)[160]. Activation of Pfkfb3 helps to maintain higher concentrations of fructose-2,6-bisphosphate which allosterically activates phosphofructokinase 1 (Pfk1). When activated in this manner, Pfk1 has an increased affinity for fructose-6-phosphate and decreased inhibition by ATP[160]. Additionally, Hif-1α is activated and there is an increase in lactate dehydrogenase (Ldhb) activity[161,162]. Together, these changes shunt glucose into lactate generation and anaerobic respiration. Evolutionarily, this is thought to occur because the cell requires a large and rapid burst of energy to undergo functions such as bacterial killing, actin

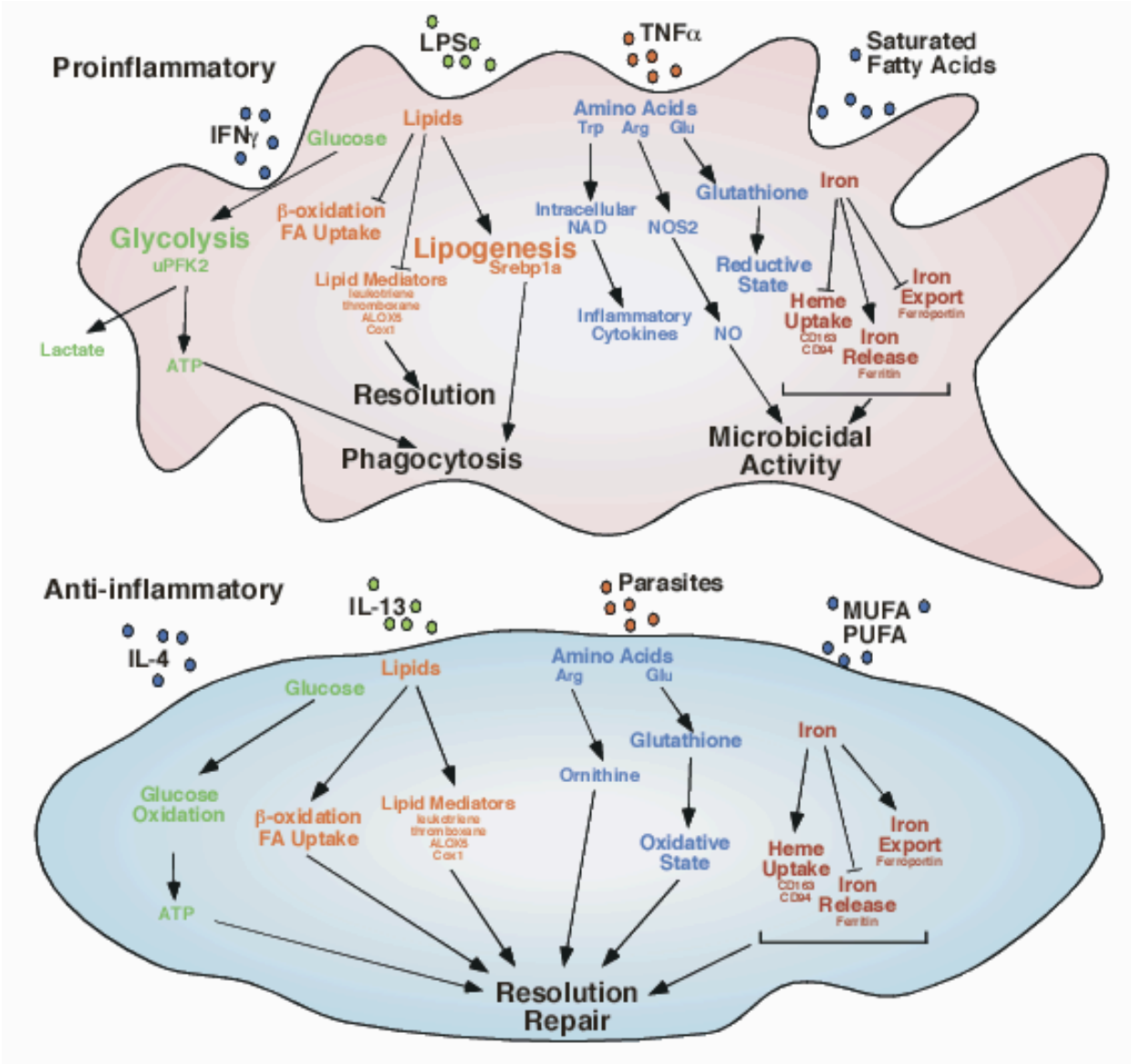


Figure 6: Dietary lipids and inflammatory mediators share common signaling pathways in the control of macrophage metabolism. The T_H1 (M1) type macrophage response, elicited by inflammatory cytokines, bacterial surface proteins, and saturated fatty acids, can induce metabolic changes that promote an anaerobic and reductive environment. The most notable change involves the switch to lactate production and anaerobic respiration. While energetically unfavorable, this state allows for rapid and large energy bursts that can be used for bacterial killing by production of ROS and other mediators. In contrast, cytokines Il-4 and Il-13, parasitic worm infection, and unsaturated fatty acids induce metabolic changes that induce an oxidative state capable of sustaining long term repair and tissue homeostasis. These macrophages rely primarily on fat burning to generate energy and produce mediators critical for resolution

polymerization, transcription and translation. In contrast to the very energy demanding proinflammatory macrophage, anti-inflammatory macrophages rely on oxidative glucose and fatty acid metabolism to generate more sustained and constant energy sources for tissue remodeling and repair[163].

ATP is a critical cofactor for intracellular energy transfer. ATP hydrolysis leads to the release of one or more high-energy phosphate groups that can be transferred to other pathways for use. ATP is critical for a majority of cellular functions from generation of macromolecules to cell division and movement. During a bacterial infection, ATP is most rapidly utilized by the macrophage for cellular kinesis and the oxidative burst[164,165]. ATP is hydrolyzed during actin polymerization, thereby allowing the cell to move to its given target. Additionally, actin plays a critical role in membrane invagination, phagosome formation, and phagosome-lysosome fusion[164]. ATP is also rapidly hydrolyzed by the vacuolar ATPase, a membrane bound protein complex that shuttles the released electrons across the phagosomal membrane to produce the oxidative burst[165].

In addition to ATP generation, NADPH is an important molecule that is required for critical enzymatic activities during bacterial killing. NADPH is generated in the pentose phosphate pathway (PPP) in the reactions catalysed by glucose 6 phosphate dehydrogenase (G6pdh) and phosphogluconate dehydrogenase (Pgd). There is evidence that immune stimulated glucose uptake is tightly coupled to increased PPP activity [159]. The PPP uses a three-enzyme oxidative process to convert glucose-6-phosphate to ribulose-5-phosphate that is used for further metabolic reactions, generating 2 molecules of NADPH from $NADP^+$ reduction. The first step of the PPP is rate-limiting because of the allosteric role of NADPH in regulating G6pdh activity[166]. NADPH can also be generated through activities of malic enzyme and isocitrate dehydrogenase,

but these enzymes do not generate enough NADPH to independently maintain cellular processes[159,166]. Once generated, NADPH is used for several pathways critical for bacterial killing. On the phagosomal membrane, NADPH is oxidized to $NADP^+$ by the enzyme NADPH oxidase to generate ROS within the phagosome (**Fig. 5a**)[167]. NADPH is also a critical cofactor for many enzymes involved in *de novo* lipogenesis, antioxidant systems, and nitric oxide regulation, which are also modulated by and alter the outcome of bacterial infections[166].

Lipids and Lipid Mediators: Lipids play a critical role in macrophage activation and function. Not only can they be taken up and oxidized for energy, as is observed in Il-4 stimulated macrophages, but lipids themselves can be generated during phagocytosis through an increase in *de novo* lipogenesis[168,169]. Lipids are also critical signaling molecules during the activation of various enzyme complexes in the cell, including the NADPH oxidase. While the origin of the lipids is unclear, they typically reside in the cellular membrane and interact with proteins to provide scaffolds for spatial activation or to directly activate target proteins. For example, PIP_3 is the best studied signaling lipid that resides in the cellular membrane and is critical for Akt activation both in the context of infection and insulin signaling in many tissue types[170]. Additional lipids have since been identified that can differentially regulate activation of proteins. For example, specificity of NADPH oxidase activation occurs through lipid binding of the organizer protein, either neutrophil cytosolic factor 1 (Ncf1, also known as p47phox) or NADPH oxidase organizer 1 (Noxo1), to lipids found on the cellular membrane (**Fig. 5b**). These membrane lipids associate with the phosphoinositide-lipid binding (PX) domain found towards the N-terminal end of either organizer protein. Several studies suggest that differences in the PX domain of p47phox and Noxo1 specify binding of these two proteins to their respective lipids,

thereby leading spatial and temporal control of NADPH complex activation[171,172]. While lipids have been shown to play a role in macrophage function, improved lipidomic techniques will help to identify the source of these lipids and the importance of unique lipid species as signaling and structural molecules.

Lipid mediators secreted from macrophages are critical for signaling cascades, cell survival and resolution. At a transcriptional level, pro- and anti-inflammatory macrophages exhibit differential regulation of enzymes involved in eicosanoid production. For example, pro-inflammatory macrophages show marked upregulation of prostaglandin-endoperoxide synthase 2 (Ptgs2, also known as Cox2), the consequence of which is an increase in inflammation. Concurrently, these cells suppress enzymes such as Ptgs1 (also known as Cox1), leukotriene A4 hydrolase, thromboxane A synthase 1, and arachidonate 5-lipoxygenase (Alox5)[173], which produce downstream lipid mediators that promote cellular repair and resolution. Conversely, in an Il-4 stimulated macrophage, Cox1 and Alox5 are upregulated as a mechanism to promote resolution and suppress inflammation[173]. Sphingosine, ceramide kinases, and sphingosine mediators are also differentially regulated by activated macrophages, thereby promoting pro-inflammatory or pro-resolution environments[173].

Amino Acids: Epidemiologic data shows that deficiencies in dietary protein or amino acids are associated with impaired immune function and increased susceptibility to infectious diseases. Amino acids, derived from the diet or endogenously synthesized, are converted to critical mediators through many complex processes. The derived products have many important roles in signaling, inflammatory responses, and resolution. For example, L-Arg is metabolized by Nos2

to produce nitric oxide (NO), an important microbicidal molecule. Alternatively, when arginase 1 (Arg1) is highly expressed, as is the case in anti-inflammatory macrophages, L-Arg is converted to ornithine, which contributes to collagen synthesis, cell proliferation, and tissue remodeling[174]. The end product of tryptophan metabolism, nicotinamide adenine dinucleotide (NAD), promotes Tnf synthesis in a sirtuin 6 (Sirt6) dependent manner[175]. Levels of glutathione, a derivative of L-cysteine, L-glutamic acid, and glycine, are differentially regulated in pro- and anti-inflammatory macrophages. As a molecule that can be oxidized and reduced, glutathione acts as a mechanism for the cell to reduce cellular toxicity[176-178]. These examples are only a few ways in which amino acids contribute to macrophage function and bacterial killing. While many of the mechanisms are clear, more work is needed to understand the intricate relationship between amino acids and cellular functions in the macrophage.

Micronutrients: Micronutrients such as vitamin D, iron, zinc, and copper have been shown to play a critical role in immune function. Epidemiological data suggest that a deficiency in any of these micronutrients makes the host more susceptible to infections. Below are two examples of the mechanism by which micronutrients can direct bacterial killing. However, many more micronutrients influence microbicidal activity, with some of the mechanisms more defined than others. During infection, macrophages undergo changes to retain iron to support bacterial killing. For example, by increasing levels of ferretin, the macrophage is able to release free iron which can be utilized during the respiratory burst to enhance free radical release[179]. Additionally, alterations in total cellular iron levels can greatly affect NADPH oxidase function and the respiratory burst. Iron loading also influences NO release and Nos2 activity. Conversely, macrophages that are resolving inflammation or repairing damaged tissue display a phenotype of

iron export that supports matrix remodeling and cellular proliferation[180]. While these mechanisms by the macrophage to regulate iron homeostasis appear to be in an effort to protect the host, they also serve to sequester iron from the pathogen. Because of the dependance of many pathogens on iron uptake from the host, it has been suggested that evolutionary mechanisms may function to sequester iron such that uptake by pathogens is restricted[179].

While the role of iron in bacterial killing is well studied, the functions of other micronutrients are less understood. Evidence suggests that both silver and copper have been used as anti-microbial agents since documentation of medical practices began. It has been shown that copper receptors on the surface of macrophages are increased post infection and copper is taken up into the macrophage[181]. There is evidence that copper helps mediate ATPase activity and contributes to the respiratory burst. This data is supported by the notion that macrophages from patients with copper deficiency display decreased levels of ROS within the phagosome that is associated with a suppressed respiratory burst[182,183]. Copper is also believed to be antimicrobial because of the toxic effects of copper in bacteria[181]. However, in an effort to survive the insult of copper, many pathogens have developed methods to promote copper export and avoid toxicity. Despite this, copper is still a potent antimicrobial agent. Because of the critical role of micronutrients in cell survival, both the pathogen and the host have developed mechanisms to utilize and defend against micronutrients. Understanding these mechanisms provides insight into metabolic and immune function.

SIGNIFICANCE AND PREVIEW

In many contexts, immune cells and metabolic tissues rely on each other in a physiological setting and dysregulation of either of these processes leads to pathologic disease

states. With current therapeutics aimed at restoring homeostasis, it is crucial to understand the mechanisms by which immune cells and metabolic tissues communicate. This dissertation seeks to elucidate the relationship between metabolic pathways, immune cells, and pathogens to help us discover novel targets for complex diseases. Additionally, understanding the effects of immunomodulatory compounds on immune activation and systemic metabolism can lend insight into the effects of therapies targeted at chronic inflammatory diseases.

In this thesis, I seek to understand the functional relationship between immune and metabolic signaling pathways in an effort to develop therapeutics for multifaceted metabolic and immune pathologies (**Fig.7**). In the first two chapters, I will detail the therapeutic potential of parasite derived host-like glycans in controlling metabolic dysfunction associated with chronic inflammation. I demonstrate in chapter 2 that administration of lacto-N-fucopentaose III (LNFPIII), a Lewis^X containing immunomodulatory glycan found in human milk and on the helminth egg improves glucose tolerance and insulin sensitivity in diet-induced obese mice. This effect is mediated partly through increased Il-10 production by LNFPIII activated macrophages and dendritic cells, which reduces white adipose tissue inflammation and sensitizes the insulin response of adipocytes. In chapter 3, I show that LNFPIII treatment up-regulates nuclear receptor Fxrα to suppress lipogenesis in the liver, conferring protection against hepatosteatosis. At the mechanistic level, the extracellular signal-regulated kinase (Erk)-AP1 pathway appears to mediate the effects of LNFPIII on both inflammatory and metabolic pathways. Together, these results suggest that LNFPIII/Lewis^X may provide novel therapeutic approaches to treat metabolic diseases. The data also suggest a potential role for modulation of metabolic pathways as a treatment for schistosomiasis and other parasitic infections. This concept led to the work done in chapter 4.

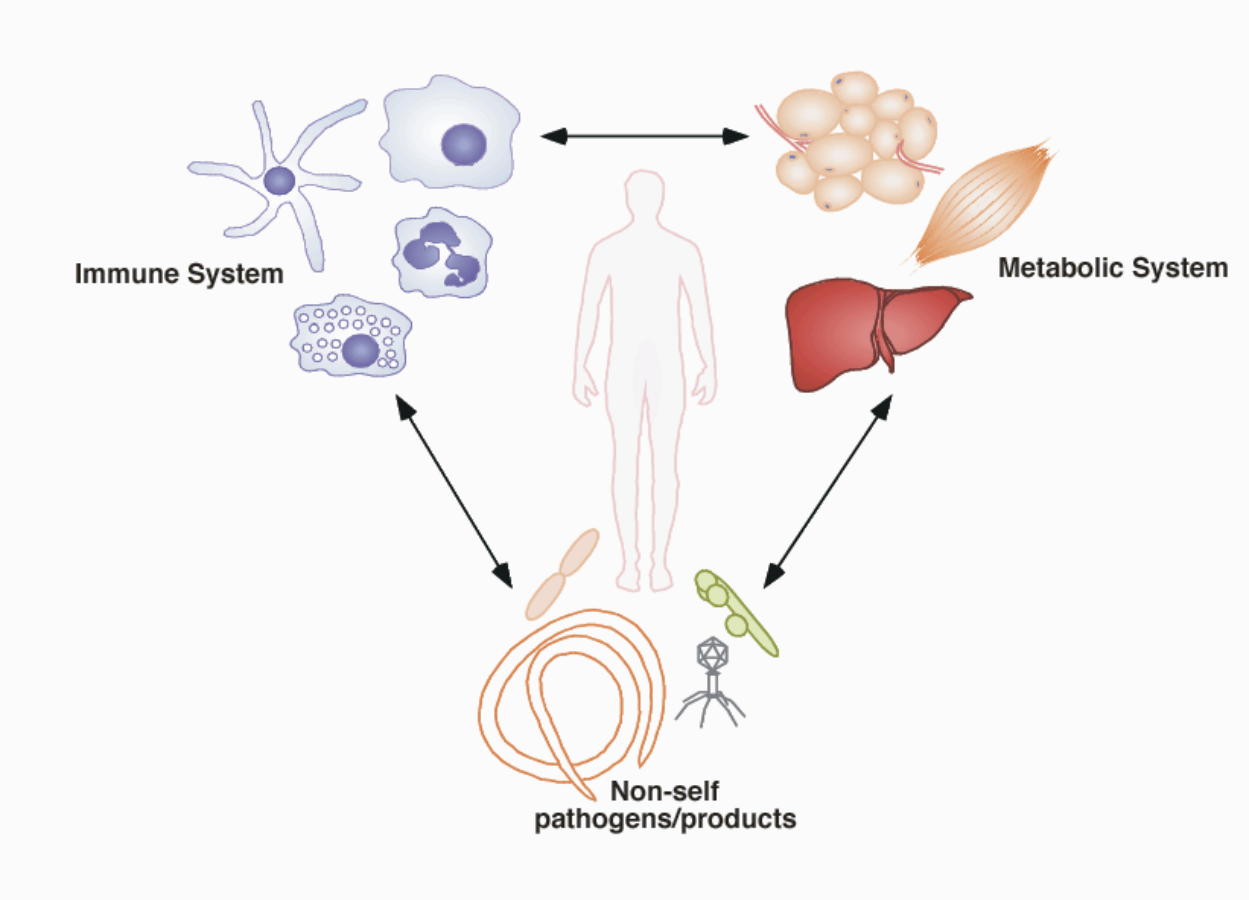


Figure 7: Immune systems, metabolic systems, and non-self pathogens interact to maintain homeostasis. The interactions of non-self pathogens with the immune system are well documented. Recent studies have shown crosstalk between immune systems and metabolic systems. In chapter 2, I show that pathogen derived products can influence immune function and subsequently alter metabolic function within the host. Chapter 3 addresses the direct interactions of non-self pathogens with the metabolic system. The interaction between metabolic regulation in the immune system and pathogens is discussed in chapter 4. Understanding the relationship between these three systems is critical for developing therapeutics that can maintain the careful homeostatic balance dictated by evolution for physiological health.

In the fourth chapter, I address the hypothesis that metabolic pathways are required for proper bacterial killing within the macrophage. In chapter 4, I show that the lipid sensing nuclear receptor Pparδ controls key genes in phagosomal ROS production to promote bacterial clearance. Lack of Pparδ in the macrophage prevents efficient killing of *Streptococcus pneumoniae*, the causative bacterium in many cases of respiratory pneumonia. In contrast, Pparδ activation improves animal survival after bacterial infection. My data reveal an unexpected role for Pparδ in the control of innate immunity and identifies a therapeutic target to improve the outcome of bacterial infections. Together, this thesis work identifies several critical mechanisms by which immune signaling and metabolic signaling pathways intersect with the potential of developing therapeutics for improving host outcome to infections and environmental pathologies.

CHAPTER 2

Immunomodulatory glycan LNFPIII improves WAT insulin sensitivity

Introduction

Metabolic syndrome is a major medical and economic concern worldwide. Chronic inflammation is a key contributor of pathologies associated with metabolic diseases, such as insulin resistance/type 2 diabetes, atherosclerosis and nonalcoholic fatty liver diseases. Although features of chronic "metabolic-related inflammation" differ from those of acute inflammatory responses to exogenous insults, studies have shown that several pathogen sensing mechanisms of innate immunity are negative regulators of insulin sensitivity[184]. For example, pattern recognition receptors and downstream effectors [e.g., toll-like receptor-4 (Tlr4), IκB kinase β (Ikkβ) and Ikkε, Tnfα and double-stranded RNA-dependent protein kinase (Pkr)] have been shown to be activated by high fat feeding and to induce metabolic diseases[71,77,80,86,87]. In addition, activation of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (Nlrp3) inflammasome[185], possibly in response to lipid metabolites (e.g., fatty acids and ceramides)[99,101], results in the cleavage of pro-caspase-1 (pro-casp-1) and release of mature Il-1β, which causes insulin resistance[99-101].

Resident macrophages and lymphocytes in white adipose tissue (WAT) and liver are believed to play important roles in metabolic-related inflammation[124,137,186-188]. As described in detail in the introduction, T_H2 cytokine driven anti-inflammatory macrophages play a critical role in maintaining local and systemic insulin sensitivity[50]. Subsequent studies have identified several sources of T helper type 2 (T_H2) cytokines (e.g., Il-4 and Il-13) within WAT that mediate alternative activation[124,126,188]. Depletion of T_H2 cytokine producing cells or the downstream mediators in macrophages leads to insulin resistance[124,134,188]. In contrast, increased T_H2 cytokine production, as seen in helminth infection, improves glucose homeostasis[126]. While it remains unclear how T_H2-biased and/or anti-inflammatory immune responses improve metabolic

homeostasis, over-expression of the anti-inflammatory cytokine Il-10 has been shown to improve insulin sensitivity[189].

The ability to drive T_H2-type and anti-inflammatory responses during helminth infection has been associated with decreased damage to host tissues, prolonging host survival[190]. During infection with *Schistosoma mansoni* (*S. mansoni*), parasite eggs trapped in host tissues such as the liver, are the main stimuli for T_H2 cytokine production in mice[191]. Injection of a saline soluble homogenate of eggs (or soluble egg antigen, SEA) is sufficient to induce T_H2-biasing of the immune response[192]. Subsequently, it was shown that glycans and glycoproteins found in SEA, such as the $Lewis^X$ containing Lacto-N-fucopentaose III (LNFPIII), GalNAcβ1-4GlcNAc (LacdiNAc), fucosylated LacdiNAc and omega-1 (a T2 Rnase), are capable of mediating the immunomodulatory activity[193-199]. Notably, LNFPIII is one of the major sugars found in human milk post-partum and is thought to play a similar protective role in the neonate[200,201]. These observations suggest that during co-evolution with human hosts, *S. mansoni* parasites expressing immunomodulatory glycans were able to escape detection and had a developmental advantage[202,203]. In fact, macrophages treated with LNFPIII exhibit a T_H2 cytokine-independent, M2-like phenotype, characterized by the expression of the M2 markers, arginase 1 (Arg1) and chitinase 3-like 3 (Ym1, also known as Chi3l3)[204]. LNFPIII and other helminth derived glycans induce Il-10 production[205], up-regulate T regulatory cell numbers[206] and inhibit bacterial lipopolysaccharide induced inflammatory responses[205]. The mechanisms through which LNFPIII exerts these anti-inflammatory activities remain poorly characterized. It has been shown that LNFPIII signals through several C-type lectin receptors (CLRs)[207] and Tlr4[208], which leads to activation of Erk. Although helminth infections polarize the immune response towards the T_H2 type, studies have shown that it reduces, rather than exacerbates, the incidence of allergic

responses, possibly because of the regulatory activity of Il-10[209,210].

In the current study, I seek to further characterize the unique immunomodulatory activity of LNFPIII and determine whether LNFPIII is effective in dampening chronic inflammation and improving metabolic function in a diet induced model of type 2 diabetes. My results show LNFPIII or SEA treatment reduced inflammation and increased insulin sensitivity in WAT in an Il-10-dependent manner.

Results

LNFPIII treatment improves insulin sensitivity

LNFPIII has been shown to induce M2-like macrophage activation, independent of T_H2 cytokines[204]. I confirmed that LNFPIII treatment up-regulated *Arg1*, *Ym1* and macrophage galactose-type C-type lectin-1 (*Mgl1*, also known as *Cd301*) expression, albeit to a lesser extent than Il-4, in macrophages (**Fig. 8a**). In contrast, LNFPIII was more effective than Il-4 in inducing *Il10* expression and release into macrophage conditioned media (CM) (**Fig. 8a** and **8b, and Supplementary Fig. 1a**). This effect was blunted when macrophages were treated with PD98059 to block the activity of Erk, which has been implicated in mediating LNFPIII signal transduction (**Fig. 8b**)[205,208,211]. I also found LNFPIII increased Il-10 production in dendritic cells in an Erk dependant manner (**Supplementary Fig. 1b**). The ability of LNFPIII to upregulate *Il10* expression was independent of Stat6 and Pparδ (known effectors of T_H2 cytokines, **Supplementary Fig. 1c**). Taken together, LNFPIII induces an anti-inflammatory state that increases Il-10 production. To determine whether the immunomodulatory activity of LNFPIII might be beneficial for treating metabolic diseases thought to be associated with chronic inflammation, I injected 8-week-old male mice (C57BL/6J) with vehicle or LNFPIII (25

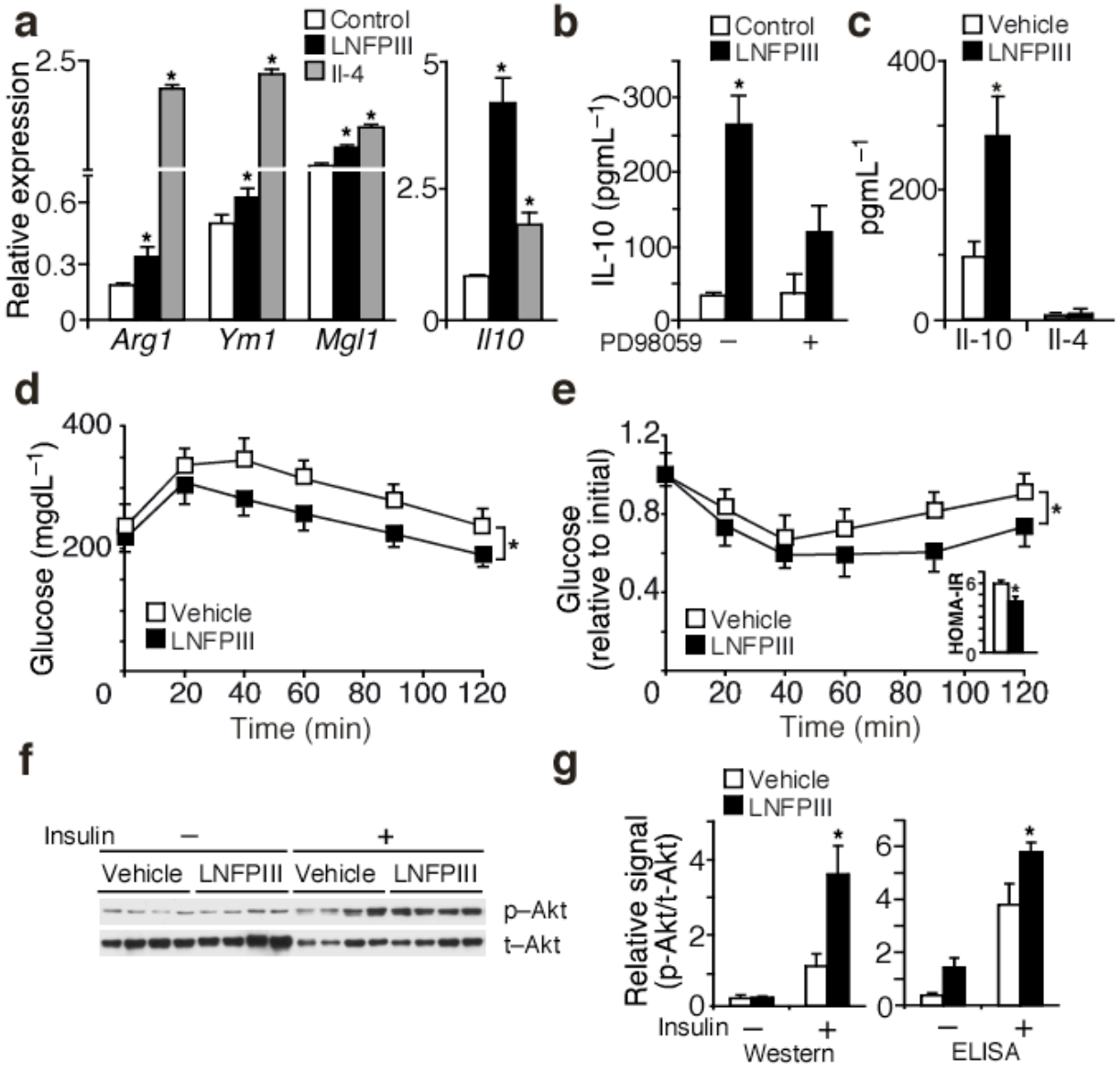


Figure 8 LNFPIII increases Il-10 production and improves insulin sensitivity. (**a**) Real-time q-PCR examining the expression of M2 (alternative activation) genes in bone marrow derived macrophages treated with Il-4 (20 ng ml^{-1}) or LNFPIII (20 μg ml^{-1}) for 24 hr. (**b**) Il-10 concentrations in conditioned medium from LNFPIII or vehicle treated macrophages determined by ELISA. PD98059 (10 μM): Erk inhibitor. (**c**) Serum Il-10 and Il-4 concentrations in vehicle or LNFPIII treated mice (n = 5–7 per treatment) (**d** and **e**) Glucose tolerance test (GTT, **d**) and insulin tolerance test (ITT, **e**) in vehicle and LNFPIII treated mice. Inset in (**e**): homeostasis model of assessment-insulin resistance (HOMA-IR). (**f**) Western blot analyses to examine insulin stimulated Akt phosphorylation in WAT from vehicle and LNFPIII treated mice (from four individual mice per treatment). P-Akt: phospho-Akt; t-Akt: total Akt. (**g**) The relative level of p-Akt to t-Akt in WAT ± insulin injection quantified by densitometry based on Western signals in (**f**) or by ELISA-based assays. Values are expressed as means ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated 3 times. *In vivo* studies were reproduced in three mouse cohorts (n = 5–7 per treatment). Insulin signaling was examined in two of the three cohorts. *$P < 0.05$ (LNFPIII or Il-4 versus vehicle control).

μg,twice a week) for 4–6 weeks after onset of high fat diet (HFD)-induced obesity and metabolic dysfunction. Consistent with the *in vitro* study, LNFPIII treated mice had higher circulating concentrations of Il-10 but the same concentration of Il-4 compared to vehicle treated mice(**Fig. 8c**). When subjected to glucose tolerance test (GTT), LNFPIII treated mice showed significantly higher glucose handling capability compared to vehicle-treated control group(**Fig. 8d**; area under the curve (AUC), vehicle=36,727.5±1,769.13 (mean ± s.e.m.); LNFPIII=29,244±1,119.7, P=0.02). LNFPIII treatment also led to higher insulin sensitivity compared to vehicle treatment, as demonstrated by reductions in fasting serum insulin concentrations (vehicle, 1.54 ± 0.07 ng ml^{-1}; LNFPIII: 1.29±0.06 ng ml^{-1}, P<0.05) and improvements in both the results of insulin tolerance tests (ITT) and the homeostasis model of assessment-insulin resistance (HOMA-IR) (**Fig. 8e**. AUC, vehicle=123.58±4.79; LNFPIII=98.23±3.30, P=0.04). Enhanced insulin signaling, based on insulin stimulated Ser473 Akt phosphorylation (p-Akt), was observed in WAT but not in liver or muscle after acute insulin injection through the portal vein (**Fig. 8f–g** and data not shown). I conducted parallel metabolic studies with SEA treatment, which increased both blood Il-10 and Il-4 concentrations (**Supplementary Fig. 1d**). HFD-fed mice treated with SEA also showed an improved insulin response compared to vehicle-treated mice, as determined by GTTs, ITTs, fasting insulin concentrations and HOMA-IR (**Supplementary Fig. 1e–g**). Treatment with LNFPIII or SEA did not affect body weight, food intake or circulating concentrations of lipids and adiponectin (**Table 1**). These studies demonstrate the potential of using glycans derived from *S. mansoni* egg extract, including LNFPIII, to improve glucose homeostasis and insulin sensitivity.

Table 1. Metabolic parameters of wt mice treated with SEA and LNFPIII

Treatment	Vehicle	SEA	Vehicle	LNFPIII
Body weight (g)	43.63 ± 1.74	41.62 ± 1.36	45.26 ± 0.94	46.86 ± 1.09
WAT/body weight	0.044 ± 0.0044	0.068 ± 0.002*	0.031 ± 0.015	0.039 ± 0.001
Liver/body weight	0.042 ± 0.004	0.041 ± 0.003	0.038 ± 0.002	0.043 ± 0.002
Triglyceride (mg dL^{-1})	102.38 ± 3.89	130.78 ± 7.52	67.17 ± 2.01	69.79 ± 1.18
Free fatty acid (mmol L^{-1})	1.06 ± 0.05	1.14 ± 0.04	1.08 ± 0.08	1.02 ± 0.03
Cholesterol (mg dL^{-1})	165.90 ± 8.59	166.78 ± 12.35	154.72 ± 14.06	144.24 ± 15.99
Glucose (mg dL^{-1})	221.67 ± 15.17	221.17 ± 8.53	236.2 ± 18.45	217 ± 12.01
Insulin (ng mL^{-1})	1.002 ± 0.156	0.615 ± 0.081*	1.545 ± 0.065	1.288 ± 0.059*
Adiponectin (mg mL^{-1})	5.92 ± 0.16	6.35 ± 0.13	5.59 ± 0.32	5.84 ± 0.10

Serum samples were collected after 6 hr fasting. *P<0.05. Values displayed as mean ± s.e.m. n=6/group

LNFPIII enhances WAT insulin signaling through Il-10

The interaction between resident macrophages and adipocytes plays an important role in adipose tissue metabolic homeostasis[124]. WAT histological analyses demonstrated that mice given LNFPIII had lower numbers of CLSs and reduced expression of *F4/80* (a macrophage marker, also known as *Emr1*; **Fig. 9a** and **9b**) compared to vehicle-treated mice. In addition, *Il10* and M2 genes (*Arg1* and *Mgl1*) were upregulated and *Tnfα* and genes encoding the inflammasome pathway (*Casp1*, *Nlrp3*, *Il18*, and *Il1b*) were down-regulated (**Fig. 9b**). Consistent with the increased insulin sensitivity, the expressions of insulin receptor β (*InsRb*), insulin receptor substrate 2 (*Irs2*), CCAAT/enhancer binding protein *α (C/ebpa)*, glucose transporter 4 (*Glut4,* also known as *Slc2a4*) and lipogenic genes were up-regulated in WAT from LNFPIII treated mice than vehicle-treated control mice (**Fig. 9c**). C/ebpα and Irs2 protein abundances were also higher in these mice than in controls (**Fig. 9d**). I obtained similar results in SEA-treated cohorts of mice (**Supplementary Fig. 2**). Of note, SEA induced *Arg1* expression in WAT to a greater extent than did LNFPIII, reflecting the fact that SEA treatment increased serum Il-4 concentrations (**Supplementary Fig. 1d**). These data support the notion that LNFPIII modulates WAT resident macrophage inflammation and improves adipose tissue metabolic homeostasis.

Both *in vitro* and *in vivo* studies demonstrated that LNFPIII induced an anti-inflammatory phenotype characterized by elevated Il-10 concentrations, a cytokine that has been shown to improve metabolic homeostasis[189]. To address whether the insulin-sensitizing effect in WAT is a direct action of the glycan or mediated by Il-10, I cultured 3T3-L1 adipocytes with or without LNFPIII or recombinant Il-10 (rIl-10). I observed that rIl-10, but not LNFPIII itself, improved insulin responsiveness, as determined by insulin-stimulated Akt phosphorylation, glucose uptake

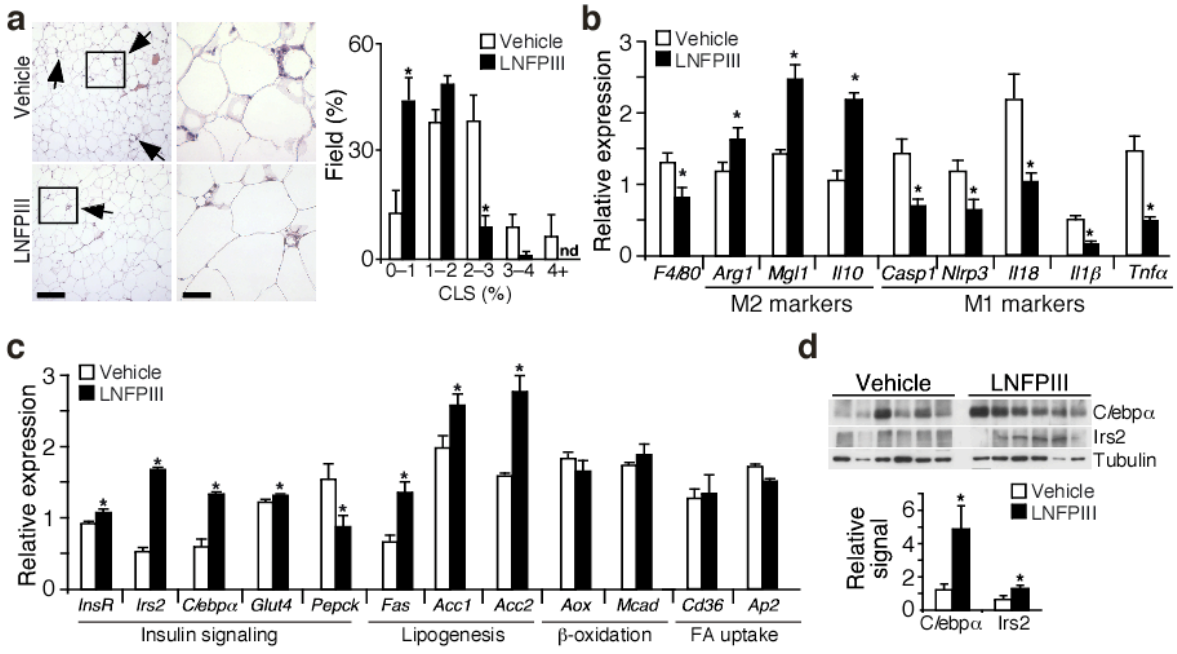


Figure 9 Reduced inflammation and enhanced insulin signaling in WAT of LNFPIII treated mice. (**a**) Left panel: WAT histology showing crown-like structures (CLS, indicated with arrows). Scale bar: left images = 200 μm; right images = 50 μm. Right panel: CLSs quantified in 90 fields from 30 sections (3 fields per section) for each individual animal (n = 4 per group). Y-axis: % fields that contains certain percentage of CLSs; X-axis: % CLS-containing adipocytes in a given field; n.d: not detected. (**b**) Real-time q-PCR analyses of M1 and M2 gene expression in WAT of vehicle- and LNFPIII-treated mice (n = 5 per treatment). (**c**) Metabolic gene expression in WAT determined by real-time q-PCR. (**d**) Western blotting showing C/ebp-α and Irs2 protein levels in WAT. Bottom panel: relative C/ebp-α and Irs2 levels normalized to tubulin. Values are expressed as means ± s.e.m. Metabolic studies were reproduced in three mouse cohorts (n = 5–7 per treatment). Crown-like structures and expression analyses were examined in one and three of the three cohorts, respectively. *P < 0.05 (LNFPIII versus vehicle).

and lipogenesis (**Fig. 10a**). Conditioned medium from LNFPIII- primed wild type (wt) macrophages, which contained higher Il-10 concentrations than vehicle-primed conditioned medium (**Fig. 8b**), was able to reproduce the insulin-sensitizing activity in 3T3-L1 adipocytes, an effect that was abrogated in conditioned medium from LNFPIII-primed *Il10*$^{-/-}$ macrophages (**Fig. 10b**). Furthermore, conditioned medium derived from LNFPIII-primed wt, but not *Il10*$^{-/-}$, macrophages induced the expression of *InsR*, *Irs2* and *C/ebpa* and suppressed *Il1b* and *Tnfa* in 3T3-L1 adipocytes (**Fig. 10c**). Reconstitution of rIl-10 in CM from *Il10*$^{-/-}$ macrophages restored its ability to improve insulin-stimulated glucose uptake and modulate metabolic and inflammatory gene expression (**Fig. 10b**,**c**). *In vivo*, LNFPIII was unable to improve insulin tolerance (**Fig. 10d** and **Table 2**), enhance insulin stimulated glucose uptake (**Fig. 10e**) and inhibit WAT inflammation (**Supplementary Fig. 3a-b**) in *Il10*$^{-/-}$ mice. In addition, *Il10*$^{-/-}$ mice were more insulin resistant than wt control mice (**Supplementary Fig. 3c**). Reciprocally, rIl-10 injections (1 μg every other day for three total doses) improved glucose tolerance and insulin sensitivity and recapitulated the WAT phenotype observed in LNFPIII-treated mice (**Supplementary Fig 3d–h** and **Table 3**). Taken together, the results suggest that Il-10 is required for LNFPIII mediated improvement in WAT insulin signaling and systemic glucose homeostasis.

Discussion

In this chapter, I demonstrate that LNFPIII treatment in obese mice shifts the immune profile to an anti-inflammatory state and decreases macrophage infiltration into WAT. This effect is, in part, driven by macrophage-derived Il-10, which inhibits inflammation and enhances the insulin response in WAT.

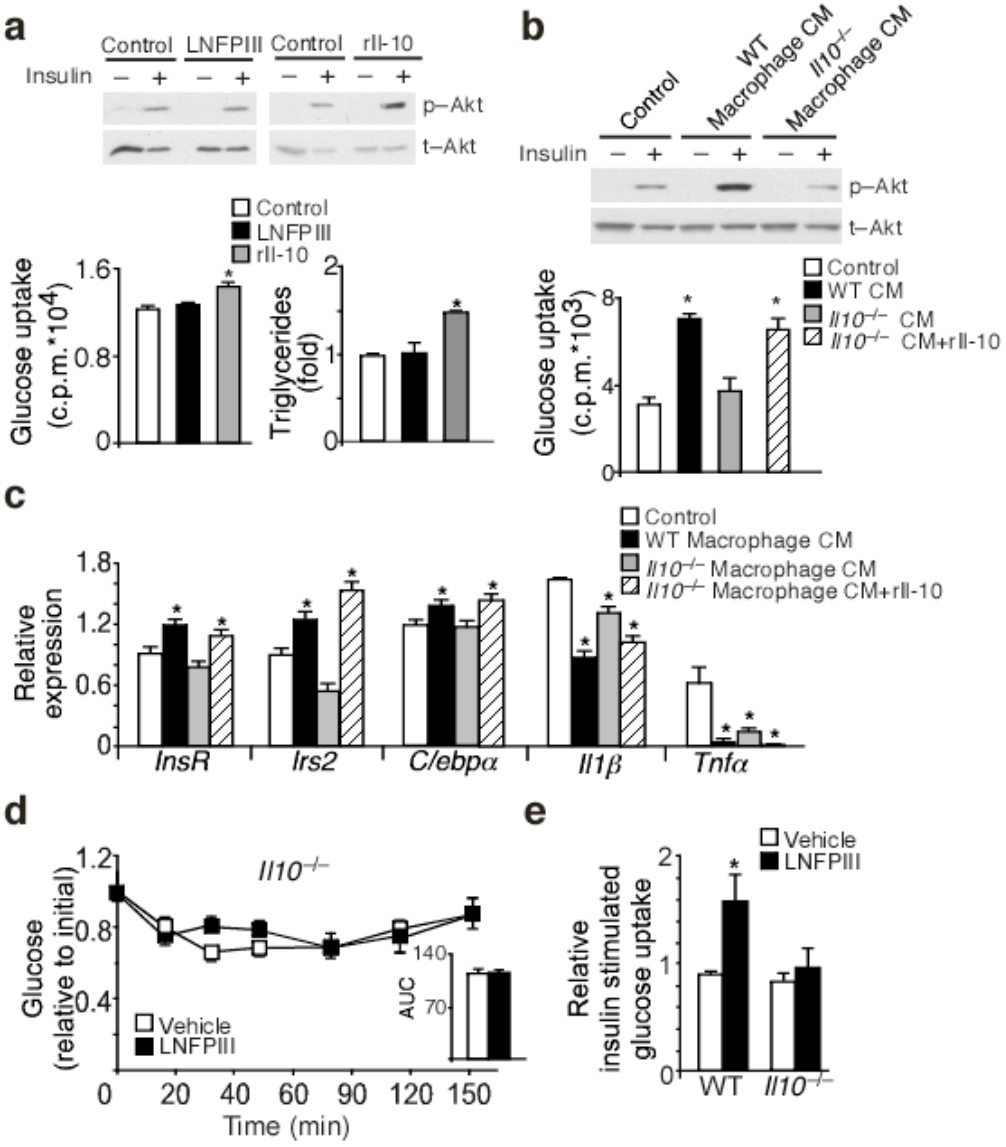


Figure 10 LNFPIII primed macrophage conditioned medium improves insulin sensitivity in 3T3L1 adipocytes in an Il-10 dependent manner. (**a**) Top panel: Western blotting showing protein levels of total Akt (t-Akt) and insulin stimulated Akt phosphorylation (p-Akt) in 3T3-L1 adipocytes treated with vehicle, LNFPIII or rIl-10 (representative samples from three experiments each with three biological replicates). Bottom left panel: insulin stimulated glucose uptake determined using radioactive 2-[H^3]deoxy-D-glucose. Bottom right panel: cellular triglyceride contents measured at day 6 of 3T3-L1 adipocyte differentiation. Vehicle controls for LNFPIII (20 μg ml^{-1}) and Il-10 (10 ng ml^{-1}) were dextran and PBS, respectively. (**b**) Top panel: Western blotting showing protein levels of t-Akt and insulin stimulated p-Akt in adipocytes treated with control and conditioned medium (CM) from LNFPIII primed wt and *Il10*$^{-/-}$ macrophages. Bottom panel: insulin stimulated glucose uptake in adipocytes. (**c**) Gene expression in 3T3-L1 adipocytes determined by real-time q-PCR. (**d**) Insulin tolerance test in vehicle and LNFPIII treated *Il10*$^{-/-}$ mice (n = 6 per treatment per genotype). AUC: area under the curve of ITT. (**e**) *Ex vivo* glucose uptake assay performed in adipose tissue slices collected before and after portal vein injection of 5 U kg^{-1} insulin. Values are expressed as means ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. Studies in *Il10*$^{-/-}$ and control mice were conducted in one cohort (n = 6). *P < 0.05 (treatment versus control).

Table 2. Metabolic parameters of *Il10*$^{-/-}$ mice treated with LNFPIII

Treatment	Vehicle	LNFPIII
Body weight (g)	33.67 ± 3.84	37.03 ± 4.14
WAT/body weight	0.019 ± 0.004	0.028 ± 0.005
Liver/body weight	0.038 ± 0.002	0.032 ± 0.003
Triglyceride (mg dL^{-1})	29.11 ± 1.11	36.14 ± 3.61
Free fatty acid (mmol L^{-1})	0.49 ± 0.05	0.54 ± 0.08
Cholesterol (mg dL^{-1})	136.36 ± 10.87	153.89 ± 19.95
Glucose (mg dL^{-1})	132.17 ± 17.36	142.5 ± 16.42
Insulin (ng mL^{-1})	0.260 ± 0.033	0.259 ± 0.048

Serum samples were collected after 6 hr fasting. *P<0.05.
Values displayed as mean ± s.e.m. n=6/group

Table 3. Metabolic parameters of wt mice treated with rIl-10

Treatment	Vehicle	rIL-10
Body weight (g)	31.93 ± 1.75	32.67 ± 1.03
WAT/body weight	0.025 ± 0.001	0.032 ± 0.001*
Liver/body weight	0.036 ± 0.001	0.035 ± 0.001
Triglyceride (mg dL^{-1})	76.69 ± 5.04	84.83 ± 4.2
Free fatty acid (mmol L^{-1})	0.57 ± 0.04	0.61 ± 0.06
Cholesterol (mg dL^{-1})	106 ± 3.80	104.21 ± 4.31
Glucose (mg dL^{-1})	226.5 ± 11.93	228 ± 8.47

Serum samples were collected after 6 hr fasting. *P<0.05.
Values displayed as mean ± s.e.m. n=6/group

The hygiene hypothesis attributes the increased incidence of autoimmune diseases and allergic responses in developed countries to reduced human contact with pathogens[212]. A special emphasis has been on the interaction between parasitic worms and humans[212,213]; in addition to the T_H2-biasing immune phenotype, helminth infections induce the proliferation of regulatory T cells and production of the anti-inflammatory cytokine Il-10[214]. My data show that LNFPIII treatment is sufficient to increase Il-10 production in macrophages and dendritic cells. Regulatory T cells are likely another major source of Il-10[214]. These cells are enriched in fat tissues from lean individuals and play a role in maintaining WAT function[186]. Anti-inflammatory pathways are thought to improve metabolic homeostasis primarily through attenuating the action of pro-inflammatory signaling (e.g., Tnfα and Il-1β). Overexpression of Il-10 in the muscle was shown to improve systemic insulin resistance through suppression of inflammation[189]. I find that in an Il-10 dependent manner, conditioned medium from LNFPIII treated wt macrophages directly enhanced insulin responses in adipocytes by up-regulation of InsR and Irs2 and increased insulin mediated glucose uptake and lipogenesis. Acute rIl-10 injection in HFD-fed mice also improved glucose homeostasis. Of note, short-term rIl-10 treatment did not appear to affect WAT macrophage infiltration (**data not shown**), suggesting that the insulin sensitizing effect of Il-10 can be separated from its anti-inflammatory activity.

In line with this notion, several studies have identified *IL10* polymorphisms associated with insulin resistance/type 2 diabetes in humans[38,215,216]. Of note, a previous study has demonstrated that deficiency in hematopoietic cell derived Il-10 in mice through transplantation of *Il10*$^{-/-}$ bone marrow did not affect high fat diet induced tissue inflammation and insulin resistance[217]. In that study, *Il10* mRNA and protein abundances were up-regulated several-fold in WAT and liver. It is unclear whether the compensatory increase in Il-10 production was from

non-hematopoietic cells or from residual wt bone marrow-derived cells. The result seems to suggest that increased Il-10 levels within metabolic tissues are sufficient to maintain metabolic homeostasis.

While adipose tissue is known to produce adipokines, such as leptin, adiponectin and resistin, to modulate systemic glucose and lipid metabolism, it is not a major tissue for glucose uptake under physiological conditions. LNFPIII treatment did not affect the expression of these adipokines in WAT (**Table 1** and **data not shown**). How, then, does increased WAT insulin sensitivity by LNFPIII lead to improvement in systemic glucose homeostasis? It has been shown that GTT and ITT in muscle-specific insulin receptor knockout (MIRKO) mice were indistinguishable from control animals, even though insulin stimulated glucose uptake was substantially reduced in muscle (the major site of glucose disposal)[218]. Subsequent studies attributed the normo-glycemic phenotype of MIRKO mice to a three-fold induction in glucose deposition to WAT[219], suggesting that, at least in mice, increased glucose uptake by adipocytes was able to sustain glucose homeostasis. Consistent with this notion, over-expression of the glucose transporter Glut4 in adipose tissues improved glucose tolerance[220]. Recently, increased glucose flux in adipocytes has been shown to up-regulate fatty acid synthesis through a novel isoform of carbohydrate-responsive-element-binding protein (Chrebp-β, also known as Mlxipl)[221]. In addition, the expression of adipose *MLXIPL* and lipogenic genes positively correlates with insulin sensitivity in humans[221-223]. My data demonstrate that LNFPIII treatment increased WAT glucose uptake and lipogenic gene expression. *Mlxipl* was also induced (**data not shown**).

While the mechanism through which fat synthesis in adipose tissues contributes to whole body homeostasis is not completely understood, a lipogenic product of adipocytes has been

linked to improved systemic metabolism[224]. The beneficial effect of LNFPIII could also be mediated by central regulation, in which reduced inflammation may lead to improved central insulin sensitivity that is known to regulate hepatic glucose production[225,226]. However, LNFPIII did not affect gluconeogenesis based on pyruvate tolerance tests and the expression of gluconeogenic genes in the liver (**data not shown**). Taken together, my results demonstrate a therapeutic potential for LNFPIII in treating components of metabolic syndrome through its ability to directly modulate both immune and metabolic pathways.

Materials and Methods

Animal experiments. Male C57BL/6J mice at 8–10 weeks of age (Jackson Laboratory) were placed on a high-fat, high-carbohydrate diet (F3282, Bio-Serv) for the duration of the experiments. Six weeks after high fat feeding, mice were injected (i.p.) twice per week with 25 μg of dextran (40 kDa, vehicle) or LNFPIII conjugated with dextran (~8–10 LNFPIII/dextran). Experiments were reproduced in 3 independent mouse cohorts (n=5–7/treatment). Three additional cohorts (n=4–6/treatment) were treated with 0.9% NaCl (vehicle) or SEA dissolved in 0.9% NaCl (25 μg/twice a week). LNFPIII and SEA were prepared as described[204,227]. Metabolic studies started 4 weeks after LNFPIII or SEA treatment and were conducted after 6hr fasting. Animals were sacrificed at the 6th week of treatment for serum and tissue collection. For rIl-10 experiments, mice were treated with PBS (vehicle) or rIl-10 (Peprotech; 1ug/every other day, i.p.) after 4 weeks of high fat diet. Body weight and food intake were monitored weekly. GTT was performed by injecting 1 g glucose per kg body weight into the peritoneum. Blood glucose was measured before and after injection at the time points indicated using a OneTouch glucose monitoring system (LifeScan). ITT was conducted similarly by injecting 1 U kg^{-1} body

weight of insulin. *In vivo* insulin signaling was determined by injecting 5 U kg^{-1} body weight of insulin through the portal vein. Pieces of liver, epididymal fat, and muscle were collected before and 10 minutes after insulin injection and rapidly stored in liquid nitrogen. Additional adipose tissue slices were immediately incubated with 2-[H^3]deoxy-D-glucose to determine insulin stimulated glucose uptake *ex vivo*[228]. To estimate crown like structures, a piece of epididymal fat pad was divided into three sections and embedded in a single paraffin block. Sequential sagittal sections were collected every 20 μm for H&E staining. HOMA-IR was calculated as described[229]. Levels of insulin, phospho-Akt, total-Akt, and Il-4 were measured using ELISA-based plates from Meso Scale Discovery. Il-10 was measured using ELISA kits (Peprotech & Meso Scale Discovery). The Dana-Farber/Harvard Cancer Center Research Pathology Core provided all histological services and preliminary assessments by a pathologist. *Il10*$^{-/-}$(male, *n*=6/genotype/treatment, C57BL/6J background) were obtained from the Jackson Laboratory. Metabolic studies and treatments in these animals were similar to those in wt C57BL/6J mice. All animal studies were approved by the Harvard Medical Area Standing Committee on Animals.

Immunoblotting experiments. I performed Western blot analyses using antibodies to detect the following proteins: p-Akt (Cell Signaling # 9271, 1:1000); t-Akt (Cell signaling # 9272, 1:1000); Irs2 (Santa Cruz # sc-8299, 1:500); C/ebp-α (Santa Cruz # sc-61, 1:500); actin (Cell Signaling # 4970, Clone 13E5, 1:1000); tubulin (Cell Signaling # 2128, Clone 9F3, 1:1000).

Primary cells, adipocyte differentiation and functional assays. Bone marrow-derived macrophages were differentiated in L929 conditioned media as previously described[124].

Dendritic cells were differentiated in the presence of macrophage colony-stimulating factor (3 ng mL^{-1}) and Il-4 (5 ng mL^{-1}). Cells were treated with dextran (control) or LNFPIII overnight. Following treatment, cells were washed and cultured in DMEM alone. Media was collected 8 hours later and 10% FBS was added, which constituted the conditioned medium. For M2 skewing experiments, macrophages were treated with dextran, LNFPIII or Il-4 overnight. Where indicated, cells were pretreated for 1 hour with PD98059 (10 μM, Cell Signaling) prior to treatments. PD98059 is an MEK1 (also called MAPKK or Erk kinase) inhibitor used to block Erk activation. 3T3L1 cells were differentiated as previously described in a cocktail containing insulin, isobutylmethylxanthine, and dexamethasone[230]. For differentiation experiments, 3T3-L1 cells were given various treatments indicated together with the differentiation cocktail. At day 2, the cocktail was removed and differentiation continued in the presence of the treatment and insulin. Triglyceride analysis was performed after 6d of differentiation. Cellular lipids were extracted with a 2:1 (v/v) chloroform:methanol solution. For insulin signaling experiments, fully differentiated 3T3-L1 adipocytes (at day 6) were given the indicated treatments for 48 hr without insulin. Cells were washed, serum starved for 2 hr and stimulated with insulin (100 nM) for 60 min. Glucose uptake was performed using 2-[H^3]deoxy-D-glucose with a 20 min insulin pre-stimulation. Cellular radioactivity was determined and normalized to protein content.

Expression analyses. For gene expression analyses, relative expression levels were determined by SYBR green-based real-time quantitative PCR (q-PCR) reactions. *36B4* levels were used for normalization. For Western blot analyses, tissue and cell lysates were prepared in the presence of protease and phosphatase inhibitors.

Statistical analyses. Statistical analyses comparing two parameters (between treatments or genotypes) in cell-based work were conducted using the two-tailed Student's *t* test. Two parameters analyses for samples from *in vivo* studies (non-gaussian distribution) were determined using the Mann-Whitney test (Figs. 8c, 8g, 9a–d, 10e, Supplementary Figs. 1d, 1g, 2b–c, 3a, 3b, 3f–h, and Tables 1–4). Statistics for multi-parameter analyses was determined by One-Way ANOVA followed by Bonferroni posthoc tests (Figs. 8a, 10a–c, and Supplementary Figs. 1a). Two-way ANOVA was used to determine statistical significance for GTT and ITT (Figs. 8d–e, 10d and Supplementary Figs. 1e–f, 3c–e). Values are presented as mean ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for one representative experiment. Experiments were repeated at least three times. $P<0.05$ was considered significant.

Author contributions

Dr. Donald Harn (University of Georgia) and Dr. Changlin Li (University of Georgia) established a collaboration on the SEA/LNFPIII project and provided us with the bioactive glycans. Dr. Tiffany Horng provided us with *Il10*$^{-/-}$ mice and reagents for studying the inflammasome. Members of the Lee laboratory provided technical assistance.

CHAPTER 3

LNFPIII improves HFD-induced hepatic fat accumulation and dysfunction

Introduction

The liver is a major target of metabolic dysregulation. While insulin resistance is the best documented pathology associated with metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) plays a significant role in the morbidity and mortality of obese patients. NAFLD is characterized as a spectrum of histological changes from hepatocyte lipid accumulation without concomitant inflammation to steatohepatitis associated with inflammation, fibrosis, and cirrhosis[231]. While inflammatory changes, with an immune cell component, are thought to lead to the onset of NAFLD in many obese patients, the mechanisms that determine the extent of liver damage are questionable. Some researchers suggest a two hit hypothesis; once inflammatory signals make the hepatocytes susceptible, a second oxidative stress leads to the transformation of the hepatocytes to fibrotic and cancerous[232]. Patients with NAFLD are prescribed interventions aimed at reducing lipid burden and inflammation[233]. However, there is currently no proven treatment for NAFLD.

Several nuclear receptors are known to control glucose and lipid metabolism in the liver. Peroxisome proliferator-activated receptor (Ppar, also known as Nr1c1)-α regulates β-oxidation and gluconeogenesis in the fasted state[128]. The liver X receptor (Lxr, also known as Nr1h3)–α – farnesoid X receptor (Fxr, also known as Nr0b2)-α pathway controls cholesterol and bile acid metabolism. Fxrα has also been shown to suppress hepatic lipogenesis in small heterodimer partner (Shp)-dependant and –independent mechanisms through suppression of sterol regulatory element binding protein (Srebp)-1c [234]. *Fxra*$^{-/-}$ mice have increased hepatic lipid storage with peripheral insulin resistance, suggesting a role for Fxrα as a potential therapeutic target for NAFLD[235]. In Chapter 3, I show that LNFPIII directly regulates the lipogenic program in

hepatocytes and prevents obesity-induced hepatic steatosis through an unexpected link to the nuclear receptor Fxrα.

Results

LNFPIII protects against diet-induced hepatic steatosis

In addition to improving WAT function, histological and triglyceride content analyses showed a strong protective effect of LNFPIII against HFD-induced hepatic lipid accumulation (**Fig. 11a**). Furthermore, overall liver function, determined by circulating levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), was significantly improved (**Fig. 11b**). In line with reduced hepatic steatosis, lipogenic genes, including fatty acid synthase (*Fas*), acetyl-CoA carboxylase1/2 (*Acc1/2*), stearoyl-CoA desaturase 1 (*Scd1*) and sterol regulatory element-binding protein 1c (*Srebp1c,* also known as *Srebf1*), were suppressed in LNFPIII-treated livers (**Fig. 11c**).

Srebp-1c is a master lipogenic transcription factor whose expression and transcriptional activity are controlled by a network of nuclear receptor signaling pathways, notably, the positive regulator Lxrα (NR1H3)[236,237] and negative regulators Fxrα (NR1H4) and its target Shp (NR0B2)[234]. The Fxrα gene consists of two major 5' regulatory regions, with the upstream and downstream promoters driving the expression of *Fxr-α1/α2* and *Fxr-α3/α4* isoforms, respectively (**Fig. 13a**)[238]. The only difference between Fxr-α1/α2 (or Fxr-α3/α4) is a 4 amino acid insertion in Fxr-α1 (or Fxr-α3). I found that expression of *Fxr-α3/α4* isoforms as well as several known Fxr target genes[238], including *Shp*, organic anion-transporting polypeptides (*Oatp,* also known as *Slco1a1*), phospholipid transfer protein (*Pltp*) and bile salt efflux pump (*Bsep,* also known as *Abcb11*), were up-regulated in livers of LNFPIII treated mice, compared to vehicle treated controls (**Fig. 11c**). *Lxrα* expression remained unchanged. Similar effects were

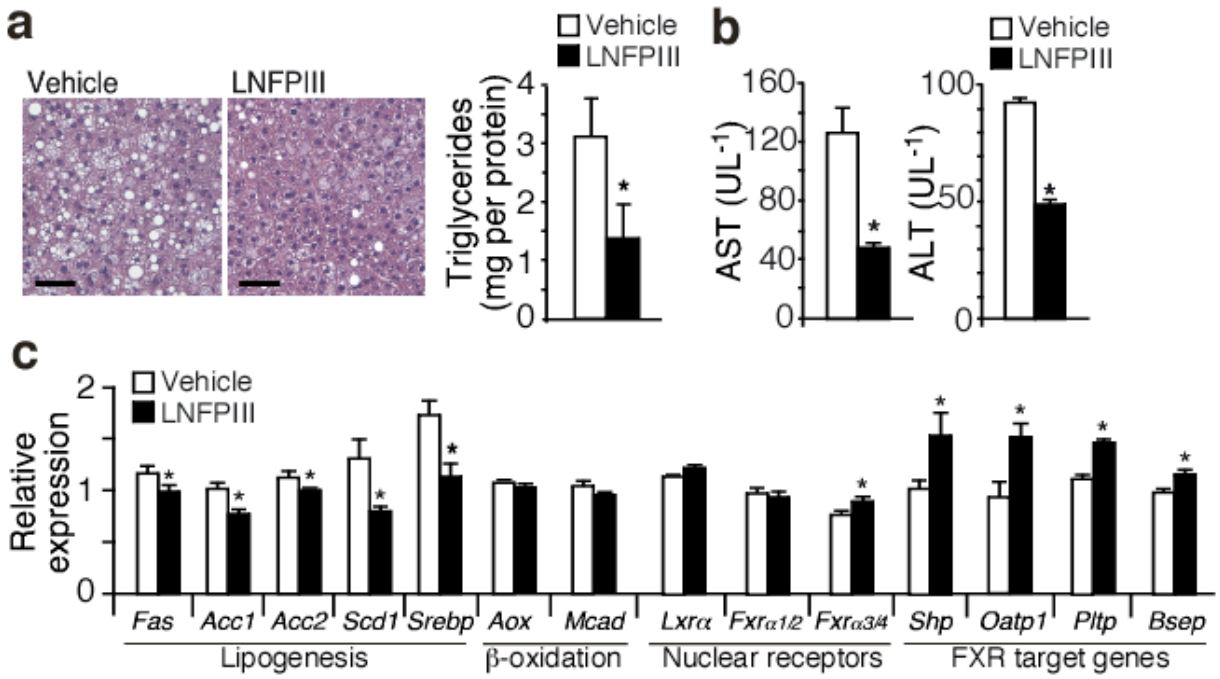


Figure 11 LNFPIII protects against high fat diet induced hepatic steatosis. (**a**) Liver histology and triglyceride content analyses to determine hepatic fat accumulation in vehicle and LNFPIII treated mice. Scale bar = 100 μm. (**b**) Circulating AST and ALT concentrations to assess liver function. (**c**) Gene expression analyses in livers from vehicle or LNFPIII treated mice (n = 5) by real-time q-PCR. Values are expressed as means ± s.e.m. Metabolic studies were reproduced in three mouse cohorts (n = 5–7 per treatment). Histology was examined in one and lipid and expression analyses were examined in three of the three cohorts. *$P < 0.05$ (LNFPIII versus vehicle control).

obtained with SEA treatment, except that SEA up-regulated both *Fxr-α1/α2* and *Fxr-α3/α4* (**Supplementary Fig. 4a–d**). Metabolic gene expression in muscle was largely unaffected by LNFPIII or SEA (**Supplementary Fig. 4e** and **data not shown**). These results indicate that LNFPIII prevents ectopic fat accumulation in the liver and regulates the expression of transcriptional factors critical for *de novo* lipogenesis.

To assess the role of LNFPIII or LNFPIII primed macrophages in hepatic lipid homeostasis, *de novo* lipogenic assays were conducted in isolated primary hepatocytes. LNFPIII, but not conditioned medium from LNFPIII-treated macrophages, suppressed lipogenesis and increased fatty acid β-oxidation compared to vehicle treatment (**Fig. 12a**). LNFPIII also induced expression of *Fxr-α3/α4* and *Shp* as well as suppressed *Srebp1c* and *Acc1* (**Fig. 12b**). As LNFPIII did not affect β-oxidation gene expression (**Fig. 11c** and **12b**), the increased fat burning was likely secondary to decreased fatty acid synthesis. These results suggest that unlike in WAT, the LNFPIII effect in the liver was not mediated by Il-10. Consistent with this notion, rIl-10 treatment did not affect hepatic *de novo* lipogenesis *in vitro* or *in vivo* (**Supplementary Fig. 5a–c**) and the protective effect of LNFPIII was preserved in *Il10*$^{-/-}$ liver (**Supplementary Fig 5d–e**).

LNFPIII suppresses hepatic *de novo* lipogenesis through FXR

The up-regulation of the Fxrα-dependent transcription program by LNFPIII in the liver suggested that LNFPIII may induce Fxrα to inhibit fat synthesis. In fact, in hepatocytes with Fxrα gene ablation or Erk inhibition by PD98059, LNFPIII was unable to inhibit *de novo* lipogenesis and increase fat oxidation (**Fig. 12c**). Furthermore, the ability of LNFPIII to reduce hepatic triglyceride accumulation, improve liver function and suppress lipogenic gene expression was lost in *Fxra*$^{-/-}$ mice (**Fig. 12d–e** and **Supplementary Fig. 5f**), while the insulin sensitizing

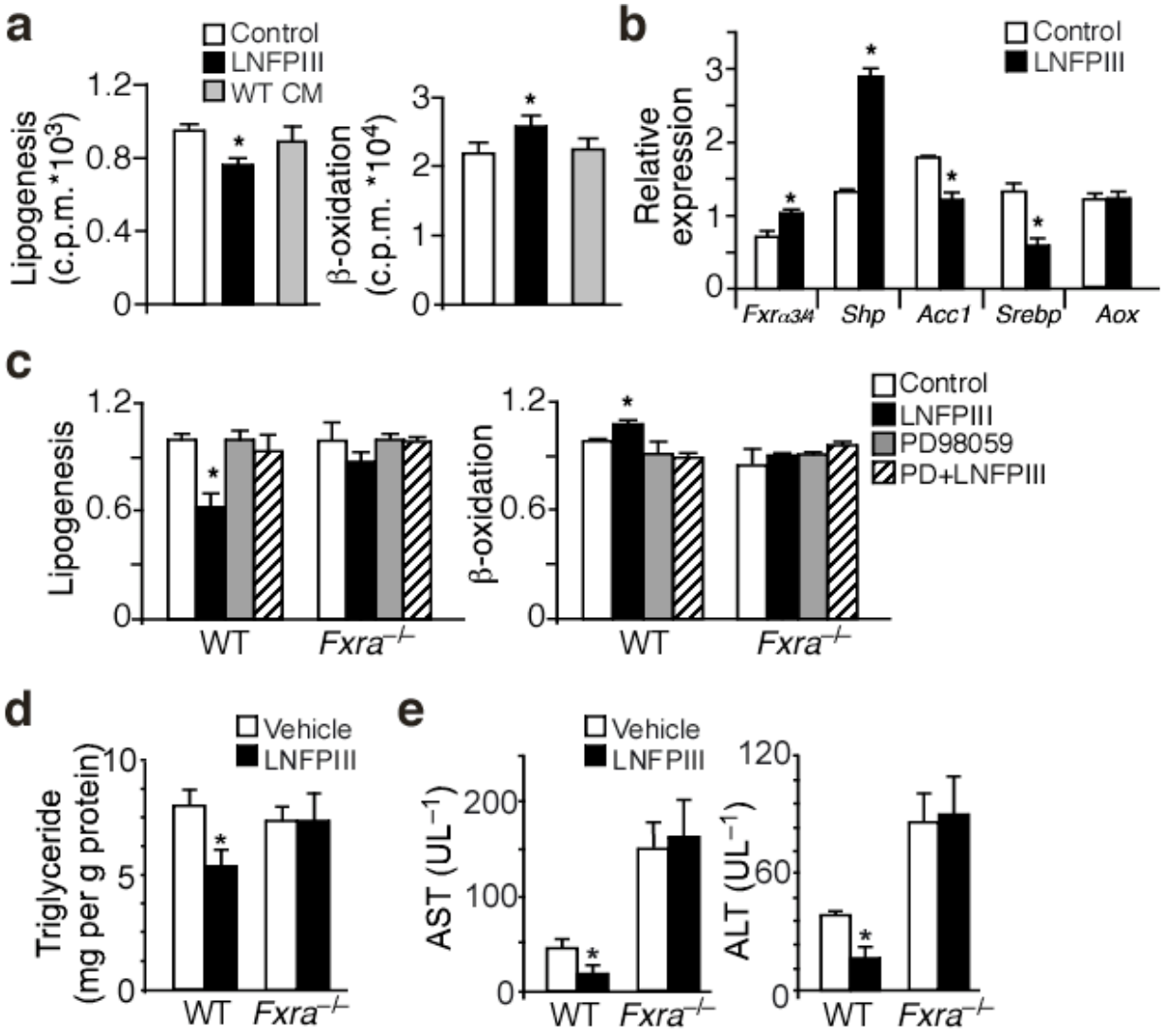


Figure 12 LNFPIII suppresses lipid synthesis through Fxr-α. (**a**) *De novo* lipogenesis (left panel) and fatty acid β-oxidation (right panel) assays in primary hepatocytes treated with vehicle, LNFPIII (20 μg ml^{-1}) or CM from LNFPIII primed wt macrophages. (**b**) Gene expression in hepatocytes treated with vehicle or LNFPIII determined by real-time q-PCR. (**c**) Lipogenic (left panel) and β-oxidation (right panel) assays in hepatocytes isolated from wild type (wt) or $Fxr\alpha^{-/-}$ mice ± LNFPIII ± PD98059. (**d** and **e**) Hepatic triglyceride content and serum AST and ALT concentrations in wt and $Fxr\alpha^{-/-}$mice ± LNFPIII treatment (n = 6). Values are expressed as means ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated 3 times. Studies for $Fxr\alpha^{-/-}$ and control mice were conducted in one cohort (n = 6 per treatment per genotype). *$P < 0.05$ (LNFPIII versus vehicle control).

activity of LNFPIII was unaffected (**Supplementary Fig. 5g–5i** and **Table 4**). Together, the results suggest LNFPIII inhibits hepatic lipogenesis in a Fxrα-dependent manner.

I next sought to determine if Fxrα is a molecular target of LNFPIII. As mentioned earlier, both human and mouse Fxrα contain two major promoters (**Fig. 13a**). Sequence comparison analyses revealed that the 5' regulatory sequences were highly conserved between mouse, rat and human Fxrα genes (**Supplementary Fig. 6a**). The activities of reporters driven by upstream (promoter 1) or downstream (promoter 2) regulatory regions were examined in HepG2 cells (human hepatoma cells). In accordance with the regulation of Fxrα in the liver or isolated hepatocytes, LNFPIII activated Fxrα promoter 2, while SEA induced the activity of both promoters (**Fig. 13a**). *In vivo*, Fxrα protein levels were higher in liver lysates from LNFPIII treated mice (**Fig. 13a**). A minimal LNFPIII responsive region (approximately 130 bp upstream of the transcriptional start site) was defined through serial deletion of promoter 2, which contained consensus binding sites for C/EBP and AP1 (**Supplementary Fig. 6a**). Site directed mutagenesis experiments further identified two overlapping AP1 sites (located between –57 and –47 bp) that were required for the induction of Fxrα promoter 2 by LNFPIII (**Fig. 13b** and **Supplemental Fig. 6b**). Similarly, inhibition of Erk activation, which is upstream of AP1, by PD98059 abolished LNFPIII induced activity of Fxrα promoter 2. In concert, LNFPIII treatment increased levels of phospho-Erk and total Erk in the liver (**Fig. 13c**) and hepatocytes (**Fig. 13d**). Similar results were observed in SEA treated liver cells (**Supplementary Fig. 6c–d**).Collectively, these findings suggest that LNFPIII regulates hepatic lipogenesis through the Erk-AP1-Fxrα axis.

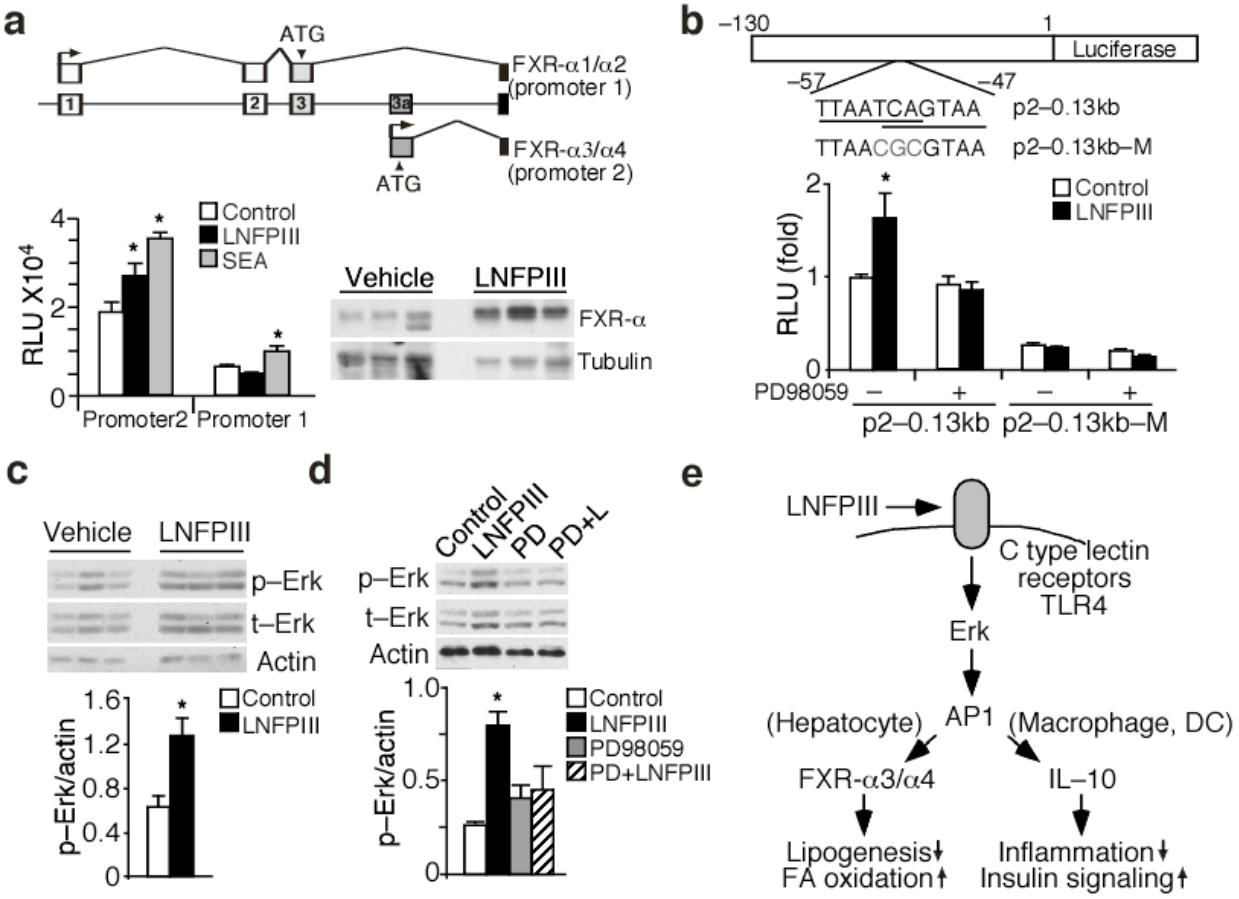


Figure 13 Induction of Fxrα activity by LNFPIII is mediated by Erk-Ap1 signaling. (**a**) Top panel: genomic structure showing alternative promoter usage by human *FXR-α1/α2* (promoter 1) and *FXR-α3/α4* (promoter 2). Bottom left: Relative activities (RLU) of luciferase reporters driven by human *FXRα* promoter 1 (~2 kb) or human *FXRα* promoter 2 (~0.13 kb) in HepG2 cells ± LNFPIII (20 μg ml^{-1}) or SEA (2 μg ml^{-1}). Bottom right: Western blotting showing Fxrα protein levels in livers from vehicle or LNFPIII treated mice. (**b**) Top panel: diagram demonstrating *FXRα* promoter 2 (p2–0.13kb) or mutant (p2–0.13kb–M) reporter constructs. The two overlapping AP1 binding sites and the mutation are shown. Bottom panel: Relative luciferase activities of p2–0.13kb and p2–0.13kb–M ± LNFPIII ± PD98059 (**c**) Erk phosphorylation (p-Erk) in livers of vehicle and LNFPIII treated mice. Bottom panel: Quantification of the Western signal. (**d**) Western blot analyses showing Erk phosphorylation in hepatocytes ± LNFPIII ± PD98059 (representative samples from three experiments each with three biological replicates). Bottom panel: Quantification of the Western signal. Values are expressed as means ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates from one of three repeats. Hepatic p-Erk was determined from one of three metabolic study cohorts (n = 5, showing 3 representative samples). *$P < 0.05$ (LNFPIII or SEA versus vehicle control). (**e**) Model for direct and indirect regulation of metabolic pathways by LNFPIII. DC: dendritic cells.

Table 4. Metabolic parameters of *Fxra*$^{-/-}$ mice treated with LNFPIII

Treatment	Vehicle	LNFPIII
Body weight (g)	40.73 ± 3.23	36.33 ± 3.54
WAT/body weight	0.029 ± 0.003	0.041 ± 0.003
Liver/body weight	0.046 ± 0.004	0.053 ± 0.011
Triglyceride (mg dL^{-1})	46.12 ± 5.22	44.11 ± 4.90
Free fatty acid (mmol L^{-1})	0.40 ± 0.02	0.49 ± 0.03
Cholesterol (mg dL^{-1})	303.18 ± 27.10	315.47 ± 8.16
Glucose (mg dL^{-1})	175 ± 4.38	150 ± 18.78
Insulin (ng mL^{-1})	0.450 ± 0.025	0.198 ± 0.026*

Serum samples were collected after 6 hr fasting. *P<0.05.
Values displayed as mean ± s.e.m. n=6/group

Discussion

In addition to the LNFPIII-IL-10 insulin-sensitizing axis in WAT described in Chapter 2, here I show a role for LNFPIII in improving hepatic function. In the liver, LNFPIII up-regulates bile acid sensing nuclear receptor FXRα and its downstream targets. Activated Fxrα signaling suppresses lipogenesis and protects against hepatic steatosis.

After entering the body, *S. mansoni* settles in the hepatic portal system where male and female worms mate and produce eggs[191]. Egg deposition in the liver is critical for induction of a systemic T_H2 response[191]. It is therefore not surprising that the liver is also a target of LNFPIII. At first glance, it seems unexpected that LNFPIII directly controls Fxrα signaling to regulate hepatic lipid metabolism. Fxrα senses endogenous bile acids and controls bile acid homeostasis by inhibiting production while increasing the recycling of bile acids in the enterohepatic system[239]. Fxrα activation also suppresses *de novo* lipogenesis through Shp-mediated inhibition of Srebp-1c in the liver[234]. Interestingly, *S. mansoni* is incapable of *de novo* synthesis of fatty acids and sterols but is able to synthesize complex lipids from precursors acquired from the host[240]. In addition, it has been shown that bile acids increase the number of deposited parasite eggs[241]. Therefore, the induction of Fxrα by LNFPIII could be the host's response to limit fatty acids and bile acids available to the worm. In contrast, bile acids facilitate lipid absorption in the intestine. Increased bile flow via Fxrα may help schistosomes to extract lipids from the digestive system of the host. Additionally, the induction of Fxrα could serve as an anti-inflammatory mechanism in the liver as Fxrα is known to trans-repress Nf-κb activity[242]. Although future studies are needed to test these potential mechanisms, these data demonstrate that LNFPIII improves overall liver function through targeting the metabolic program.

The signal transduction pathway of LNFPIII has not been fully characterized. In dendritic cells, LNFPIII is recognized by multiple CLRs, including DC-SIGN (also known as Cd209), Mgl1 and mannose receptor. In addition, Erk is proposed to be a downstream effector of LNFPIII[205,243]. Erk/AP1 has been shown to regulate *Il10* expression[211]. Consistent with this observation, the ability of LNFPIII to induce Il-10 production in the macrophage was blocked by Erk inhibition. Notably, I find that primary hepatocytes also express CLRs, such as DC-SIGN (**data not shown**) and LNFPIII is able to activate Erk in the liver, which mediates Fxrα transcriptional regulation and the subsequent suppression of lipogenesis. The gene products of the two promoters (Fxr-α1/α2 versus Fxr-α3/α4) differ by several amino acids located at the N-terminus. Functional differences between isoforms have not been reported[238]. My data suggests that the induction of Fxr-α3/α4 by LNFPIII is sufficient to suppress lipogenesis. While LNFPIII only activates the downstream Fxrα promoter, SEA induces the activities of both promoters. This is not unexpected, since SEA is known to contain multiple bioactive glycans that could signal through different pathways[243]. Together, my data show that helminths may utilize the Erk-AP1 signaling pathway to modulate host metabolism and immune responses (**Fig. 6e**). My study suggests that the M2-like, anti-inflammatory activity of LNFPIII, which is likely the product of helminth's survival strategy, may be used to treat metabolic disorders, such as insulin resistance and nonalcoholic fatty liver diseases.

Materials and Methods

All methods are the same as in Chapter 2. Additional methods utilized in Chapter 3 are detailed below.

Animal experiments. Serum and tissue lipids, ALT and AST were measured using commercial kits as described previously[244]. *Fxra*$^{-/-}$ mice (male, *n*=6/genotype/treatment, C57BL/6J background) were obtained from the Jackson Laboratory. Metabolic studies and treatments in these animals were similar to those in wt C57BL/6J mice. All animal studies were approved by the Harvard Medical Area Standing Committee on Animals.

Immunoblotting experiments. I performed Western blot analyses using antibodies to detect the following proteins: Fxrα (R&D systems, # PP-A9033A-00, Clone A9033A, 1:500 and Santa Cruz Biotechnology # sc-1204, 1:500); p-Erk (Cell signaling # 9101, 1:1000); t-Erk (Cell signaling # 4695, 1:1000).

Primary cells and functional assays. Primary hepatocytes were isolated as previously described[244,245]. Hepatocytes were allowed to attach overnight in William's E, 5% FBS, followed by treatments for 24 hr. For *de novo* lipogenesis, cells were labeled with ^{14}C-acetate and lipids were extracted with a 2:1 (v/v) chloroform:methanol mixture 6 hrs later. β-oxidation was conducted as described previously[245]. For LNFPIII signaling in hepatocytes, attached hepatocytes were pretreated with Erk inhibitor where indicated for 1 hr followed by a 30 min incubation with dextran or LNFPIII.

Expression analyses. The four Fxrα (Nr1h4) isoforms were based on previous reports, which were originally designated as Fxr-α1/α2 and Fxr-β1/β2[238,246]. The latter were renamed as Fxr-α3/α4 to avoid confusion with the rodent Fxrβ (Nr1h5)[238]. For reporter assays, the upstream promoter 1 and downstream promoter 2 regions of human FXRα gene were cloned into the

pGL3-basic luciferase reporter construct and transfected into HepG2 cells in a 96-well format. A β-galactosidase reporter construct was used as a transfection control.

Statistical analyses. Statistical analyses comparing two parameters (between treatments or genotypes) in cell-based work were conducted using the two-tailed Student's *t* test. Two parameters analyses for samples from *in vivo* studies (non-gaussian distribution) were determined using the Mann-Whitney test (11a–c, 12d–e, 13c, Supplementary Figs. 4a–d, 5d–e, 5i and Tables 1–4). Statistics for multi-parameter analyses was determined by One-Way ANOVA followed by Bonferroni posthoc tests (Figs. 12a, 12c, 13a, 13d and Supplementary Figs. 5a, 6c–d). Two-way ANOVA was used to determine statistical significance for GTT and ITT (Supplementary Figs. 5f–g). Values are presented as mean ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for one representative experiment. Experiments were repeated at least three times. $P<0.05$ was considered significant.

Author contributions

Members of the Lee laboratory provided technical assistance.

CHAPTER 4

A role for PPARδ and PPARγ in macrophage bacterial killing

Introduction

It is well established that patients with altered metabolic function such as those with type II diabetes mellitus or elderly people, are more likely to die from bacterial or fungal infections[16,247,248]. Respiratory infections are the fifth leading cause of disease in people aged 65 and older[249]. Vaccines have also proven less effective in these populations[250]. To date, several immunological biomarkers have been associated with the increased risk of infections in these susceptible populations. However, the significance of these immunological changes is poorly understood. Simultaneously, these same populations exhibit a systemically decreased metabolic rate, increased fat accumulation, and increased oxidative stress[251]. Metabolic changes have also been observed in the macrophage[252]. Functionally, myeloid-derived cells from elderly patients and patients with metabolic syndrome have decreased bactericidal capacity and respiratory burst[253-257]. These processes are critically dependent on gross metabolic state, implicating macrophage metabolic dysregulation in the susceptibility to infection. Identifying signaling nodes critical for regulating metabolic function in the macrophage can provide a novel mechanism to improve host bactericidal activity in susceptible populations.

When activated by pathogens or proinflammatory cytokines, macrophages activate signaling cascades that lead to changes in energy producing processes within the cell, including an increased dependance on anaerobic respiration and increased flux through the pentose phosphate pathway (PPP)[116,159]. High-energy molecules, such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NAD(P)H), produced in these processes are crucial for the substrate driven activities of enzymes involved in various processes such as phagocytosis, movement, and cytokine production. In contrast, anti-inflammatory macrophages

display a distinct metabolic phenotype characterized by the reliance on oxidative metabolism for sustained energy for repair and resolution[163].

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors critical for maintaining metabolic homeostasis by sensing extracellular nutrients and modulating intracellular metabolism. The Ppar family of receptors consists of Pparα (Nr1c1), Pparδ (Nr1c2) and Pparγ (Nr1c3) and is best known for sensing fatty acids and regulating metabolic processes[128]. In the macrophage, Pparδ and Pparγ regulate oxidative metabolism and anti-inflammatory macrophage functions, such as apoptotic cell clearance[124,134,258]. Given the established role of PPARs in cellular metabolism, immune cell function and organelle regulation, I hypothesized that Pparδ and Pparγ are critical for coordinating metabolic and immune responses for efficient bacterial killing. Using the gram-positive bacteria *Streptococcus pneumoniae* (*S.pneumoniae*), I show here that Pparδ regulates phagosomal ROS production and subsequent bacterial killing in the macrophage. My data also suggest that Pparγ deficient macrophages are unable to efficiently kill bacteria. Pharmacological interventions that increase Pparδ activity improve the survival of mice infected with *S.pneumoniae*, suggesting that modulating host metabolic sensing pathways can have positive outcomes in various patient populations that have age- or metabolically-weakened immune systems.

Results

Lack of Pparδ in the macrophage inhibits bacterial killing

Given the importance of metabolic pathways in bacterial killing, the role of Pparδ as a lipid sensing nuclear receptor makes it an interesting candidate to integrate metabolic and immune signals and regulate bacterial killing. In fact, macrophages treated with *S.pneumoniae*

exhibited increased expression of *Pparδ* and its known target gene, solute carrier family 25, member 20 (*Slc25a20*) (**Supplementary Fig. 7**). To elucidate a critical role for Pparδ during bacterial infection, I treated fully differentiated PparδKO and wt bone marrow derived macrophages with *S.pneumoniae* to determine killing efficiency in a gentamicin-protection assay. Macrophages lacking Pparδ had approximately two times as many bacteria remaining after two hours compared to wt macrophages (**Fig 14a**). Because of the known role of Pparδ as a metabolic regulator and the critical function of metabolites in efficient bacterial killing[128,164,166], I investigated various metabolic pathways that may be dysregulated. Expression of genes in the pentose phosphate pathway [glucose-6-phosphate dehydrogenase (*G6pdh*) and phosphogluconate dehydrogenase (*Pgd)*] and the mitochondrial β-oxidation pathway [cytochrome C oxidase (*Cox*)-*2* and *Cox3,* ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (*ATP5o*), and cytochrome B (*Cytb*)] was unchanged in wt macrophages compared to knockout cells. Lactate dehydrogenase b (*Ldhb*), the enzyme critical for the macrophage switch to anaerobic respiration during bacterial killing, was also unchanged during infection. (**Supplementary Fig 8a**). Functional assays revealed an inability of PparδKO macrophages to increase glucose uptake during bacterial infection (**Supplementary Fig. 8b**). However, this did not translate into a defect in mitochondrial number, lactate production or ATP levels (**Supplementary Fig. 8c-e**). Therefore, I turned my attention to steps critical for successful phagocytosis (**Fig. 4**).

I found no difference in uptake or binding of bacteria by the macrophage in wt and PparδKO macrophages (**Fig. 14a**). Rate of phagocytosis and number of phagosomes generated by bacterial uptake was unaffected by reduced Pparδ abundances (**Fig. 14b-c**). There was also no defect observed in fusion of the phagosome to the lysosome (**data not shown**). Once the

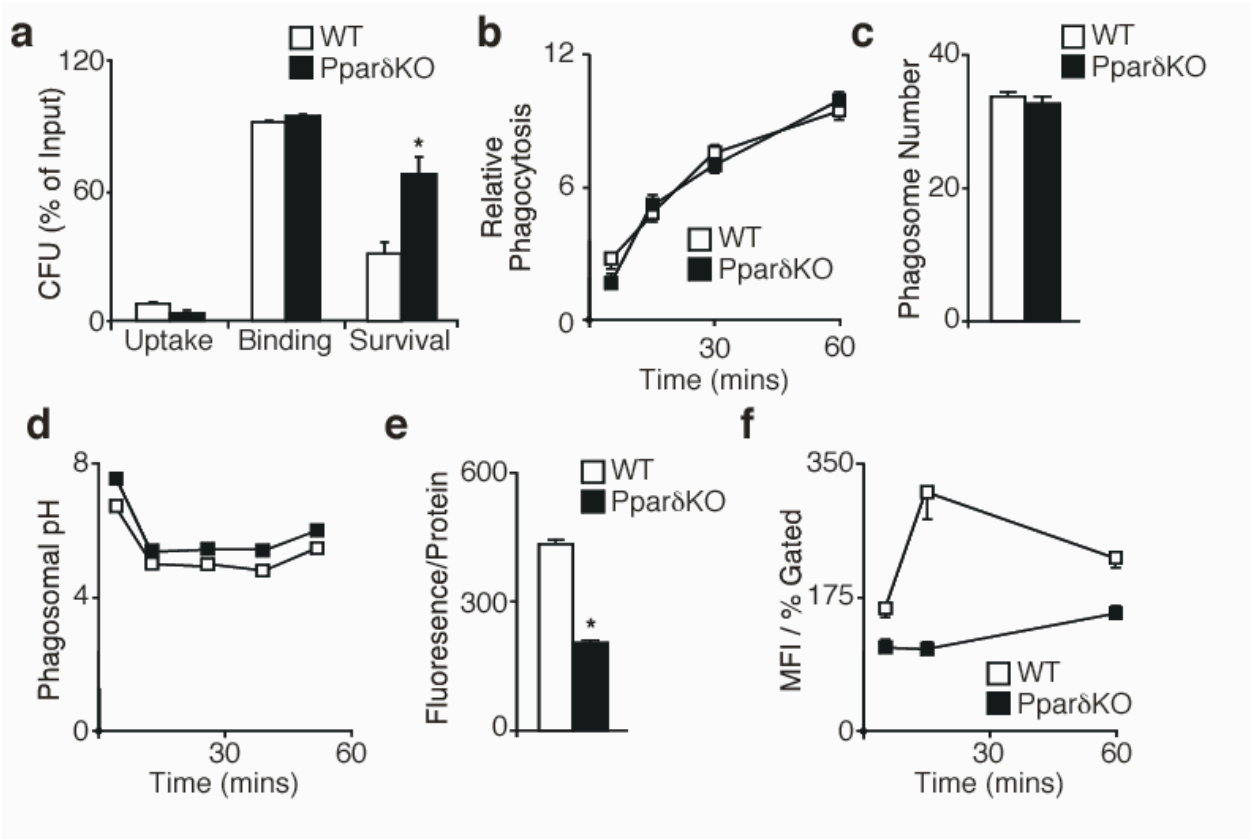


Figure 14: PPARδ promotes efficient bacterial killing. (**a**) *In vitro* killing assay in wt and PPARδKO macrophages. Primary bone marrow derived macrophages were infected with *S.pneumoniae* (10 bacteria: 1 macrophage) to determine intracellular killing *in vitro*. (**b**) Phagocytosis assay in wt and PPARδKO macrophages. Macrophages were infected with heat-killed *S.pneumoniae* labelled with Alexa Fluor 647 (25 bacteria: 1 macrophage) for the indicated times and fluorescence was determined by FACS. (**c**) Number of phagosomes found in heat-killed Alexa Fluor 647: *S.pneumoniae* infected wt and PPARδKO macrophages 60 minutes after infection. Phagosome number was determined by dividing the mean fluorescence intensity (MFI) of the macrophage (with ingested bacteria) by the MFI of a single bacterium. (**d** and **e**) Phagosome acidification is not defective in PPARδKO macrophages. (**d**) Acidification was determined by infecting macrophages with heat killed *S.pneumoniae* dually labeled with FITC (pH sensitive) and Alexa Fluor 647 (pH insensitive). Fluorescence was quantified by FACS and pH was caluculated from the ratio of Alexa Fluor 647: FITC in the unknown samples and that of a known standard curve. (**e**) Phagosomal pH was determined using pHrodo (Invitrogen). (**f**) ROS production is blunted in PPARδKO macrophages. FcOxyburst was used to track the phagosomal oxidative burst by FACS analysis.

phagosome is properly formed, critical enzyme complexes help to acidify the phagosome and produce ROS to help degrade bacteria[259]. In PparδKO macrophages, both phagosomal acidification and ROS production were reduced compared to wt macrophages. (**Fig. 14d–f**). Taken together, my data reveal a novel role for Pparδ in bacterial killing possibly through changes in phagosomal regulation.

PPARδ regulates expression of phagosomal genes

To elucidate a mechanism for Pparδ regulation of phagosomal ROS production, gene expression of phagosomal genes was analyzed in stably transformed wt and PparδKO macrophages prior to and during *S.pneumoniae* infection. Gene expression revealed that some genes from the NADPH oxidase family (*Nox2*, *Noxo1*, and *Noxa1*) were all significantly decreased. Interestingly some of the associated proteins, such as p47phox and p22phox were upregulated in knockout cells compared to wt cells. No difference was observed in gene expression of components of the vacuolar ATPase complex (**Fig. 15a**). The data suggest a role for Pparδ in NADPH oxidase regulation.

To determine whether members of the NADPH complex are direct targets of Pparδ, I first studied Pparδ activity during infection with *S.pneumoniae* by assessing ligand binding activity of Pparδ. When infected with *S.pneumoniae,* macrophages that contained a Gal4-Pparδ ligand binding domain fusion contruct had significantly higher luciferase activity than cells containing a Gal4 control plasmid (**Fig. 15b**). I then generated luciferase constructs with promoters 2kb upstream of the transcription start site of both *Noxo1* and *Nox2*. I observed that Pparδ ligand, GW501516, was able to increase luciferase activity of the 2kb *Noxo1* promoter, but not the 2kb

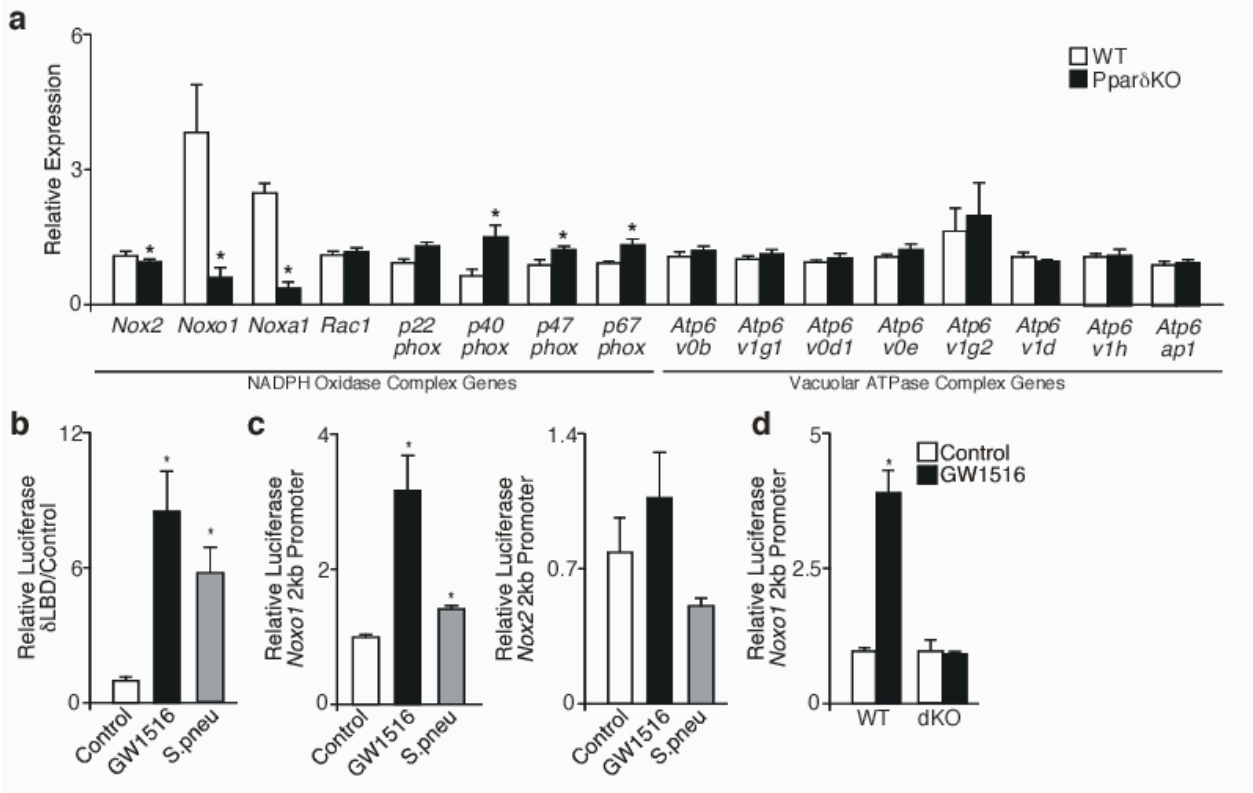


Figure 15: Pparδ regulates phagosome function by regulating Noxo1 transcription. (**a**) Expression of NADPH associated proteins Noxo1 and Noxa1 were reduced in PparδKO macrophages. Expression of genes that comprise the vacuolar ATPase and the NADPH oxidase complex were analyzed. (**b**) *S.pneumoniae* increases activity of the ligand binding domain (LBD) of Pparδ. Macrophages were transfected with Gal4-Pparδ LBD along with a Gal4 responsive luciferase construct. After transfection, macrophages were infected with *S.pneumoniae* for 12 hours. (**c**) Pparδ ligand, GW501516, increases luciferase activity of the *Noxo1* promoter, but not the *Nox2* promoter. 2kb of the *Noxo1* or *Nox2* promoter upstream of the transcriptional start site was cloned into a plasmid upstream of the luciferase gene. Transfection was done in Raw264.7 macrophages. (**d**) Pparδ ligand is unable to increase *Noxo1* transcription in the absence of Pparδ. Transfection was done in stably transformed wt and PparδKO macrophages.

Nox2 promoter (**Fig. 15c**). This effect was lost in cells lacking *Pparδ* (**Fig. 15d**). Together my data suggest that during bacterial infection Pparδ regulates *Noxo1* transcription.

Pparδ promotes bacterial killing *in vivo*

To understand if Pparδ regulation of phagosomal ROS production contributes to bacterial killing *in vivo*, I measured ROS production in isolated alveolar macrophages from wt and macrophage specific Pparδ knockout mice. Similar to my *in vitro* data, Pparδ knockout alveolar macrophages were unable to produce phagosomal ROS (**Fig. 16a**). Oral gavage with GW501516 for three days prior to alveolar macrophage isolation increased ROS production in wt macrophages, but not in Pparδ knockout macrophages (**Fig. 16a**). To understand if defects in phagosomal ROS resulted in an inability to kill bacteria *in vivo*, myeloid specific Pparδ knockout mice ($Pparδ^{LysM}$; $Pparδ^{fl/fl}$ used as wt controls) were infected intranasally with *S.pneumoniae* and surviving bacterial burden was measured from bronchoalveolar lavage fluid (Balf) 24 hours post infection. (**Fig. 16b**). Circulating cell numbers were not different in infected wt or $Pparδ^{LysM}$ mice, suggesting that $Pparδ^{LysM}$ mice have no defect in recruitment of cells to the site of infection. Differential cell counts of Balf indicated that the number of neutrophils was significantly higher in knockout mice compared to wt mice, in agreement with the increase in bacterial counts in the lung (**Supplementary Fig. 9a-c**). No differences were observed in Tnfα, while Il-6 concentrations were significantly higher in Balf of knockout mice compared to wt mice (**Supplementary Fig. 9d**). Gene expression from infected alveolar macrophages 24 post infection indicated a defect in both *Noxo1* and *Noxa1* expression, similar to the results observed *in vitro* (**Fig. 16c**). The results indicate a critical role for Pparδ in regulating phagosome function and efficient bacterial killing *in vivo*.

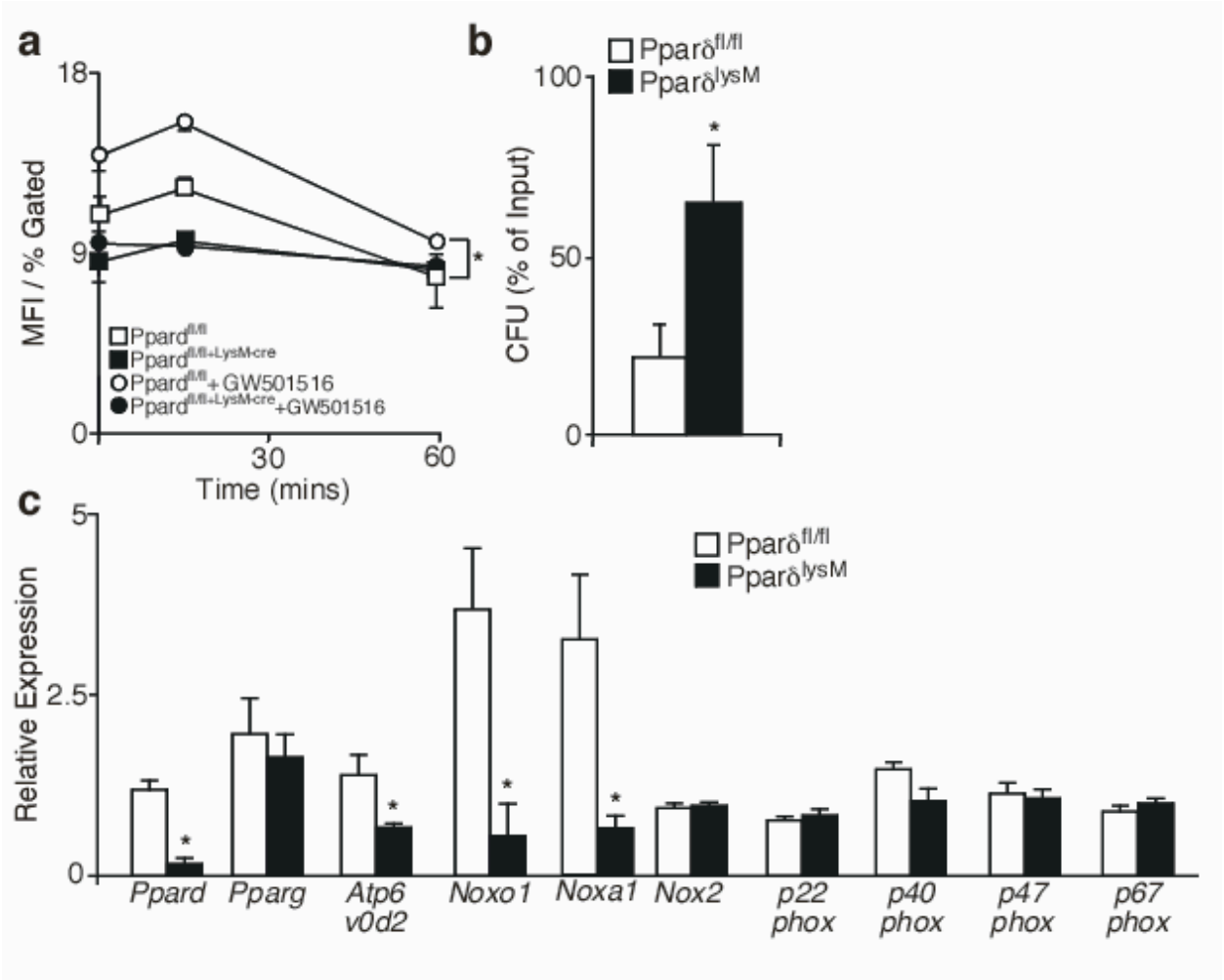


Figure 16: Alveolar macrophages from PPARδ^{lysM} mice have a defect in ROS production and are unable to kill bacteria. (**a**) Three day *in vivo* treatment of PPARδ ligand, GW501516, is able to increase ROS production in alveolar macrophages from wt mice. This effect is lost in knockout mice. Mice were gavaged with 2mg/kg/day of GW501516. Alveolar macrophages were isolated and ROS was determined with FxOxyburst. (**b**) 24 hour *in vivo* killing assay. Wt and PPARδ^{lysM} mice were infected intranasally with *S.pneumoniae* for 24hrs and percent bacterial survival was determined. (**c**) Gene expression from lavage fluid from mice 24 hours after infection with *S.pneumoniae*.

PPARδ ligand, GW501516, improves macrophage bacterial killing

I next wanted to investigate whether ligand treatment *in vivo* could improve bacterial killing. Mice were gavaged with GW501516 for 3 days (2 mg kg^{-1} per day) and infected with *S.pneumoniae* for 24 hours. Surviving bacteria was determined from Balf. There were less bacteria remaining in the lungs of ligand treated mice compared to vehicle treated mice (**Fig. 17a**). Gene expression analyses of Balf cells showed an increase in expression of *Pparδ* and its target gene *Slc25a20*, confirming ligand efficacy. The expression of *Noxo1* was also increased (**Fig. 17b**). Defective bacterial killing in $Ppar\delta^{LysM}$ mice was associated with earlier death relative to wt mice. Ligand treatment did not improve survival in knockout mice. However, with ligand treatment, no death was observed in wt mice (**Fig. 17c**). Taken together, my data suggest that Pparδ regulates phagosomal ROS production and *Noxo1* transcription. Ligands that target Pparδ improve bacterial killing suggesting a role for activators that target metabolic sensors in the treatment of bacterial infections.

Epidemiological data has shown that neonates, elderly people (65+), and people with type 2 diabetes are more susceptible to bacterial infections[16,260,261]. In neonatal and elderly mice, expression of *Slc25a20* and *Noxo1* was significantly decreased in unstimulated macrophages (**Fig. 17d-e**). This decrease in gene expression correlated with a decrease in ROS production *ex vivo* (**Fig. 17f**). Three-day ligand treatment in three-year-old mice was associated with a 50% increase in ROS production, although ligand was unable to completely rescue ROS production to the levels of two-month-old mice. Together, I show here that ligand treatment can be an effective method to increase ROS production in older mice that have defects in phagosomal gene expression.

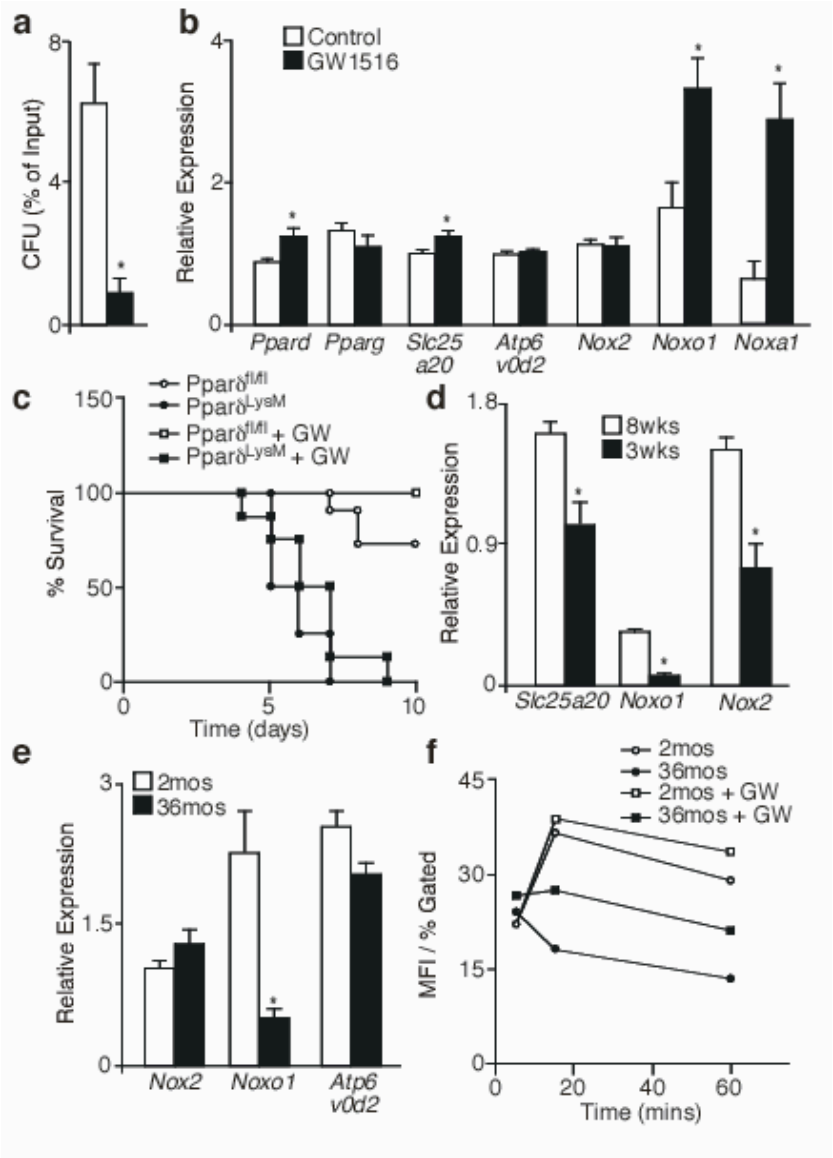


Figure 17: Ligand activation of PPARδ improves bacterial killing *in vivo*. (**a**) Oral gavage of GW501516 improves bacterial killing *in vivo*. 24 hours post infection, lavage fluid was isolated and analyzed for live bacterial forming colonies. (**b**) Gene expression analyses on lavage fluid indicate that ligand activation of PPARδ increases expression of known target gene (Slc25a) and phagosomal markers (NOX2 and NOXO1). Mice were gavaged with GW501516 for three days prior to intranasal infection. (**c**) Survival curves were determined for wt and macrophage specific PPARδ knockout mice with and without ligand treatment. Wt and PPARδ^{lysM} mice were gavaged with GW501516 orally for 3 days prior to and throughout the course of infection. On day 0, mice were infected with 10^5 CFU of *S.pneumoniae* and were monitored daily for signs of death and changes in body weight. (**d**) Gene expression of alveolar macrophages from uninfected 3wk or 8wk old mice. (**e**) Gene expression of alveolar macrophages from uninfected 2mos or 36mos old mice. (**f**) Three day *in vivo* treatment of PPARδ ligand, GW501516, is able to increase ROS production in alveolar macrophages from both young and old wt mice. Mice were gavaged with 2mg/kg/day of GW501516. Alveolar macrophages were isolated and ROS was determined with FxOxyburst.

Pparγ also influences macrophage bactericidal activity

The inability of Pparδ ligand to completely rescue the defects in phagosomal ROS production in three-year-old mice implicated another receptor in regulation of ROS production. Iturned my attention to the role of Pparγ in the same processes. During a bacterial infection, Pparδ and Pparγ become transcriptionally activated (**Fig. 18a**). However, this regulation appears to be temporal with maximal *Pparg* expression appearing at 1 hour post infection followed by *Ppard* expression peaking at about 9 hours post infection (**Fig. 18a**). Additionally, Pparγ ligand activation with troglitazone, led to increases in expression of genes critical for anaerobic respiration and lactate production (*Ldhb*) and the pentose phosphate pathway (*Pgd*). Additionally, genes involved in phagosomal ROS production, also shown to be regulated by Pparδ, were increased by troglitazone treatment (**Fig. 18b**). However, analysis of uninfected alveolar macrophages from $Ppar\delta^{LysM}$ or $Ppar\gamma^{LysM}$ mice suggests a differential role for both receptors (**Fig. 18c**). In alveolar macrophages from $Ppar\delta^{LysM}$ mice, *Ppard* expression is reduced about 95% while there are no differences observed in Pparγ expression compared to wt mice. In these cells, I only observe differences in the expression of phagosomal genes. However, in $Ppar\gamma^{LysM}$ mice, expression of both *Ppard* and *Pparg* was reduced about 50% compared to wt macrophages. Additionally, expression of both phagosomal and metabolic genes was reduced significantly, with the reduction in phagosomal gene expression presumably due to a decrease in *Ppard* expression (**Fig. 18d**). Preliminary data suggest a role for Pparγ in regulating the metabolic switch from aerobic to anaerobic respiration, thereby producing high-energy molecules such at ATP and NADPH that are critical for bacterial killing.

To access the role of Pparγ in bacterial killing, I performed *in vitro* and *in vivo* bacterial

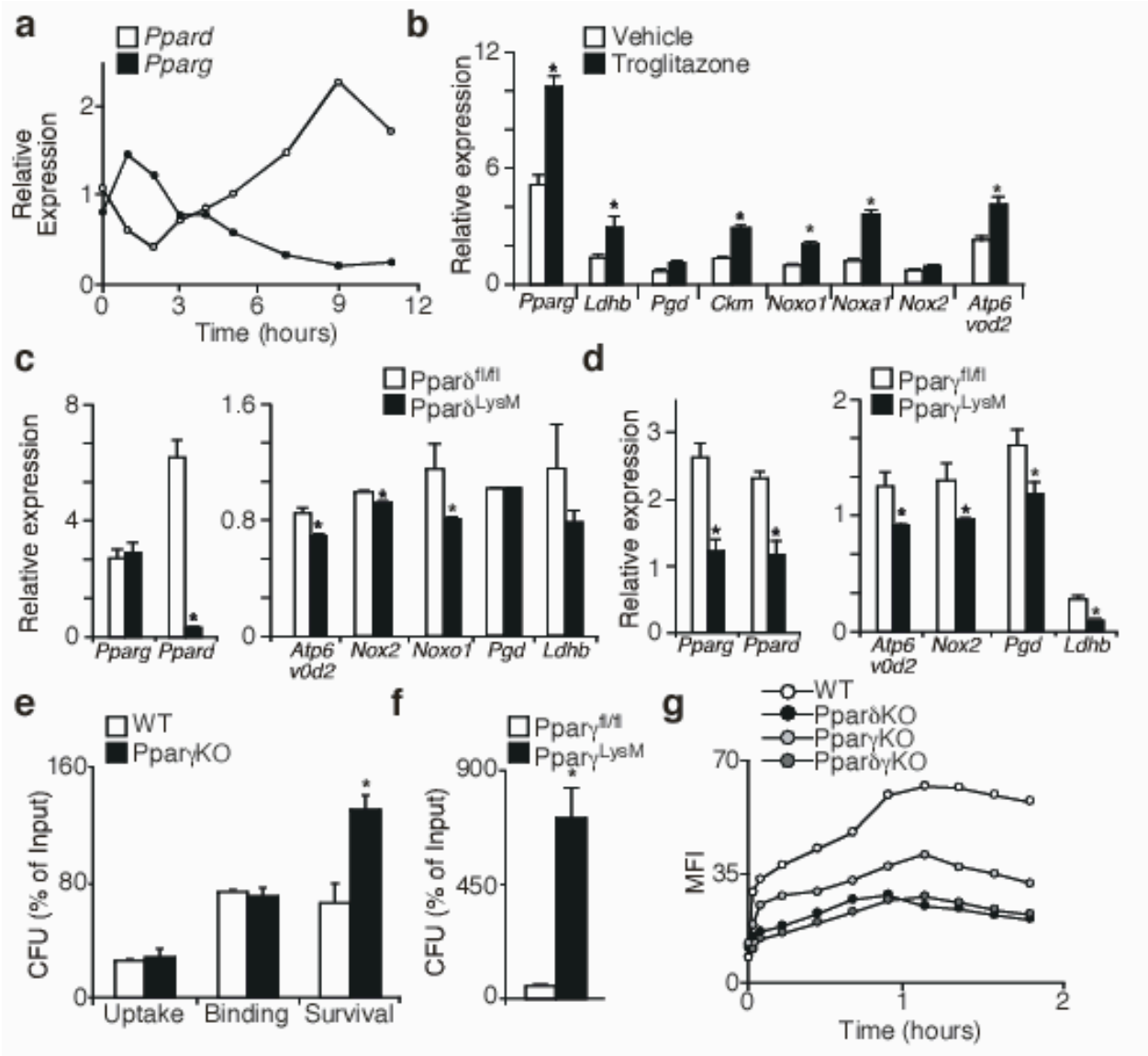


Figure 18: PPARγ also contributes to macrophage bactericidal activity. (**a**) PPARδ and PPARγ expression is increased during macrophage bacterial infection. BMDM were treated with *S.pneumoniae* for the indicated times. (**b**) Gene expression analysis of PPARγ overexpressing macrophages treated with or without ligand. Troglitazone is able to increase expression of metabolic and phagosomal genes. (**c** and **d**) Gene expression of alveolar macrophages from uninfected PPARδ^{lysM} or PPARγ^{lysM} mice. (**e**) *In vitro* killing assay in wt and PPARγKO macrophages. Primary bone marrow derived macrophages were infected with *S.pneumoniae* (10 bacteria: 1 macrophage) to determine intracellular killing *in vitro*. (**f**) 24 hour *in vivo* killing assay. Wt and PPARγ^{lysM} mice were infected intranasally with *S.pneumoniae* for 24hrs and percent bacterial survival was determined. (**g**) Phagosomal ROS production is lost in knockout macrophages. ROS was determined with FcOxyburst (Invitrogen).

killing assays. PparγKO macrophages also displayed similar rates of uptake and binding of bacteria compared to wt cells. PparγKO macrophages had 50% more surviving bacteria remaining in the macrophage compared to wt macrophages after 2 hours (**Fig. 18e**). Similar results were obtained *in vivo* when Pparγ^{LysM} mice were infected intranasally with *S.pneumoniae*. 24 hours post infection, Pparγ^{LysM} mice had significantly more bacteria in the lungs compared to wt mice (**Fig. 18f**). These mice had similar numbers of cells within the lavage fluid. Differential cell counts of the Balf suggested increased neutrophil percentages, in accordance with increases in bacteria number (**Supplementary Fig. 10a)**. Mechanistically, macrophages from knockout macrophages were also defective in Fc mediated ROS production **(Fig. 18g)**. As expected from the single knockout phenotypes, mice with macrophage specific deletion of both Pparδ and Pparγ (Pparδγ^{LysM}) are defective in both *in vitro* and *in vivo* bacterial killing (**Supplementary Fig. 10b-d)**. These mice are also unable to survive a bacterial challenge (**Supplementary Fig. 10e)**. It is important to note that the double knockout mice display an increase in cell number in the Balf post infection, a phenotype not observed in either of the single knockout mice. Both Pparδ and Pparγ appear to display similar functional deficiencies, as all three types of mice exhibit similar defects in phagosomal ROS production. Taken together, it is clear that Pparγ plays a critical role in macrophage bacterial killing.

Discussion

In Chapter 4, I investigated the role of lipid sensing nuclear receptors in bacterial killing. Here, I show that both Pparδ and Pparγ have a significant role in bacterial killing. I show that Pparδ regulates phagosomal ROS production and transcriptional activation of *Noxo1*. Lack of Pparδ in the macrophage contributes to inefficient bacterial killing. Pparγ^{LysM} mice also exhibit

defects in bacterial killing *in vitro* and *in vivo*. The mechanism of action for Pparγ regulation of bacterial killing is unclear.

Pparδ is a lipid sensing nuclear receptor. Despite its role in regulating metabolic processes in other cells, Pparδ does not appear to regulate these pathways during bacterial killing. As such, I have elucidated a mechanism by which Pparδ regulates phagosomal ROS production. The defects in ROS production lead to alterations in the ability of the macrophage to kill bacteria. This is observed *in vitro* and *in vivo* suggesting that there is a cell autonomous defect that contributes to the inability of macrophages lacking Pparδ to kill bacteria. I suggest here that the defect arises from an inability of Pparδ to regulate *Noxo1* transcription. Noxo1 is a homologue of p47phox, an organizer protein that has been shown to associate with the Nox2 complex to mediate proper phagosomal ROS production (**Fig. 5**). Phosphorylation of the AIR domain in response to pathogen ligation of surface receptors activates p47phox allowing for a tight regulation of ROS production in the phagosome[156]. Lack of the AIR domain in Noxo1 is thought to lead to constitutive activation and therefore contributes to the activity of the more ubiquitous oxidases, Nox1 and Nox3[156,172]. To date, a role for Noxo1 in phagosomal ROS production and bacterial killing has not been shown.

Recently it was observed that Noxo1 is expressed in macrophages and that mRNA is induced in the presence of inflammatory agents such as LPS and Tnfα, suggesting that transcriptional upregulation of Noxo1 may play a role in the bactericidal activity of the macrophage[171,262]. Through *in vitro* assays, I show that Pparδ ligand, GW501516, is able to increase activity of the promoter of *Noxo1*. This effect was lost in cells that did not contain Pparδ. Analysis of the promoter of *Noxo1* for PPAR binding elements revealed no sites that

match previously described patterns. One possible explanation is that Pparδ is activating a signaling cascade that converges on *Noxo1* upregulation. However, the functional consequences of increases in *Noxo1* transcriptional activation remain unclear. Several studies showed that Noxo1 can associate with Nox2 and p22phox[263-265]. Because these were done *in vitro* by overexpressing all of the proteins, it is arguable that the results are due to a forced association of the complexes. To address this question *in vitro* and *in vivo*, I am currently generating reagents that will allow for overexpression or knockdown Noxo1 in both wt and PparδKO macrophages. With these tools, I hope to link the role of Noxo1 to Pparδ regulation of phagosomal ROS production.

Functional activation of Pparδ occurs when ligand binds to the ligand-binding domain of the protein and allows for association with promoter elements within target genes. My data show that stimulation of the macrophage with live or heat-killed bacteria can generate an endogenous ligand that activates Pparδ. It is possible that lipids on the surface of the bacterium could act as ligands, despite the fact that Pparδ is physically separated from the bacterium by at least the phagosomal membrane. It is unclear how bacteria are able to increase Pparδ activity and more work needs to be done to understand this regulation.

In addition to the spatial disconnect between Pparδ and the bacterium, there is also a temporal disconnect. Activation of the macrophage occurs within seconds. The oxidative burst occurs within 15 minutes. Bacterial killing begins as early as 30 minutes after initiation of ingestion[266]. However, Pparδ, as a transcription factor, needs to be activated, initiate transcription and translation, and those proteins need to be used to effect a change. This process takes much longer than the time it takes to observe an oxidative burst or even to observe

bacterial killing. Rapid transcriptional activation has been observed as in the case of Nf-κb[161], and a similar mechanism may be regulating rapid activation of Pparδ. Another explanation is that deficiencies in Pparδ manifest as defects in target proteins and lipids in the cell prior to bacterial infection. Pparδ could also be acting as a signaling molecule, instead of a transcription factor. Regardless of the mechanism, it is clear that Pparδ is able to affect phagosomal ROS production, either through an extremely rapid signal transduction cascade, a non-transcriptional mechanism, or prior deficiences in the cell. Further investigation will help to address this question.

While the focus of this chapter was on Pparδ, I also presented data suggesting that Pparγ has a similar functional defect in bacterial killing. While PparγKO macrophages were also unable to increase phagosomal ROS production, this defect was not as significant as the defect in PparδKO macrophages. The data suggest that although redundant in function, Pparδ and Pparγ may regulate different pathways that regulate bacterial killing. Preliminary data from uninfected macrophages suggest that Pparδ may play a role in phagosomal regulation, while Pparγ appears to regulate the metabolic pathways that would generate ATP and NADPH. This data is supported by the fact that PparγKO macrophage exhibit defects in ATP and NADPH levels (**Data not shown**). Additionally, mice that are deficient in both receptors show similar phenotypes to both single knockout mice. However until a clear mechanism for Pparγ is determined, the interactions between Pparδ and Pparγ remain unclear.

Using PparδγKO macrophages, I isolated phagosomes and identified several potential bands of interest whose expression was decreased in phagosomes from knockout compared to wt macrophages. These proteins could be mediating the defects observed in phagosomal ROS production. Using mass spectrophotometry, I can identify these bands in an unbiased manner

(**Supplementary Fig. 11**). Additionally, a comparison of phagosomal composition from PparδKO, PparγKO, and PparδγKO could help to identify pathways that are differentially regulated by the two receptors. Coomassie stain of the phagosomal proteins also showed reduced levels of protein in ATP pretreated phagosome samples from wt macrophages, as previously described in the literature[267]. This effect was lost in PparδγKO, suggesting that ATP availability may be influencing phagosome function (**Supplementary Fig. 11**).

Neonates, elderly patients, and people with type 2 diabetes have increased rates of morbidity from bacterial and fungal infections compared to a young, healthy population, suggesting a common defect in immune function[16,260,261,268]. A previous study showed that mice with higher activity of nulear receptor co-repressors display decreased Pparδ activity and exhibit pathologies associated with aging. Polymorphisms in these same co-repressors are associated with type 2 diabetes and increased insulin resistance[269]. Age-associated changes in Ppar nuclear protein, mRNA levels, and promoter binding were also observed in rat kidneys[251,270]. I show that in alveolar macrophages from both young and old mice, expression of phagosomal genes is decreased. I also observe that in older mice, ROS production is decreased. Pparδ ligand can increase phagosomal ROS production in these mice. Ligand treatment is also able to improve bacterial killing and survival in eight-week-old mice. Together, I suggest a novel mechanism to increase host bacterial killing potential to improve killing efficiency. Further studies will determine if ligand treatment post infection can recapitulate these same findings. Additionally, studies should be conducted addressing the ability of combination treatments involving Pparδ ligands and antibiotics in the treatment of bacterial infections. While Pparδ ligand appears to improve bacterial killing, it is also likely that use of multiple ligands to improve several pathways of host killing could be much more potent. If these pathways regulate bacterial killing

in the human macrophage, it would also be exciting to see if similar ligands could be used to treat other types of bacterial, viral, or fungal stresses. In recent years, a lack of novel antibiotics and the rise of multi-drug resistant pathogens have led to the immediate need for novel therapeutics (CDC, WHO). The link between Pparδ and phagosomal function provides a novel mechanism to improve bacterial killing.

Material and Methods

All methods are the same as in Chapter 2 and 3. Additional methods utilized in Chapter 4 are detailed below.

Bacterial growth and manipulation. *Streptococcus pneumoniae,* serotype 3 was obtained from American Type Culture Collection. Bacteria were cultured as described previously [271]. For large volumes of *S.pneumoniae*, bacteria was cultured in Tryptic Soy Broth for 8 hours at 37^{o}C without shaking. Fluorescent magnetic beads (BM570, Bangs Laboratory) were conjugated to *S.pneumoniae* as previously described[272]. *S.pneumoniae* was heat-killed by incubation at 50^{o}C for 40mins.

Animal experiments. Pparδ and Pparγ floxed animals were generated as described previously[273,274]. Pparδ and Pparγ mice were crossed to lysozyme cre mice (Jackson laboratory) to generate Pparδ^{LysM}, Pparγ^{LysM}, and Pparδγ^{LysM} mice. Pparδ$^{fl/fl}$, Pparγ$^{fl/fl}$, and Pparδγ$^{fl/fl}$ mice were used as wt controls. All mice were maintained on a C57BL/6 background. For *in vivo* killing assays, mice were anesthetized with a ketamine/xylazine solution and infected intranasally with 10^{6} CFU *S.pneumoniae.* 24 hours post infection, mice were sacrificed with

isofluorane and Balf was extracted[271]. For survival experiments, mice were infected intranasally with 10^5 CFU *S.pneumoniae*. Mice were observed for signs of distress twice a day for 2 weeks[271]. Ligand was gavaged for the indicated amount of time at a dose of 2mg kg^{-1} per day. All animal studies were approved by the Harvard Medical Area Standing Committee on Animals.

Primary cells and stable cell lines. Bone marrow derived macrophages were differentiated as described previously and in chapter 2. Stables lines were created from bone marrow of all the different knockout and control mouse lines using a CreJ2 viral system[275,276]. A retroviral Cre virus was then used to generate clonal wt and knockout cell lines. Retroviral vectors were also used to generate cell lines overexpressing Pparδ or Pparγ. Alveolar macrophages were obtained from mice by lavage after sacrifice.

Functional assays. Gentamicin protection assays were used for *in vitro* bacterial killing[277,278]. Briefly, macrophages were incubated with *S.pneumoniae*. After 1 hour, cells were washed and gentamicin (200μg/ml) was used to kill extracellular bacteria. Cells were allowed to proceed with killing for an additional hour. Colony forming units (CFU) were determined as a measure of bacterial survival. ROS assays were done using FcOxyburst according to manufacturers protocols using either FACS or a 96-well luminometer (Invitrogen). pH was determined with *E.coli* labelled pHRODO (Invitrogen). ATP and lactate were measured using commercially available kits. Phagocytosis and phagosome number were determined FACS using Alexa Fluor 647 labeled heat killed *S.pneumoniae*[279]. Phagosomal pH was measured by FACS using heat killed *S.pneumoniae* that was doubly labeled with Alexa Fluor 647 and FITC[279]. Phagosomes

were isolated as previously described[280]. Briefly, macrophages were incubated with magnetic beads and allowed to phagocytose. Post infection, cells were collected and samples were homogenized manually. Beads were isolated from supernatants and analyzed by Western blot. Equal fluorescence was loaded. Mitotracker (Invitrogen) was used to determine mitochondrial number. Glucose uptake was determined as previously described.

Immunoblotting experiments. I performed Western blot analyses using the following antibodies: v-ATPase D2 (Santa Cruz # sc-69111, Clone S-20, 1:500); gp91phox (Nox2, Santa Cruz # sc-5827, Clone C-15, 1:500); Lamp1 (Santa Cruz # sc-19992, Clone 1D4B, 1:1000); Actin (Cell Signaling #4970, Clone 13E5, 1:1000).

Statistical analyses. Statistical analyses comparing two parameters (between treatments or genotypes) in cell-based work were conducted using the two-tailed Student's *t* test. Two parameters analyses for samples from *in vivo* studies (non-gaussian distribution) were determined using the Mann-Whitney test (Figs. 14b-c, 15a,b,d,e, 16c-d, f and Supplementary Figs. 9a-d, 10a, 10c-d). Statistics for multi-parameter analyses was determined by One-Way ANOVA followed by Bonferroni posthoc tests (Figs. 13c-e, 5c, 6a, 6d). Two-way ANOVA was used to determine statistical significance for GTT and ITT (Figs. 14a, 15f,). Kaplan-Meier statistics were used for the survival curves (Fig. 15c and Supplementary Fig. 10e). Values are presented as mean ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for one representative experiment. Experiments were repeated at least three times. $P<0.05$ was considered significant.

Author Contributions

Dr. Les Kobzik and Dr. Zhiping Yang taught us the protocols for *S.pneumoniae* infections and provided me with reagents and technical support. Dr. Lynda Stuart and Dr. Anna Sokolovska taught me fluorescent phagosome assays.

CHAPTER 5

Concluding Remarks

Summary and Significance

A critical balance exists between immune and metabolic pathways within the host. In the course of aging or in states of gluttony and energy storage, this homeostasis becomes dysregulated leading to a plethora of diseases characterized by chronic inflammation, poor immune responses, and metabolic stress. With improved hygiene and access to better healthcare, the world population is rapidly aging[249]. Associated with this increase in an elderly population is an increase in the incidence of cancer, the metabolic syndrome, and infectious diseases[249]. Efforts to identify effective therapies for treatment of associated pathologies are paramount (ADA, WHO). Understanding the homeostatic balance between immune systems, metabolic systems, and non-self pathogens can help to inform the development of immuno-metabolic therapeutics to treat immunological and metabolic diseases.

In this thesis, I addressed three previously uncharacterized interactions that can help to deal with the growing burdens of metabolic and infectious diseases. In chapter 2, I showed that immune modulation by pathogenic products can alter metabolic function. Here, I demonstrate the utility of LNFPIII as an immunomodulatory therapeutic. In chapter 3, I describe a previously unidentified observation that helminth derived glycans can modulate hepatic lipogenic function. The role of this appears to be evolutionary and provides a novel mechanism to treat fatty liver diseases. This observation also suggests that agonists or antagonists of host metabolic pathways can be used treat infectious diseases. In this example, use of Fxr modulators could be developed as a novel treatment for schistosomiasis. This notion of altering metabolic pathways to treat infectious diseases serves as the foundation of the work described in chapter 4. My data suggest that phagosomal ROS production is dependent on Pparδ activity. In old mice or in genetic deletion of Pparδ, where Pparδ activity is low or nonexistant, macrophages have decreased

phagosomal ROS production, leading to increased bacterial burden. Increasing Pparδ activity through ligand activation proved to be a potential mechanism to improve bacterial killing in susceptible murine populations. Together, the data in these three chapters support the concept that altering metabolic pathways can be critical in treating infectious diseases. The data also show that altering immune states can directly influence metabolic outcomes. However, these studies are only the beginning of an exciting area of research that has potential for application to many different organ systems, immune platforms, and infectious agents. By understanding these interactions, we can work to develop novel therapeutics that can influence both metabolic and immunological dysfunctions. Below are areas of research that should be addressed to improve the understanding of these critical pathways.

Current state of research: Role of inflammation in metabolic dysregulation

Macrophages have evolved to sense dietary fats. The role of cholesterol in macrophage pro-inflammatory responses and atherosclerosis is well established[42,281]. Recent studies have further demonstrated that fatty acids have a broad spectrum of activities on macrophage activation. This is particularly relevant in obesity, in which the interaction of adipocytes and macrophages through fatty acid dependent mechanisms is critical to metabolic homeostasis (**Fig. 3**). Dietary ω-3 fatty acids and their derivatives, including EPA, DHA and resolvins, can inhibit inflammatory responses through GPCRs or PPARs[145,146] and as such, represent an attractive means for dietary intervention to reduce metabolic inflammation. In addition to dietary fats, studies have suggested that some amino acids, such as arginine and glutamine, promote insulin sensitivity and insulin secretion[282-284]. However, epidemiological data also suggests that levels of branched chain amino acids are associated with diabetes as early as 12 years before onset of frank

insulin resistance[285,286]. The role of amino acid sensing pathways in macrophage function and metabolic inflammation is controversial. In cultured macrophages, glutamine and arginine are critical for cytokine secretion, eicosanoid production and phagocytic uptake during macrophage activation[287]. Mammalian target of rapamycin (mTOR), a major amino acid sensing molecule, is anti-inflammatory in the macrophage. However, mTOR inhibition by rapamycin blocks MCP1 production, despite its pro-inflammatory phenotype[288]. Additional work is needed to clarify the roles of amino acids in metabolic syndrome and infectious diseases and whether these effects are mediated, in part, through macrophage activation.

While it is evident that metabolic stress (e.g., fatty acids, ceramides and ox-LDL/cholesterol) induces macrophage inflammation, the detailed mechanisms through which pro-inflammatory signaling pathways cause metabolic diseases, notably in the setting of insulin resistance, remain unclear. For example, contradictory results have challenged the role of myeloid Jnk1 in insulin resistance[289,290]. It appears that the initiation of a chronic inflammatory cycle may be due to loss of anti-inflammatory and/or regulatory signaling. However, the beneficial effects associated with alternative activation also require further investigation, as T_H2 cytokines are known to mediate allergic responses. Recent reports have implicated several other immune cell types, such as T lymphocytes, mast cells, natural killer cells and eosinophils, in modulation of insulin sensitivity[126,186-188,291-293]. For example, T cell deficient $Rag^{-/-}$ mice are more susceptible to diet induced obesity and adoptive transfer of $Cd4^{+}Foxp3^{+}$ T regulatory cells restores energy balance in these mice[186,188,294]. These studies indicate that the interactions between the innate and adaptive immune systems also contribute to the onset of the metabolic syndrome and implicate an auto-immune response in the associated pathologies. What have not been described are the

temporal recruitment and activation of various immune cells in metabolic tissues and the role of their interaction in metabolic diseases.

Other anti-inflammatory or immunomodulatory mechanisms may be relevant in maintaining metabolic homeostasis. Certain miRNAs are up-regulated by TLR signaling and act through Nf-κb pathways to dampen the inflammatory response[295,296]. miRNAs have also been implicated in atherosclerotic plaque initiation and expansion[297]. In addition, gut flora has been shown to modulate energy utilization efficiency and adiposity and contribute to the onset of metabolic diseases [298,299]. Helminth infection is known to deactivate the immune response and mice infected with parasitic worms exhibit improved insulin sensitivity, suggesting potential use of helminthic antigens for therapies. Lastly, exercise reduces blood inflammatory markers and improves metabolic parameters. The so-called myokines produced by muscle after physical activity may modulate macrophage activation[300-303]. In fact, exercise induced improvements in the inflammatory profile were found to be independent of weight loss[304,305]. Through understanding these different aspects of metabolic-related immune responses, it may be possible to design drugs to specifically target the feed-forward loop of metabolic stress-induced inflammation without unwanted side effects, such as immuno-suppression or allergic responses.

Immunomodulation as a therapeutic for chronic inflammation

An attractive method to treat chronic inflammatory pathways is through immunomodulation. However, immunomodulatory therapeutics must be developed cautiously because of a limited understanding in the intricate relationship between metabolic tissues (e.g., muscle, liver, and WAT) and immune cells. Use of T_H2 cytokines such as Il-4 and Il-13 prove problematic due to their role in onset of allergies. It is also important to avoid therapeutics that

would suppress the immune system, thereby compromising host defenses. Salicylates, inhibitors of the Nf-κb inflammatory response, have also shown promise in improving glycemic parameters in non-diabetic obsese adults, although immuno-suppressive side-effects have not been addressed[306].

The human host and several parasites co-evolved, with evidence suggesting that parasites developed glycans to mimick host mechanisms of immune suppression in an effort to live symbiotically for decades with the host[199]. In fact, many of the glycans used by parasites to maintain symbiotic relationships are found in human breast milk and other sources[202]. Epidemiological data suggest that children who are breast-fed are less likely to develop allergies, auto-immune diseases, and metabolic syndrome[307-309]. This implicates loss of glycan-derived immunomodulation in the development of auto-immune and inflammatory diseases. It also suggests an evolutionary mechanism to modulate the immune system and suppress inflammatory signaling. While the data in chapter 2 suggests that LNFPIII can be a promising therapeutic for chronic inflammatory diseases, more research is needed to elucidate the long term effects of glycan treatment and the most efficacious age for treatment.

When treating HFD-insulin resistant mice with LNFPIII, I observe a small, yet insignificant increase in body weight that is associated with a significant increase in WAT mass. This increase in weight is most likely due to the ability of insulin sensitive adipose tissue to store more lipids. This phenotype is observed in other therapeutics that improve WAT insulin sensitivity, such as Pparγ agonists and sulfonylureas[310,311]. It is unclear if long term treatment with LNFPIII will significantly influence body weight or lead to other unwanted side effects. I also observe recurrence of insulin resistance after glycan withdrawl (**Data not shown**). However, young children who are exposed by breast-feeding to these same glycans prior to the formation of their

adult immune systems appear to sustain the beneficial effects of immunomodulation. This would suggest that the glycans provide a type of "memory" to innate immune cells. Additional work understanding the temporal regulation of LNFPIII immunomodulation can help to understand the role of pathogen interactions in the formation of a strong immune system.

Current state of research: Role of metabolic pathways in immune dysfunction

The incidence of the metabolic syndrome and susceptibility to infections increases with age[312]. Age has also been associated with a decline in immunological function[313,314]. What remains unclear is whether the decline in immunological function is due to a loss of metabolic regulation in immune cells. At a systemic level, older patients have a slowed metabolic rate and display signs of metabolic dysregulation[315]. My research suggests that metabolic dysregulation in the macrophage, through fatty acid sensing receptor deletion, has adverse affects on bacterial infection.

The role of metabolic pathways in macrophage function has been shown previously. Rate of *de novo* lipogenesis is increased in the macrophage upon stimulation by a pathogen. Srebp1a, a receptor from the sterol regulatory binding protein family of receptors known to regulate lipid and cholesterol metabolism, is very highly expressed in the macrophage and Nf-κb has been shown to bind to an element in the promoter to induce transcription during bacterial stimulation[169]. In fact, lack of Srebp1a protects mice from infection with *Salmonella typhimurium*, although this effect was proposed to be due to suppression of a heightened cytokine storm[169]. Flux of glucose through the pentose phosphate pathway (PPP) is also increased during bacterial stimulation[159]. Decreased glucose-6-phosphate dehydrogenase (G6pdh) activity is associated with decreased NADPH oxidase activity in neutrophils[316]. Studies

looking at the role of other metabolic regulators, such as liver x receptor, farnesoid x receptor, mammalian target of rapamycin (mTor), and insulin receptor, suggest that many receptors in the macrophage sense metabolic status and affect macrophage function by altering cellular functions, the most commonly described being cytokine production[42,140,317-320].

Metabolic modulators in the treatment of infections

The critical relationship between metabolic pathways and immune cell functions, suggests that development of therapeutics modulating metabolic pathways can be used to treat infectious diseases. In chapter 4, I show that Ppar agonists can improve bacterial killing and survival in eight-week-old mice. Here, I suggest a novel mechanism to increase host bacterial killing potential to improve killing by modulating metabolic pathways. Further studies will determine if ligand treatment post infection can recapitulate these findings. Additionally, studies should be conducted addressing the ability of combination treatments involving Pparδ ligands and antibiotics. While Pparδ ligand appears to improve bacterial killing, it is possible that use of multiple ligands to improve several pathways of host killing could be much more potent.

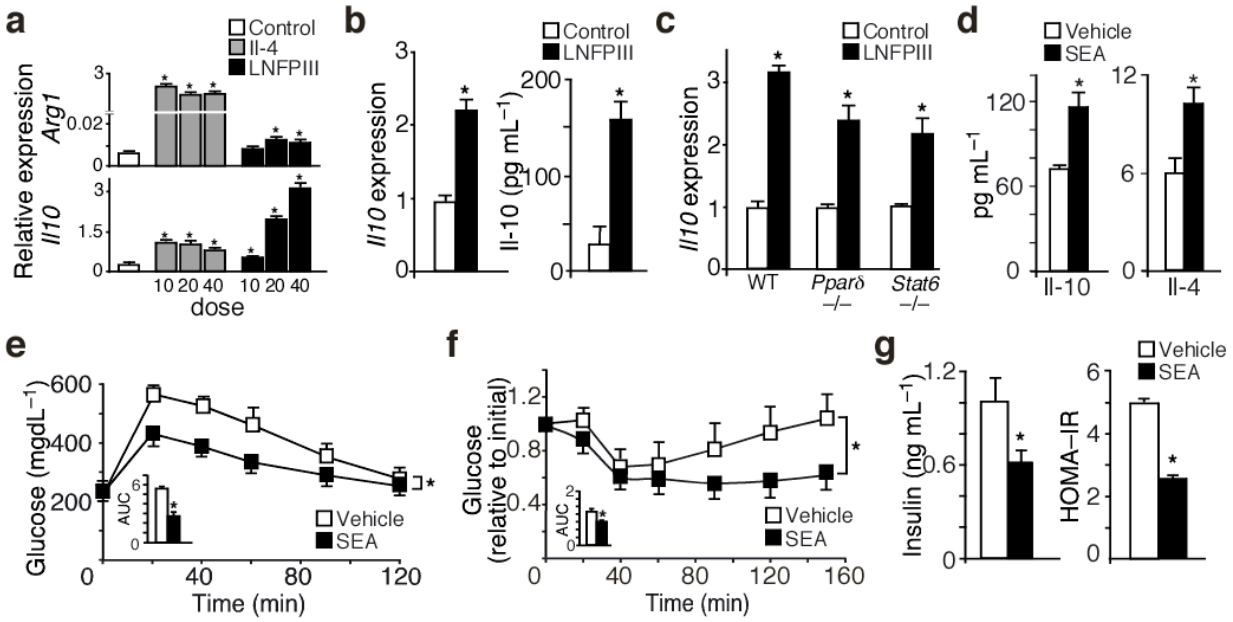
Lastly, the translation of these studies to humans should be addressed. An important concern is that metabolic pathways are regulated differently in murine and human macrophages. For example. PPARα, a third isoform of the PPAR family of receptors, has more of a contribution to macrophage function in humans than in mice[140]. In the murine macrophage, Pparα expression is much lower than Pparδ or Pparγ and the contribution of Pparα to murine macrophage function is limited[140]. In the human and murine macrophage, PPARα ligand was shown to increase NADPH oxidase function and ROS production[321]. If these pathways regulate bacterial killing in the human macrophage, it would also be exciting to see if similar ligands

could be used to treat other types of bacterial, viral, or fungal stresses. In recent years, a lack of novel antibiotics and the rise of multi-drug resistant pathogens have led to the immediate need for novel therapeutics (CDC, WHO). Through understanding these different aspects of metabolic-related immune responses, it may be possible to design drugs to specifically improve metabolite-regulate immune function and outcomes of infectious responses.

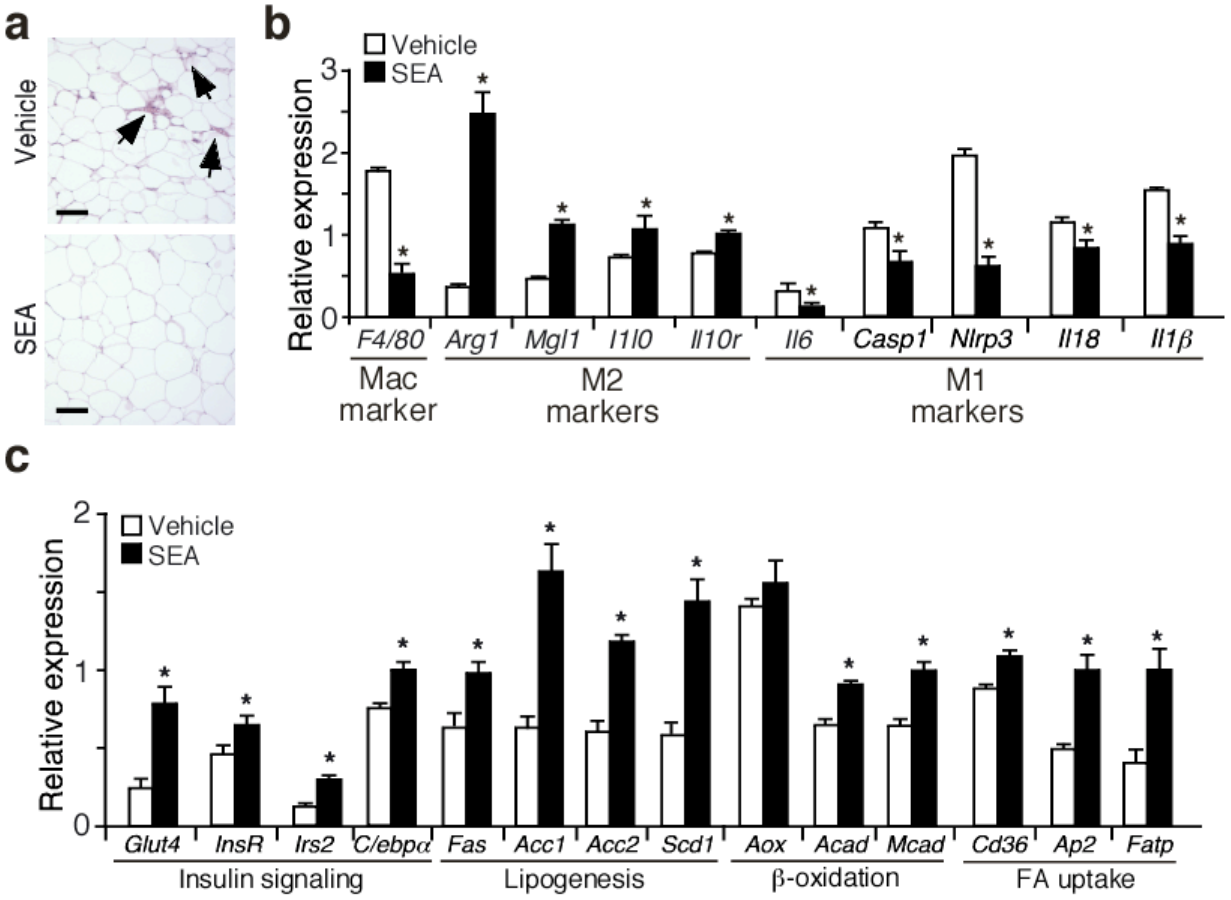
Concluding thoughts

In this thesis I have shown that metabolic pathways can be manipulated to treat infectious diseases. I have also shown that proper functioning of immune pathways contributes to metabolic homeostasis. Further investigation into these pathways will help to understand the roles that metabolic, immune, and pathogenic signaling play in maintaining physiological homeostasis in an effort to prevent pathological dysregulation.

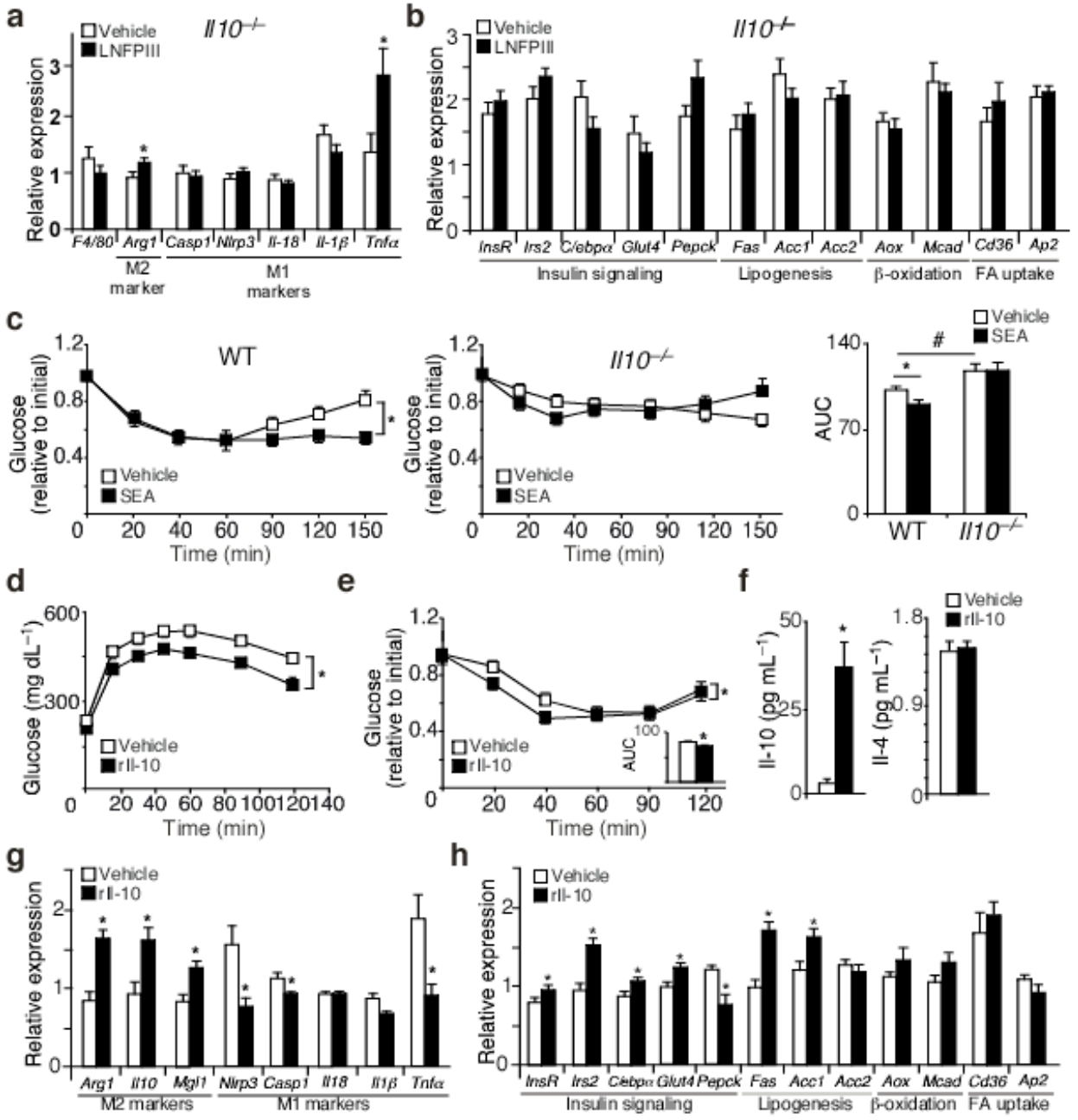
APPENDIX



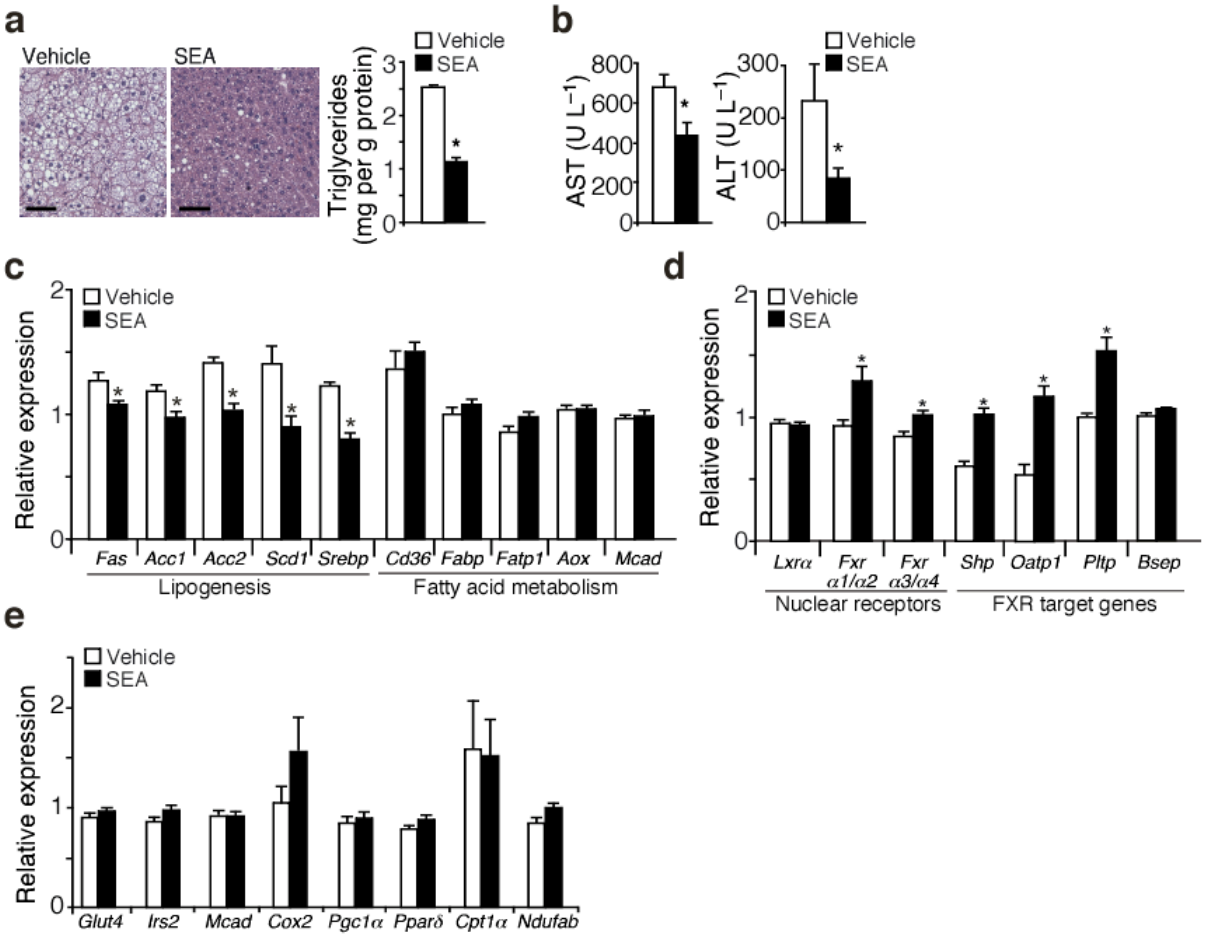
Supplementary Figure 1 Induction of Il-10 by LNFPIII is independent of T_H2 cytokines. (**a**) LNFPIII induces Il-10 and Arg1 expression in a dose-dependent manner. Bone marrow derived macrophages were treated overnight with 10, 20 or 40 ng mL^{-1} of Il-4 or 10, 20 or 40 μg mL^{-1} of LNFPIII. Gene expression was determined by RT q-PCR. (**b**) Regulation of Il-10 expression (left panel) and production (right panel) by LNFPIII (20 μg mL^{-1}) in dendritic cells (**c**) LNFPIII induced Il-10 expression is independent of Pparδ and Stat6. Wild type (wt), *Ppard*$^{-/-}$ and *Stat6*$^{-/-}$ macrophages were given 20 μg mL^{-1} LNFPIII overnight. (**d**) SEA treatment increases levels of circulating Il-10 and Il-4 *in vivo*. Serum Il-10 and Il-4 concentrations were measured in vehicle or SEA treated mice (n=4–6/treatment) used for metabolic studies. (**e** and **f**) Improved glucose tolerance (GTT, **e**) and insulin sensitivity (ITT, **f**) in mice treated with SEA. Male C57BL/6J mice (n=4–6) fed a high fat diet for 6 weeks were given vehicle or SEA (25 μg/twice a week). GTT and ITT were conducted at 4th and 5th weeks, respectively, after treatment. Inset: area under the curve (AUC). (**g**) SEA treatment decreases fasting insulin levels and increases systemic insulin sensitivity determined by HOMA-IR. Values are expressed as means ± s.e.m.. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. *In vivo* studies were reproduced in three mouse cohorts (n=4–6/treatment). *P<0.05 (SEA versus vehicle control).



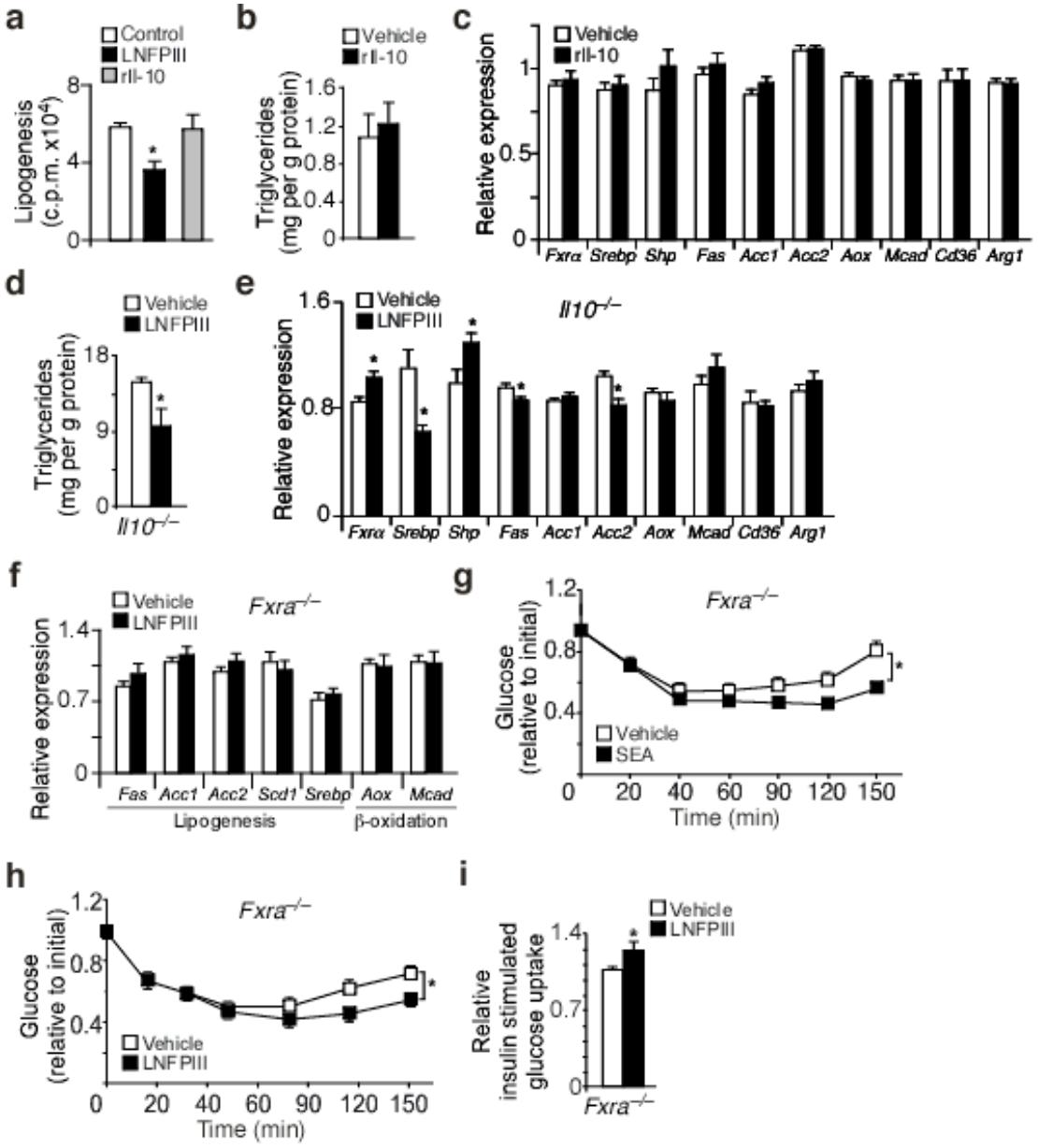
Supplementary Figure 2 SEA reduces inflammation and enhances metabolic homeostasis in WAT. (**a**) Histological analysis showing less crown-like structures in SEA treated WAT. Arrows indicate crown-like structures. Scale bar=100 μm. (**b**) Increased M2 and decreased M1 gene expression in SEA treated mice, compared to vehicle treated controls (*n*=5/treatment). (**c**) Metabolic gene expression in WAT from vehicle or SEA treated mice. WAT was collected at the end of metabolic studies. Gene expression was determined by real-time q-PCR. Values are expressed as means ± s.e.m. Metabolic studies were reproduced in three mouse cohorts (*n*=4–6/treatment). Crown-like structures and expression analyses were examined in one and two of the three cohorts, respectively. **P*<0.05 (SEA versus vehicle control).



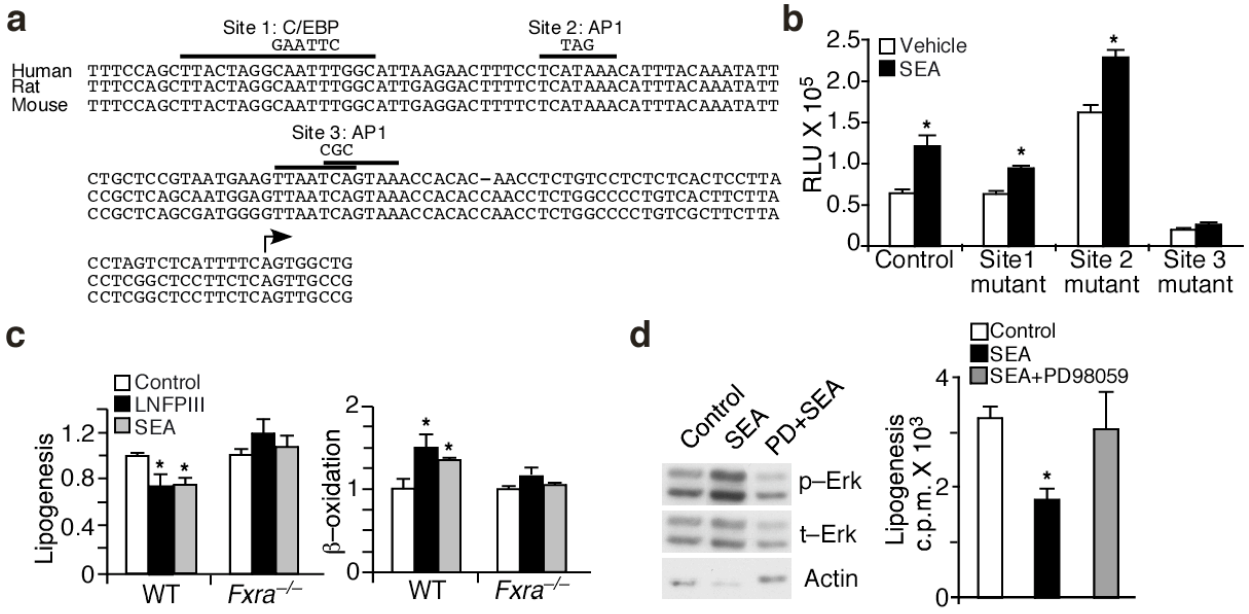
Supplementary Figure 3 The insulin sensitizing activity of LNFPIII/SEA is mediated by Il-10. (**a** and **b**) The effect of LNFPIII on inflammatory and metabolic gene expression in WAT is abrogated in *Il10*$^{-/-}$ mice (*n*=6/treatment). Mice were injected intraperitoneally with vehicle of LNFPIII for 6 weeks following 6 weeks on high fat diet. (**c**) The insulin sensitizing effect of SEA is lost in *Il10*$^{-/-}$ mice. Left and middle panels: ITT in wt and *Il10*$^{-/-}$ mice (*n*=6/treatment) treated with vehicle or SEA. Right panel: area under the curve of ITT. (**d**) GTT and (**e**) ITT showing rIl-10 treatment improves glucose tolerance and insulin sensitivity. Male C57BL/6J mice (*n*=5) fed a HFD for 4 weeks were given three treatments of PBS (vehicle) or rIl-10 (1 ug/every other day). (**f**) rIl-10 treatment increases serum Il-10, but not Il-4 levels. (**g**) WAT exhibits reduced inflammatory and increased M2 gene expression following rIl-10 treatment. (h) rIl-10 increases expression of insulin signaling and lipogenic genes in WAT. Values are expressed as means ± s.e.m. Studies using *Il10*$^{-/-}$ and control mice (*n*=6/treatment/genotype) and in rIl-10 treatment (*n*=6) were conducted in 1 cohort. **P*<0.05 (treatment versus vehicle control); #*P*<0.05 (*Il10*$^{-/-}$ versus wt).



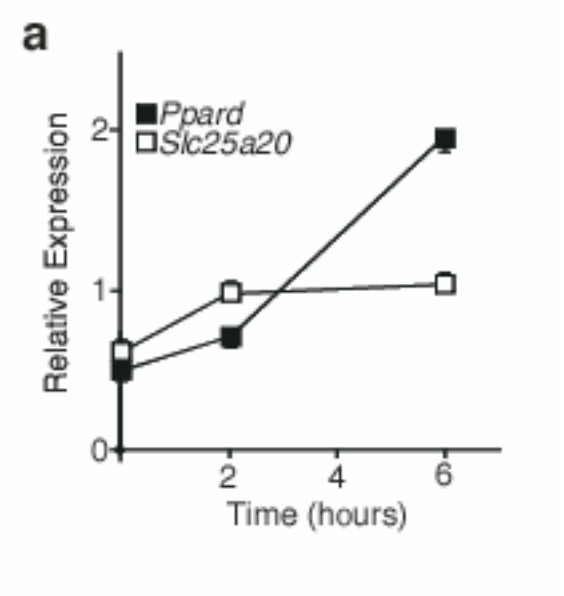
Supplementary Figure 4 SEA protects against hepatic steatosis and suppresses lipogenic gene expression. (**a**) Representative liver histology images from vehicle or SEA treated mice (n=5). Scale bar= 100 μm. Right panel: hepatic triglyceride content. (**b**) SEA improves overall liver function as assessed by circulating AST and ALT levels. (**c** and **d**) Expression analyses of metabolic genes and nuclear receptor signaling pathways known to regulate lipogenesis in livers from vehicle or SEA treated mice (n=5) by real-time q-PCR. (**e**) Gene expression in muscle from vehicle or SEA treated mice determined by real-time q-PCR. Values are expressed as means ± s.e.m. Metabolic studies were reproduced in 3 mouse cohorts (n=4–6/treatment). Histology was examined in one and lipid and expression analyses were examined in two of the three cohorts. *P<0.05 (SEA versus vehicle control).



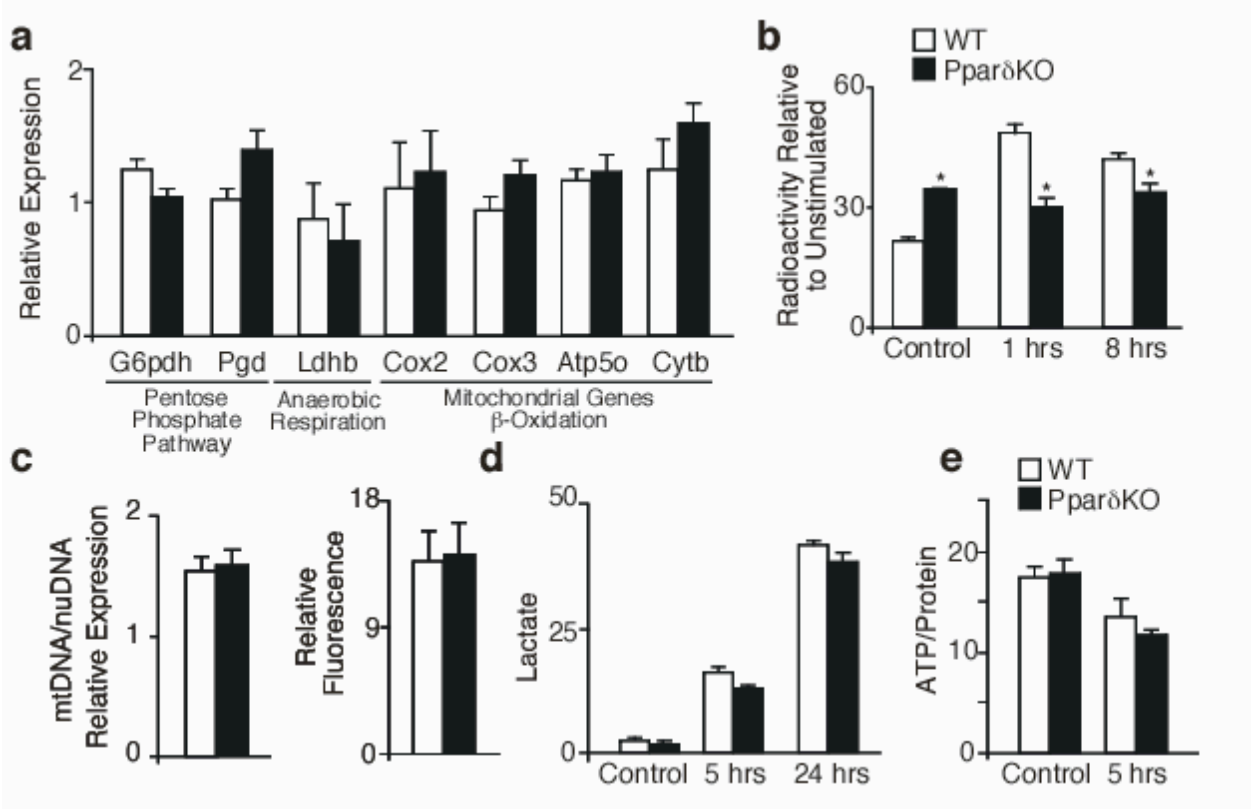
Supplementary Figure 5 LNFPIII, but not Il-10, suppresses *de novo* lipogenesis in the liver. (**a**) LNFPIII has a direct effect on lipid metabolism *ex vivo* in primary hepatocytes. Hepatocytes were treated with vehicle, LNFPIII (20 μg ml^{-1}) or Il-10 (10 ng ml^{-1}) for 24 hr. Cells were washed and subjected to lipogenesis assays. (**b** and **c**) rIl-10 treatment (n=5/treatment) does not affect the triglyceride content or lipogenic gene expression in the liver. (**d** and **e**) LNFPIII treatment in *Il10*$^{-/-}$ mice (n=6) reduces hepatic triglyceride content and suppresses lipogenic gene expression. (**f**) Gene expression analyses in livers from *Fxra*$^{-/-}$ mice treated with vehicle or LNFPIII (n=6) by real-time q-PCR. (**g**) ITT in *Fxra*$^{-/-}$ mice (n=6) treated with SEA. (**h**) LNFPIII treatment improves insulin sensitivity determined by ITT in *Fxra*$^{-/-}$ mice (n=6). (**i**) *Ex vivo* insulin stimulated glucose uptake in adipose tissue slices. Adipose tissue slices were collected before and after portal vein injection of 5U kg^{-1} insulin and immediately subjected to glucose uptake assays. Values are expressed as means ± s.e.m. For *in vitro* assays, the mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. Studies using rIl-10 (n=6), *Il10*$^{-/-}$ (n=6), *Fxra*$^{-/-}$ (n=6) and corresponding controls were performed in 1 mouse cohort. *P<0.05 (treatment versus control).



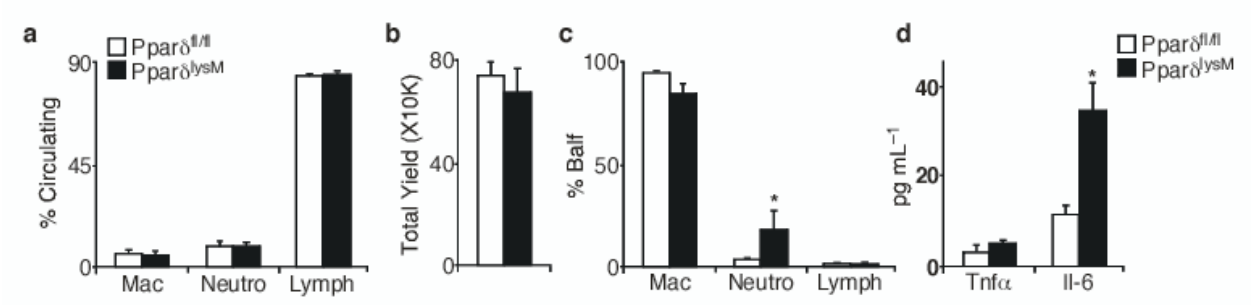
Supplementary Figure 6 (**a**) Sequence comparison of the 5' proximal regulatory region of human, rat and mouse Fxrα downstream promoter (promoter 2). The transcriptional start site is indicated with an arrow. Putative binding sites for C/EBP (site 1) and AP1 (site 2 and site 3) are highlighted. Sequences mutated for the reporter assays are indicated above the highlighted binding sites. (**b**) Reporter assays showing that the induction of Fxrα promoter 2 is mediated by AP1 binding sequences in site 3. Mutations in sites 1 and 2 had no effect. Similar results were obtained with LNFPIII treatment (data not shown). (**c**) LNFPIII (20 μg ml^{-1}) or SEA (2 μg ml^{-1}) modulate hepatic lipid metabolism in an Fxrα-dependent manner. Functional assays were conducted in hepatocytes isolated from wild type (wt) or *Fxra*$^{-/-}$ mice 24 hr after treatments. (**d**) Inhibition of Erk activation blocks the activity of SEA in suppressing lipogenesis in hepatocytes. Left panel: Western blot analyses showing that PD98059 inhibits Erk phosphorylation (p-Erk). Cells were pretreated with PD98059 for 1 hr prior to overnight SEA treatment. p-Erk: phospho-Erk; t-Erk: total Erk. Actin was included as a loading control. Right panel: *in vitro de novo* lipogenesis assays in hepatocytes. Values are expressed as means ± s.e.m. Mean and s.e.m. were determined from 3–4 biological replicates for a representative experiment. Experiments were repeated three times. **P*<0.05 (SEA/LNFPIII versus vehicle control).



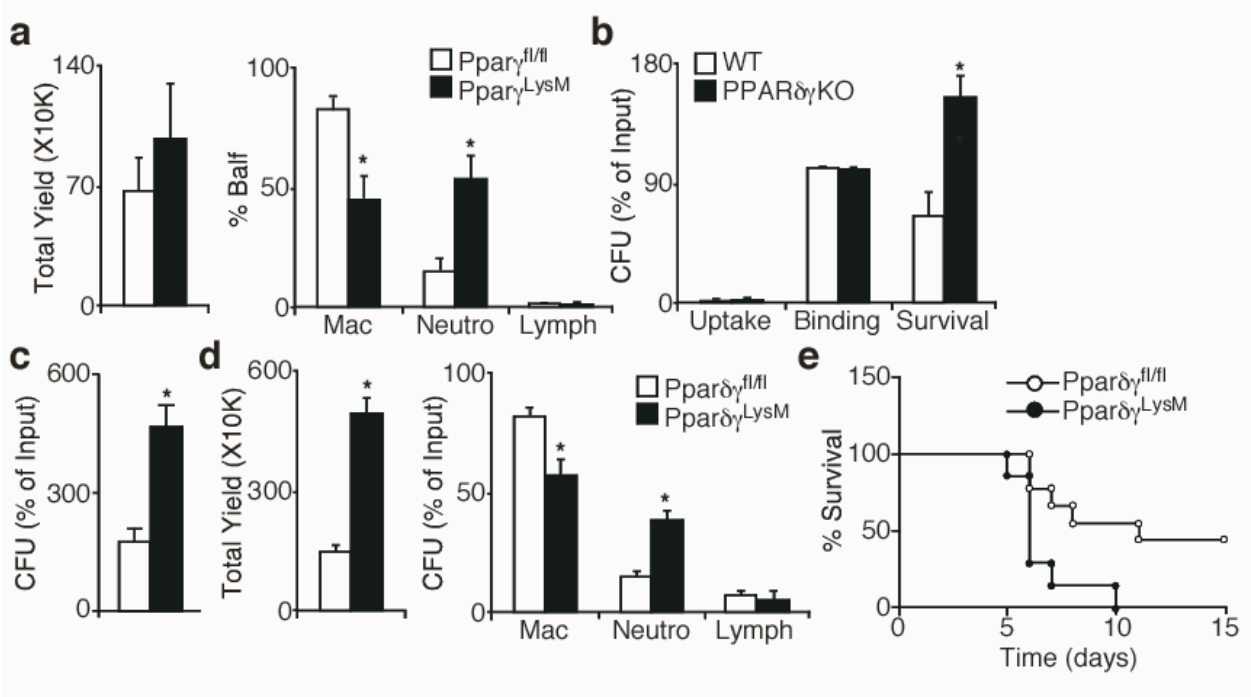
Supplementary Figure 7: Pparδ expression is increased in macrophages during infection with *S.pneumoniae*. (**a**) Expression of *Pparδ* and its target gene *Slc25a20* was determined by RT qPCR in macrophages infected with *S.pneumoniae* for the indicated time.



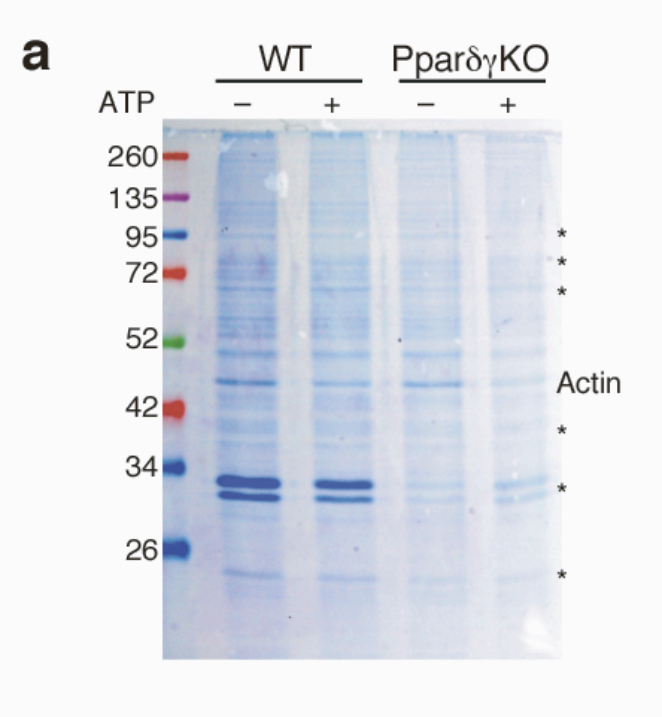
Supplementary Figure 8: PparδKO macrophages do not have defects in mitochondrial number, lactate production, and ATP generation *in vitro*. (**a**) Gene expression of metabolic enzymes in wt and PparδKO macrophages. (**b**) PparδKO macrophages have decreased glucose uptake. Cells were incubated with insulin followed by incubation with 2-[H^3]deoxy-D-glucose. (**c**) PparδKO macrophages have an equal number of mitochondria compared to wt macrophages. Mitochondrial number was determined by normalizing a mitochondrial gene (ND1) and a nuclear gene (36B4). Mitochondria were also quantified by mitotracker staining and fluorescence quantification (**d**) Pparδ knockout macrophages infected with *E.coli* do not have a defect in lactate production. (**e**) ATP production by wt or PparδKO macrophages during infection is equal.



Supplementary Figure 9: Complete cell counts in wt and Pparδ^{LysM} mice show no defects in recruitment. (**a**) Complete blood counts from blood of infected mice. (**b**) Total number of cells obtained from wt and Pparδ^{LysM} mice after 24 of infection. Mice were intranasally with *S.pneumoniae* and cells were counted 24 hours post infection. (**c**) Differential cell counts were determined on bronchoalveolar lavage fluid (Balf) from infected mice. (**d**) Cytokines were measured in Balf 24 hours post infection.



Supplementary Figure 10: Pparδγ^{LysM} mice have a defect in bacterial killing. (**a**) Total cell number was determined from lavage fluid of Pparγ^{LysM} mice infected intranasally with *S.pneumoniae* for 24 hours. Macrophages, neutrophils, and lymphocyte cell counts were determined in alveolar lavage fluid from mice infected with *S.pneumonia* for 24 hours. (**b**) *In vitro* killing assay in wt and PparδγKO macrophages. Primary bone marrow derived macrophages were infected with *S.pneumonia* (10 bacteria: 1 macrophage) to determine intracellular killing *in vitro*. (**c**) 24 hour *in vivo* killing assay. wt and Pparγ^{lysM} mice were infected intranasally with *S.pneumoniae* for 24hrs and percent bacterial survival was determined. (**d**) Total cell number was determined from lavage fluid of Pparγ^{LysM} mice infected intranasally with *S.pneumoniae* for 24 hours. Macrophages, neutrophils, and lymphocyte cell counts were determined in alveolar lavage fluid. (**e**) Survival of wt or Pparδγ^{LysM} mice. Mice were infected with bacteria and survival was followed for 2 weeks.



Supplementary Figure 11: Phagosome isolation from wt and Pparδγ^{Lysm} mice. (**a**) Macrophages were infected with magnetic beads and phagosomes were isolated after homogenization. ATP was used to disrupt binding of actin and other scaffolding proteins. Stars mark bands of potential interest.

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